Assessment of the role of early life exposure to endocrine disrupting compounds in programming of obesity in a mouse model

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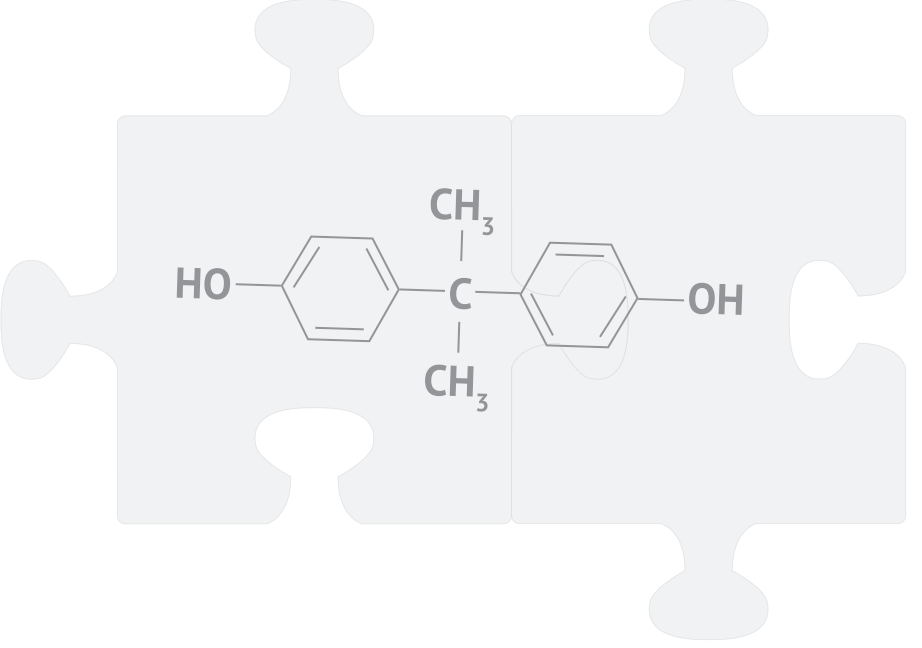
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Chapter 2

Programming of metabolic effects in C57BL/6JxFVB mice by exposure to bisphenol A during gestation and lactation

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Abstract

The global rise in prevalence of obesity is not fully explained by genetics or life style factors. The developmental origins of health and disease paradigm suggest that environmental factors during early life could play a role. In this perspective, perinatal exposure to bisphenol A (BPA) has been indicated as a programming factor for obesity and related metabolic disorders later in life. Here we study early life programming by BPA using an experimental design that is relevant for human exposure. C57BL/6JxFVB hybrid mice were exposed during gestation and lactation via maternal feed to 8 non-toxic doses (0–3000 μg/kg body weight/day (μg/kg bw/d)) of BPA. After weaning, offspring were followed for 20 weeks without further exposure. Adult male offspring showed dose-dependent increases of body and liver weights, no effects on fat pad weights and a dose-dependent decrease in circulating glucagon. Female offspring showed a dose-dependent decrease in body weight, liver, muscle and fat pad weights, adipocyte size, serum lipids, serum leptin and adiponectin. Physical activity was decreased in exposed males and suggested to be increased in exposed females. Brown adipose tissue showed slightly increased lipid accumulation in males and lipid depletion in females, and *Ucp1* expression was dose-dependently increased in females. The effects in females were more reliable and robust than in males due to wide confidence intervals and potential confounding by litter size for male data. The lowest derived lower bound of 90% confidence interval of the benchmark dose (BMDL) of 233 μg/kg bw/d (for interscapular weight in females) was below the proposed BMDL of 3633 μg/kg bw/d as a basis for tolerable daily intake. Although these results suggest that BPA can program for an altered metabolic phenotype, the sexual dimorphism of effects and diversity of outcomes among studies similar in design as the present study do not mark BPA as a specific obesogen. The consistency within the complex of observed metabolic effects suggests that upstream key element(s) in energy homeostasis are modified. Sex-dependent factors contribute to the final phenotypic outcome.

Introduction

Obesity has reached pandemic proportions in adults (OECD, 2010), and the incidence is also increasing in children (Oken and Gillman, 2003). This development is of high concern because most obese children become obese adults and childhood obesity is associated with a shorter life expectancy. Furthermore, the condition is associated with a range of metabolic disorders including insulin resistance, type 2 diabetes, and dyslipidaemia.

Obesity is a complex disorder as many factors are involved in its pathogenesis. In recent years it has become apparent that lifestyle changes, involving consumption of energy-dense foods and insufficient physical activity, even in combination with a predisposed genetic background, cannot fully explain the current obesity pandemic (McAllister et al., 2009). Therefore, other determining factors have been considered, including the developmental origins of health and disease (DOHaD) paradigm (Gluckman and Hanson, 2004). According to this principle, exposure to environmental factors during specific sensitive periods of development, mainly *in utero* and immediately after birth, can interfere with maternal hormonal and nutritional signaling to the developing organism. The organism then responds to these new signals by adapting its phenotype, e.g. through changed metabolic setpoints, resulting in a permanent or long-term change in the structure or function of the organism (Gluckman et al., 2005; Lucas, 1991). This process is called programming (Lucas, 1991) and a subsequent modification of various functions and systems in the body, including metabolic homeostasis and endocrine and reproductive functions, can ultimately predispose an individual to chronic diseases later in life, e.g. obesity and related metabolic disorders (Oken and Gillman, 2003). Many early life determinants for obesity and related metabolic disorders have now been extensively studied, of which malnutrition, maternal overweight and gestational diabetes are important examples (Barker, 1995; Boney et al., 2005; Dabelea et al., 2000; Hales, 1997). In this respect, studies of the Dutch Hunger Winter cohort have shown that a nutrition deficiency during pregnancy leads to a low birth weight that is a risk factor for an increased susceptibility to metabolic disorders later in life (Painter et al., 2005; Ravelli et al., 1998, 1999).

Exposure to endocrine disrupting compounds (EDCs) has been proposed as another important early life determinant, since increased production rates of these compounds over time coincide with the increase in incidence of obesity (Baillie-Hamilton, 2002).

An EDC is "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle" (EDSTAC, 1998). Along these lines, some EDCs may specifically alter energy homeostasis and appetite regulation, which are both important for weight control, and such EDCs are termed as environmental obesogens of which tributyltin chloride (Grun et al., 2006) and diethylstilbestrol (Newbold et al., 2005) are prototypes. Obesogens could then have a direct disrupting effect, or could act through programming of a developing organism toward increased susceptibility to develop obesity later in life (Grun and Blumberg, 2007). A troubling issue with EDCs is that many of such substances are ubiquitously present in the environment and are considered to exert their effects, including on programming, at low, environmentally relevant exposure levels (Casals-Casas and Desvergne, 2011).

Bisphenol A (BPA) is a suspected obesogen. BPA is used as a monomer in the production of polycarbonate plastics and epoxy resins. It is present in many consumer products from which it can leach, such as plastic water bottles, food containers, can linings, and thermal paper (Brotons et al., 1995), and humans are continuously exposed from these sources to low levels of BPA, mainly via the oral route. Average exposure levels (sum of oral and dermal) for adults are around 0.2 μg/kg body weight/day (μg/kg bw/d), highest exposure levels seen in teenagers are 1.5 μg/kg bw/d and a temporary tolerable daily intake has been set at 5 μg/kg bw/d (EFSA, 2014). Exposure starts early in life since BPA can cross the placenta (Schonfelder et al., 2002). After birth, infants may be exposed via breast milk (Kuruto-Niwa et al., 2007; Otaka et al., 2003; Sun et al., 2004; Ye et al., 2006), or via plastic baby bottles (Vandenberg et al., 2007). In humans, BPA has a short half-life, in the range of hours (Volkel et al., 2002). Measurable BPA in human serum as repeatedly reported comes with uncertainties, because typical serum BPA concentrations are orders of magnitude lower than levels measurable by modern analytical methods (Teeguarden et al., 2013).

Toxicity of BPA has been studied extensively in terms of classical toxicological paradigms, including hormonal activity (particularly estrogenicity), and there is evidence for conclusions in at least some toxicological domains as reviewed in detail by Chapin et al. (2008) and Willhite et al. (2008). Much controversy remains over

possible obesogenic effects of BPA, due to inconsistent results from epidemiological and animal studies. Epidemiological studies in adults reported an association of actual BPA levels in urine with cardiometabolic disorders (Lang et al., 2008; Melzer et al., 2010), or with obesity in children and adolescents (Trasande et al., 2012), but the validity of these results from the cross-sectional NHANES data were afterwards disputed (LaKind et al., 2012). Other epidemiological studies indicate inconsistent findings for an association between prenatal BPA exposure and a low birth weight, a predictor of obesity later in life (Harley et al., 2013; Lee et al., 2008; Miao et al., 2011; Padmanabhan et al., 2008; Wolff et al., 2008). Animal studies have also shown variable effects of early life exposure to BPA on body weight e.g. (Honma et al., 2002; Miyawaki et al., 2007; Ryan et al., 2010b) possibly due to variation in experimental conditions, such as dosing regimes, animal species and strains, and timing of evaluation of effects. Altogether, research until now does not allow for general and consistent conclusions regarding the hazard of low dose exposure to BPA, including the translation of experimental results to humans. These uncertainties have led to new research and policy initiatives around the world.

In view of the general concern about EDC-induced programming of obesity and the particular uncertainties associated with BPA in this context, we aimed to investigate the hypothesis that early life exposure to BPA can program the organism for increased sensitivity to develop overweight and related metabolic impairment later in life. The present study may contribute to the BPA hazard database and provide further support for improved decisionmaking, because we aimed to model human exposure conditions. Specifically, we applied gestational and lactational exposure via maternal feed in a dose-response design in mice, using a dose range of 0–3000 µg/kg bw/d, which is below the proposed lowest derived lower bound of 90% confidence interval of the benchmark dose (BMDL) of 3633 µg/kg bw/d for systemic effects in adults and offspring in a reproductive study in mice (Tyl et al., 2008) calculated by EFSA (2014) and including a dose (3 µg/kg bw/d) approaching highest estimated human exposure levels (up to1.5 µg/kg bw/d; EFSA, 2014). The adult phenotype of the offspring with the focus on metabolic profile was analyzed in detail after a latency period of 20 weeks.

Methods

Test chemical and test diets

BPA (purity > 99%; CAS No. 80–05–7, Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in soy oil, by constant stirring overnight at room temperature. This master solution was serially diluted with a factor 3–3.3. The thus obtained 7 solutions and a blank soy oil were mixed with the diet (NIH-07 diet, Research Diet Services, Wijk bij Duurstede, The Netherlands) before pelleting, aiming at concentrations of 0, 0.017, 0.056, 0.17, 0.56, 1.7, 5.6, and 16.7 mg/kg BPA in feed, which corresponded to 0, 3, 10, 30, 100, 300, 1000, and 3000 µg/kg bw/d based on calculations with standard average food consumption of 4.5 g per mouse per day and a standard average body weight of 25 g per mouse. BPA concentrations in test diets were confirmed by isotope dilution gas chromatography-mass spectrometry after extraction with methanol (8402, JT Baker, Deventer, The Netherlands), cleanup with solid phase extraction (Oasis HLB, WAT106202, Waters, Breda, The Netherlands) and derivatization using trimethylsilane (1391, Sigma-Aldrich, Zwijndrecht, The Netherlands). The limit of detection (LOD) was defined as 2x the average absolute blank level, which in view of the small data set is different than the definition of the LOD in the serum analysis. The nonpurified soy-based NIH-07 diet was chosen because it was originally designed to optimize gestation, lactation, and growth of rodents. This diet has also low levels of natural phytoestrogens, which have been shown to promote normal physiology in mice, in contrast to phytoestrogen free diets (Ruhlen et al., 2008). A cleaned up extract of the diet was checked for estrogenic and anti-estrogenic activity with an estrogen receptor mediated reporter gene ER-LUC assay (Rogers and Denison, 2000). The feed contained 306 pg estradiol equivalents per gram diet (most likely phytoestrogens), and did not contain any estrogen receptor antagonistic activity.

As females, in contrast to males, showed no body weight increase at the age of 17 weeks, we investigated whether an obesogenic response could be triggered in females by a high fat diet challenge. A high fat diet (D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands), containing 45 kcal% fat (lard) compared to 15 kcal% fat in the NIH-07 diet, was given to all female F1 mice during the final period of the study (17–23 weeks of age).

Experimental conditions

Obesity-prone (Michel et al., 2005; Surwit et al., 1988) nulliparous female C57BL/6J mice (Charles River, Sulzfeld, Germany) were mated with male FVB mice (GPL, Bilthoven, The Netherlands) to produce hybrid offspring for which comprehensive background information of phenotype and development is available in our lab (Dollé et al., 2011) and which would enable distinction of parental alleles in eventual follow-up molecular studies. For practical reasons, dams were divided over two time groups with a distance of one week. Dose groups were equally represented in each of these two time groups. Mice were maintained under specific pathogen-free conditions with a target ambient temperature of 21 °C, humidity of 60% and with a 12 h/12 h light/dark cycle. F0 males were single housed in standard Macrolontype II cages with polycarbonate bottles and were fed standard lab chow (CRM, Tecnilab-BMI, Someren, The Netherlands). To minimize environmental exposure to BPA, F0 females and all offspring were housed in polysulfone cages (Tecnilab-BMI, Someren, The Netherlands). Polysulfone does, in contrast to polycarbonate, not undergo hydrolysis in hot water during routine cleaning, so only residual BPA from the manufacturing process can be extracted from the surface, and this should be washed out after several cleaning cycles as we applied before actual use of the cages. Drinking water was supplied in glass bottles with rubber stoppers. Cages had spruce/fir wood bedding (Lignocel S 8–15; Tecnilab-BMI, Someren, The Netherlands) and aspen wood shavings (Lignocel 9 S) for cage enrichment. Both feed and water were supplied *ad libitum*.

After an acclimatization period of 4 weeks, female F0 mice were fed experimental diets explained above starting 2 weeks before mating, and continued during mating (1 week), gestation (3 weeks), and lactation (3 weeks). Each dose group contained four F0 females, which were mated in pairs with one F0 male for each pair. BPA concentrations in sera of dams and of surplus offspring sacrificed at weaning, closely around postnatal day (PND) 21, were measured to confirm internal exposure. After weaning, all offspring were fed the control NIH-07 diet. For every dose group on average 8 mice per sex (range 4–10, evenly recruited from available litters) were included for follow-up through juvenile and adult stages and housed as mixed litter groups of 4–5 animals per cage (two cages per sex per dose group). At the age of 5 weeks and continuing until the end of the study, body weight was measured weekly. Food consumption in offspring could not be recorded reliably due to high spillage.

At the age of 23 weeks, after being fasted for 16 h to induce a general basic metabolic state, mice were sacrificed under ketamine/xylazine anesthesia by eye bleed, to obtain a maximal serum blood volume for analytical purposes. During necropsy, body length (nose-tail base) was measured, and a selection of organs was weighed, including adrenal glands, brain, liver, femur, quadriceps femoris muscle, pancreas, interscapular fat, perigonadal fat, perirenal fat, mesenterial fat, and subcutaneous fat (both rostral and caudal mammary gland fat). Organs and tissues were partly snap-frozen in liquid nitrogen and stored at –80 °C and the remaining tissues fixated in formalin (see below).

This study was approved by the Animal Experimentation Ethical Committee of our institute under permit number 200900208, and carried out in accordance with prevailing legislation.

Glucose tolerance test

At 18 weeks of age a glucose tolerance test (GTT) was performed in control and top dose (3000 μg/kg bw/d) males and females (n = 8–9 per group). Mice were fasted for 16 h before a baseline blood sample was taken (0 min). Subsequently, D-glucose (Sigma, Zwijndrecht, The Netherlands) was injected i.p. at a concentration of 1.5 g/kg bw. Glucose was measured in tail vein blood at 15, 30, 60 and 120 min after glucose administration using the FreeStyle Lite meter and test strips (Abbott, Hoofddorp, The Netherlands). The experiment was performed over two morning sessions, with animals matched by age (is per time group, see above) per session, and 1.25 h between the first and last tested animal in each session. Small clusters of animals of different experimental groups were alternatively treated.

Spontaneous locomotor activity

At the age of 19–21 weeks, 1–2 cages per control and top dose group, each cage with 4 animals, were transferred to polysulfone cages mounted on LABORAS platforms (Metris BV, Hoofddorp, The Netherlands) with access to feed and water *ad libitum*. After an acclimatization period of minimal 6 h, activity of the mice was continuously registered on four parallel platforms for 4.5 (females) or 6.5 days (males), starting at the beginning of the dark phase (6.30 PM) of the first day. Through platform sensors and customized software, physical activity of the animals was registered and expressed as kinetic energy indices per cage per 15 min.

Histopathology

Dissected organs were partly or entirely fixed in 4% formalin for 24 h (except femur), subsequently placed in 70% alcohol and routinely embedded in paraffin, sectioned and stained with hematoxylin and eosin. After routine histopathological reading of the sections, the adipocyte size in perirenal white adipose tissue (WAT) was measured as the average cell diameter of 9–20 cells touched by a standard grid line on a representative area of the section. A proxy for the number of adipocytes in that fat pad was then calculated by dividing its weight (*W*) by the average cell volume (*V*) as derived from the measured cell diameter (*D*), using the formula $W/V = W/([D/2c]^3 \times 4\pi/3)$, where c is a correction factor of 0.79 to estimate the real mean cell diameter from the measured average cell diameter along a random cross-sectional line. Lipid accumulation in brown adipose tissue (BAT) adipocytes was scored semi-quantitatively in the interscapular fat depot (see Table 2).

Serum chemistry

For the analysis of BPA levels in sera from dams and pups at the time of weaning (closely around PND21), serum samples were pooled from 3 to 4 dams and 6 to 7 pups per dose group, with the exception of pups from the highest exposure group (3000 µg/kg bw/d), which were analyzed individually. Total BPA (free + conjugated) was analyzed according to a previously described method (Geens et al., 2009). Briefly, after deconjugation with β-glucuronidase/sulphatase followed by solid phase extraction and derivatization with pentafluorobenzoylchloride, total BPA was quantified by isotope dilution gas chromatography with mass spectrometric detection. BPA-d_{16} was used as an internal standard. Recoveries of bovine serum samples of 1 ml spiked with 6.6 ng/ml BPA ranged from 86 to 123%. The LOD (1.1–1.9 ng/ml) was defined as three times the standard deviation of the blanks.

Serum lipids were analyzed on a Beckman Coulter LX20 Clinical Chemistry Analyzer, using Beckman reagent kits for total cholesterol (CHOL), triglycerides (TGs), and high-density lipoproteins cholesterol (HDL-C) (Beckman Coulter B.V., Woerden, The Netherlands), and a Wako reagent kit for free fatty acids (FFAs) (Wako Chemicals GmbH, Neuss, Germany).

Milliplex kits (Millipore Corporation, Billerica, MA, USA) were used according to the manufacturer's protocol to measure serum adiponectin, ghrelin, glucagon, insulin, leptin and pancreatic peptide YY-36 (PYY-36).

Gene expression analysis

Total RNA was extracted from the tissue of interest using the RNeasy Lipid Tissue Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentrations and qualities were determined using a NanoDrop Spectrophotometer (Isogen Life Science B.V., De Meern, The Netherlands) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), respectively. cDNA was produced with the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. TaqMan Gene Expression Assays (*Ucp1*: Mm01244861_m1; *Cidea*:Mm00432554_m1) were performed with 10 ng cDNA and TaqMan Fast Universal PCR Master mix (Life Technologies, Carlsbad, CA, USA) in 10 µl total volumes using the 7500 Fast Real-Time PCR System, according to manufacturer's instructions. Relative quantification was performed by the comparative CT method (ddCt) in Microsoft Excel. *Ucp1* is a marker of energy expenditure through thermogenesis, and contributes to regulation of body weight (Kozak et al., 2010). *Cidea* is a marker of BAT adipocytes (Zhou et al., 2003), and was used as a normalizer for the contents of BAT adipocytes in the tissue extracts.

Statistical analyses

Data covering the entire study population were analyzed for statistically significant dose-responses using the benchmark dose (BMD) approach (Slob, 2002) with the PROAST software versions 36.x–37.x (www.rivm.nl/proast). In this approach, optimal models from the exponential and Hill families are fitted to the data, and a BMD with its 5% lower and upper bounds of the 90% confidence interval (BMDL, BMDU) is derived from the fitted models at a predefined benchmark response (critical effect size, CES). By default, the CES used in this study was 5% for continuous data, as proposed by the European Food Safety Authority (EFSA, 2009). The goodness-of-fit was determined by the log-likelihood of each model within a family of models. The optimal model selected for each family was the model with the lowest number of parameters which gave the best significant fit. Clustered analysis of individual animals from the same litter was applied. In the evaluation of results, data which did not produce a statistically significant dose-response with both exponential and Hill models, were not deemed sufficiently informative for robust conclusions. Furthermore, data that produced dose-responses with a wide confidence interval (BMDU/BMDL ratio >100) were not considered suitable to derive a valid BMD.

Some measures included only control and top dose animals, and could therefore not be analyzed as dose-responses. Thus GTT was evaluated by repeated-measures or nested (to account for litter covariance) two-way ANOVAs (Graphpad Prism 5.0, R) to detect differences at the different time points, and between the areas under the curve. *Ucp1* expression, WAT adipocyte size and the proxy for cell number were also tested with a nested ANOVA (R). A Student's *t*-test was used to compare exposed and controls for the activity measurement, and differences in distribution of BAT histopathology scores between experimental groups were tested for statistically significance in a two-tailed Fisher's exact test. Association between some parameters was analyzed by linear regression analysis and results expressed as a Pearson correlation coefficient (*r*).

Results

Exposure assessment

Actual BPA levels in 3 highest doses of feed were 1.8, 5.3, and 14 mg/kg, which corresponded well with the nominal levels of 1.7, 5.6, and 16.7 mg BPA/kg feed. BPA concentrations in the feed spiked at lower levels were below the limit of detection (LOD). No BPA was detected in the control feed.

Internal total BPA levels in pooled serum of dams of the four highest dose groups at the time of weaning were 2.2, 3.4, 12, and 50 ng/mL, respectively. Serum total BPA levels in dams of the lowest three dose groups and the control group did not exceed the LOD of 1.2–1.9 ng/mL.

Total BPA was measured in individual serum samples of pups at weaning from the 3000 µg/kg bw/d group. Due to the extremely small sample volumes available, typically 25–100 µL, serum samples from the 300 µg/kg bw/d group were pooled. These analyses indicated internal doses in pups ranging from 83 to 240 ng/ml serum in the highest dose group, and 24–26 ng/ml in the 300 µg/kg bw/d group.

General toxicity and reproduction parameters

In dams, dietary exposure to BPA had no effect on measurements of general toxicity, notably not on mortality, body weight, weight gain and food consumption. Parental behavior was also normal.

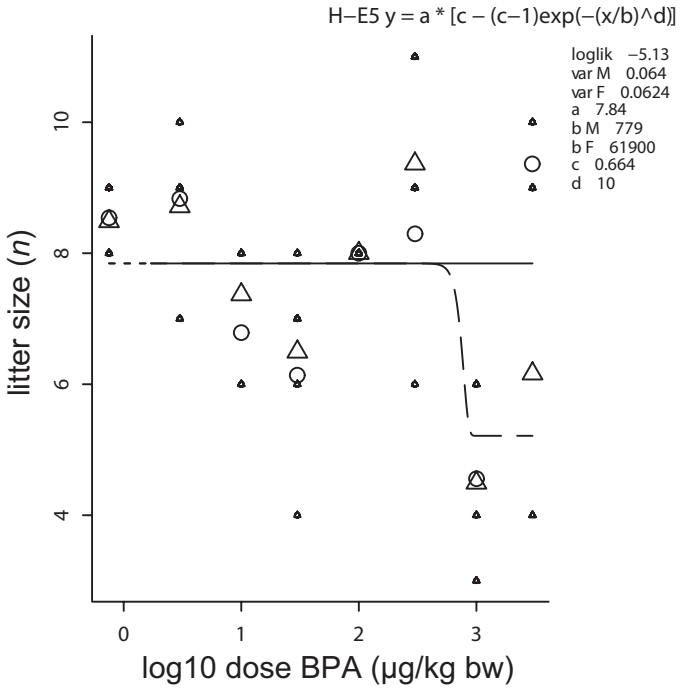

Figure 1. Distribution of litter sizes after postnatal sampling. In males (*triangles*, *dotted line*), litters from which individuals could be sampled for follow-up showed significant skewing toward larger sizes at higher doses. In females (*circles*, *solid line*), distribution of litter sizes from which individuals were sampled was evenly over doses. Explanation of the dose-response graph is in Figure 2 legend.

Average mating success rate was 84%, yielding 26 litters with an average litter size of 7.5 (range 3–11). The overall F/M sex ratio in the F1 generation was 0.9 and the overall survival rate was 96%. None of these reproduction parameters showed an effect of BPA. Still, at high doses, male pups for follow-up after weaning were mainly available from small litters, and small litters were therefore overrepresented at these high doses (Figure 1). For females, litter sizes were evenly distributed over doses.

Body weights

From week 6 (w6) onwards, males showed a persistent dose-dependent increase of body weight (Figure 2A, 21 weeks of age, final full data set), although with a wide confidence interval, arising from highly variable weights within dose groups. Growth analyzed as the ratio of body weights over the trajectory between onset of body weight effect and end of the study (w21/w6) did not differ, resulting in similar weight gain during that period across dose groups (Figure 2B), indicating that body

weight differences that were present shortly after weaning were not progressive over time. There were no effects of BPA on metrics of body size (body length, femur length, femur weight; Table 1), and when body weight at the end of the study was expressed relative to femur length as a robust measure of body size, there was also no effect, supporting that body weight moved in parallel with body size.

Because of overrepresentation of small litters in the highest dose groups in males available for follow-up (Figure 1), the effect of litter size on various endpoints in males was also analyzed. Thus, a litter size dependent effect on body weight (weeks 3–23), liver weight and body length (end of study) was found, all statistically significant in both exponential and Hill models. These effects were more defined by large litters (n = 10–11) than by small litters, as shown for example with body weight at weaning in Figure 2C.

In contrast to males, females showed a dose-dependent decrease of body weight from week 8 onwards (shown at termination of the standard diet regime, 17 weeks of age in Figure 2D). The dose-related lower body weight remained after the shift to high fat diet at 17 weeks, which was introduced to test a BPA related changed sensitivity for such a diet. Growth rates expressed as ratio body weight w17/w8 (start of high fat diet/onset of body weight effect) showed a dose-dependent decrease (Figure 2E) and the BMDL are 11.2 μg/kg/d, although the BMDU/BMDL ratio of 172 did not fully met the acceptance criterion (Table 1). Zooming in on the w17/w8 period the decrease in growth occurred at the early phase during w13/w8 and was no longer apparent during w17/w13 and neither under high fat diet (ratio w21/w18; data not shown). As in males, there were no effects on metrics of body size (body length, femur length, femur weight; Table 1), but there was a dose-dependent decrease of body weight relative to femur length (Figure 2F), supporting that the reduced body weight in BPA exposed females was due to reduced body mass rather than to reduced body size.

Metabolic homeostasis

Results for glucose tolerance, physical activity and *Ucp1* expression are given in Table 2. In the GTT, both area under the curve and baseline glucose levels between control and exposed males did not statistically differ. In terms of energy expenditure, the expended energy index derived from measured physical activity was statistically significantly lower in top dose males compared to control males. Expression of *Ucp1* in BAT, as a measure of expended energy in thermoregulation, showed no effect in males.

There was no effect of perinatal BPA on glucose clearance between top dose females and control females. A modest increase of physical activity was suggested in exposed females and although this was a robust 108 h cumulative measurement, it could not be statistically tested because of availability of only a single control unit. Dose-response analysis of *Ucp1* expression in BAT, which was done after a suggested effect in comparing top dose with control females, showed a significant dose-dependent increase (Figure 3).

Figure 2. Dose-responses of body weight, growth, and related parameters. Analyses in males and females are in the top row (A-C) and bottom row (D-F), respectively. (A, D) Dose-responses of body weight, in males at 21 w of age (end of study), in females at 17 w of age, that is at 18 and 14 weeks after cessation of BPA exposure respectively, and in females before the onset of high fat diet. (B, E) Analysis of growth with no dose-related effect in males from weeks 6–21, expressed as the ratio of body weight w21/w6, and with a significant dose-dependent decrease of growth in females between weeks 8–17 (expressed as ratio w17/w8). (C) Significant litter size dependent decrease of body weight at weaning (3 w) in males. (F) Significant dose-dependent decrease of body weight relative to femur length in females at 23 w (end of the study). The function of the curves is shown in the top line in the upper right corner of each graph, followed by parameters of significance and shape of the curve. CES, critical effect size. CED, CEDLbt, CEDUbt are the critical effect dose with its lower and upper bound of the 90% confidence interval; which are in the text indicated as BMD, BMDL and BMDU. Small symbols: individuals, large symbols: geometric mean (per dose). The analysis was done with PROAST version 37.9.

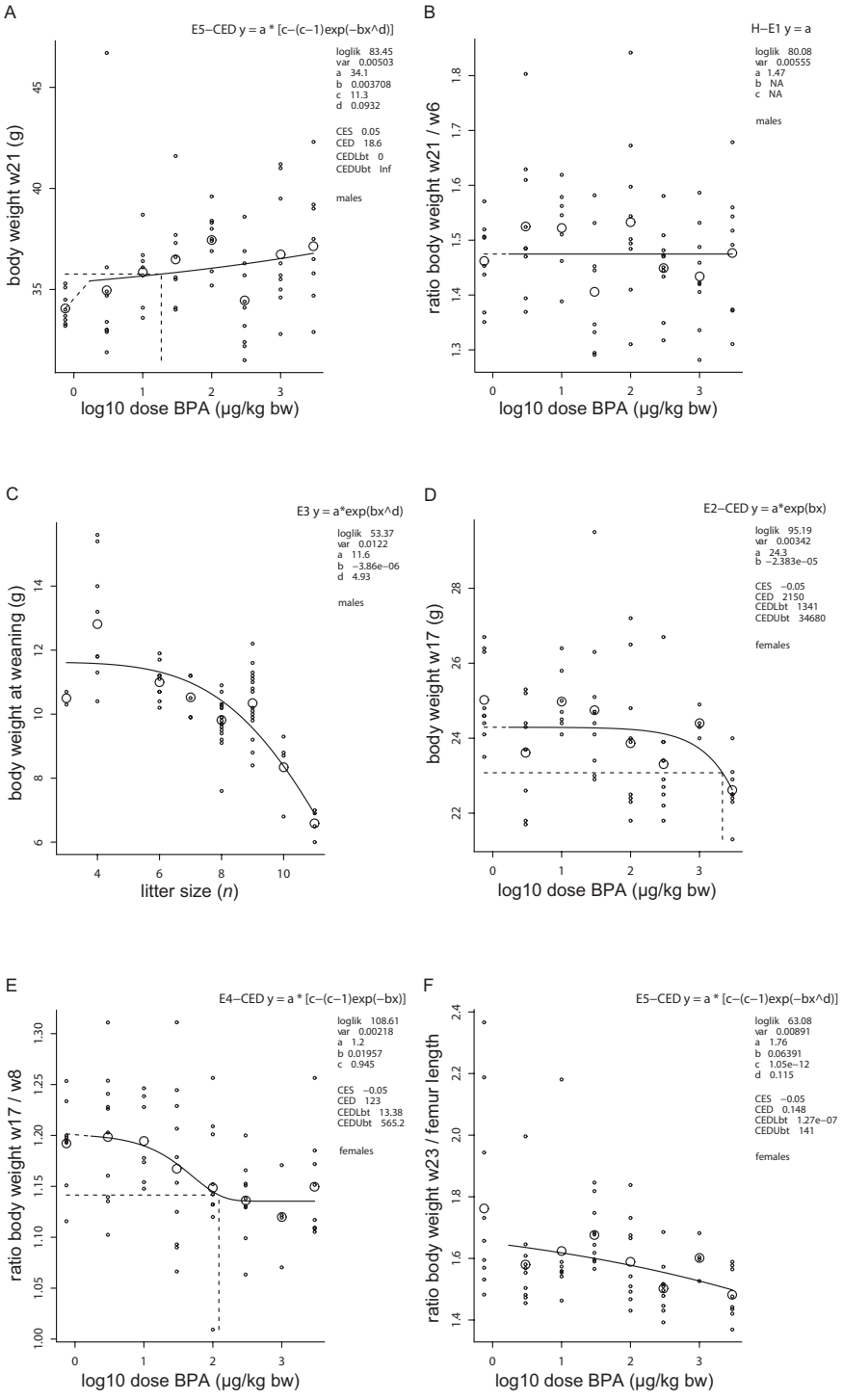
A
E5–CED y = a * [c–(c–1)exp(–bx^d)]
loglik 83.45
var 0.00503
a 34.1
b 0.003708
c 11.3
d 0.0932
CES 0.05
CED 18.6
CEDLbt 0
CEDUbt Inf
males
body weight w21 (g)
log10 dose BPA (µg/kg bw)
B
H–E1 y = a
loglik 80.08
var 0.00555
a 1.47
b NA
c NA
males
ratio body weight w21 / w6
log10 dose BPA (µg/kg bw)
C
E3 y = a*exp(bx^d)
loglik 53.37
var 0.0122
a 11.6
b −3.86e−06
d 4.93
males
body weight at weaning (g)
litter size (n)
D
E2–CED y = a*exp(bx)
loglik 95.19
var 0.00342
a 24.3
b −2.383e−05
CES −0.05
CED 2150
CEDLbt 1341
CEDUbt 34680
females
body weight w17 (g)
log10 dose BPA (µg/kg bw)
E
E4–CED y = a * [c–(c–1)exp(–bx)]
loglik 108.61
var 0.00218
a 1.2
b 0.01957
c 0.945
CES −0.05
CED 123
CEDLbt 13.38
CEDUbt 565.2
females
ratio body weight w17 / w8
log10 dose BPA (µg/kg bw)
F
E5–CED y = a * [c–(c–1)exp(–bx^d)]
loglik 63.08
var 0.00891
a 1.76
b 0.06391
c 1.05e−12
d 0.115
CES −0.05
CED 0.148
CEDLbt 1.27e−07
CEDUbt 141
females
ratio body weight w23 / femur length
log10 dose BPA (µg/kg bw)

Table 1. Dose-response and correlations with body weight at necropsy for organ and body metrics and serum parameters

	Males				Females			
	Dose-response	BMDL (µg/kg bw/d)	Max effect size (%)	Relative to body weight	Dose-response	BMDL (µg/kg bw/d)	Max effect size (%)	Relative to body weight
Body weight Week 21	↑	ni	8.0		↓	781	–11	
Body size								
Body length	–				–			
Femur length	–				–			
Body weight / femur length	–				↓	ni	–15	
Growth								
Week 21/6	–							
Week 17/8					↓	ni [2]	–5.5	
Week 21/18					–			
Organ weights								
Brain	–				–			
Femur	–				–			
Liver	↑	1.7	16	–	↓	583	–16	–
Quadriceps femoris muscle	–				↓	649	–12	–
Fat pad weights								
Interscapular	–				↓	233	–26	–
Mesenterial	– / ↑	ni			–			
Perigonadal	–				↓	ni	–55	–
Perirenal	–				↓	ni	–63	↓
Subcutaneous mammary - caudal	–				↓	ni	–34	↓
Subcutaneous mammary - rostral	–				–			
Sum fat pads	–				↓	ni	–47	–
White adipocyte size	–				↓	11.7	–17	
Ucp1 expression					↑	ni	31	
Serum lipids								
Cholesterol	–				–			
Free fatty acids	–				↓	ni	–59	
High-density lipoproteins	–				–			

Table 1. Continued

	Males				Females			
	Dose-response	BMDL (µg/kg bw/d)	Max effect size (%)	Relative to body weight	Dose-response	BMDL (µg/kg bw/d)	Max effect size (%)	Relative to body weight
Triglycerides	–				↓	ni	–66	
Serum hormones								
Adiponectin	–				↓	ni	–29	
Glucagon	↓	ni [1]	–54		–			
Insulin	–				–			
Leptin	–				↓	ni	–77	

↑,↓,- statistically significant increase, decrease dose-responses, or absence of effect. A single sign or value is given when exponential (E) and Hill (H) modeling outcomes are the same; different outcomes with E and H are indicated with the / separator. A BMDL (lowest 5% lower confidence bound of the BMD at a critical effect size of 5%) is only given in case of a small confidence interval (BMDU/BMDL ratios < 100); BMDL data with a wider confidence interval are not considered informative (ni) for risk assessment. A maximum (max) effect size is derived from the c-parameter if present in the selected dose-response models, otherwise calculated as a difference between top dose and control (background) values and the reported value is an average of E and H max effect sizes. Organ and fat pad weights showing a statistically significant dose-response are also analyzed relative to body weight, to detect interdependency of these parameters. µg/kg bw/d = µg BPA/kg body weight/day

[1] Five out of 68 glucagon values in males are below detection limit of the assay and replaced with 0.9 x lowest detected value. The BMDL for glucagon is 0.26 µg/kg bw/d, although the BMDU/BMDL ratio for glucagon is 270, thus just beyond the arbitrary validation value of 100.

[2] The BMDL for growth w17/w8 in females is 11.2 µg/kg bw/d, although BMDU/BMDL ratio is 172, thus just beyond the arbitrary validation value of 100.

Table 2. Metabolic parameters tested in control and top dose animals

	Males	Females
Glucose tolerance test	–	–
Physical activity	↓	↑ *
Ucp1 expression (thermoregulation)	–	↑ #

Statistical significance was tested with a nested ANOVA for the glucose tolerance test, and *Ucp1* expression and a Student's *t*-test was used for physical activity in males.

* This effect was only observed as a trend since statistics could not be performed due to one available control unit.

After a statistical difference was observed in control and top dose females, all female samples were tested and a dose-response was observed, see Figure. 3.

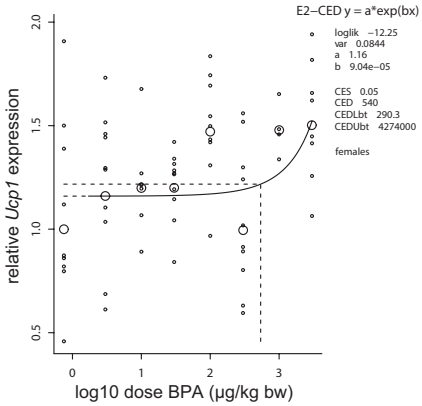

Figure 3. Thermoregulation in brown adipose tissue in females. Females showed a significant dose-dependent increase of *Ucp1* expression in brown adipose tissue, relative to expression of *Cidea* as correction for non-brown adipocyte cells in the analyzed tissue fragment. *Ucp1* is a marker of thermoregulatory energy expenditure in the animal. Explanation of the dose-response graph is in Figure 2 legend.

Organ and fat pad metrics

In males, among all measured organs during necropsy, only liver weight showed a dose-dependent effect (Figure 4A) and the BMDL is 1.7 µg/kg bw/d (Table 1). This effect on liver weight was not independent of body weight, because it did not persist when expressed relative to body weight. Particularly the absence of effects in any metrics of adiposity (weight of fat pads, adipocyte size, see below) did not support increased fat mass as a background for the suggested increased body weight. The observation that in some parameters only Hill but not exponential models could provide a statistically significant dose-response indicates that these data did not contain sufficient information to be conclusive.

In females, there was a dose-dependent decrease of liver weight (Figure 4B), muscle weight (quadriceps femoris muscle), and various fat pads (interscapular, perigonadal, perirenal, caudal subcutaneous), as well as sum fat pads. BMDLs of all parameters, except for white adipocyte size (11.7 µg/kg/d), are in a close range of 233–781 µg/kg bw/d, with 233 µg/kg bw/d for decreased interscapular weight as the lowest BMDL. Again, the effect in liver weight, and in this case also muscle weight and weight of some fat pads, was not independent of body weight, in view of absence of effect when these parameters were analyzed as measures relative to body weight (Table 1). The decrease of perirenal and caudal subcutaneous fat pads remained intact after correction for body weight, suggesting that these particular fat pads had a relative higher decrease compared to body weight. Body length and femur length did not show an effect of BPA exposure.

Details of body metrics are given in Supplementary Table 1.

Histopathology

In males, perirenal WAT adipocyte size tested significantly (p = 0.0397) between all exposed (61.9 ± 10.9 µm, n = 59) and controls (51.6 ± 7.6 µm, n = 7), but this data set did not show a statistically significant dose-response (Table 1; Supplementary Table 1). Semi-quantitative scoring of interscapular BAT revealed a mild trend (p = 0.0769) for a higher distribution of hypertrophied BAT cells in top dose males compared to control males (Table 3).

In females, the size of perirenal WAT adipocytes showed a statistically significant dose-dependent decrease (Table 1) indicating hypotrophy. The interscapular BAT cells in top dose females also revealed hypotrophy when compared to control females (Table 3). The proxy for WAT adipocyte number in the perirenal fat pad showed no dose-response in either sex and no difference between control and exposed (0.35 ± 0.09 µg/µm^3, n = 7 and 0.36 ± 0.19 µg/µm^3, n = 59 in males; 0.34 ± 0.22 µg/µm^3, n = 8 and 0.29 ± 0.18 µg/µm^3, n = 55 in females). Size differences in WAT and BAT are illustrated in Figure 5.

Histopathological examination of the liver, quadriceps femoris muscle, thyroid gland, adrenals and pancreatic islets of males did not reveal any effects, including lipid accumulation.

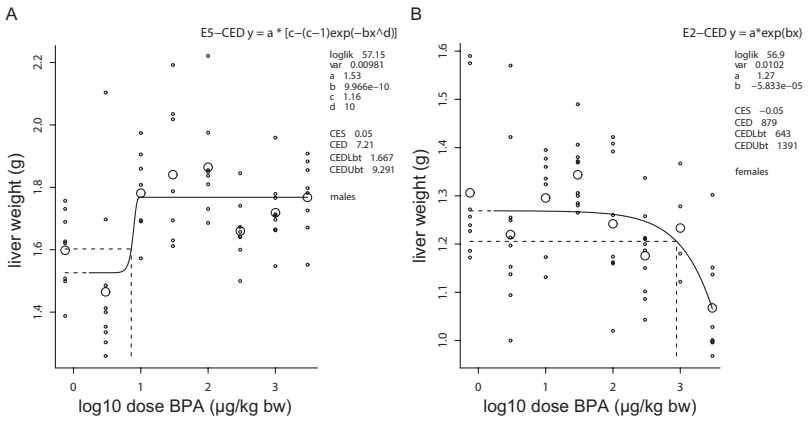

Figure 4. Liver weight. Significant dose-dependent increase of liver weight in males (A), and decrease in females (B). Explanation of the dose-response graphs is in Figure 2 legend.

Table 3. Histopathology scores of BAT for control and top dose animals

	Males		Females		
	score 1	score 2	score 0	score 1	score 2
Control	8	0	0	2	7
Top dose	4	4	4	2	2 *

Scores were defined through a first blinded screening of sections and represent no (score 0), moderate (score 1), or strong (score 2) lipid accumulation. In this distribution table, numbers are counts of perinatally BPA exposed individuals with a given score.
* The distribution in the top dose groups in females is statistically significant ($p < 0.05$) in a two-tailed Fisher's exact test (using combined scores for females).

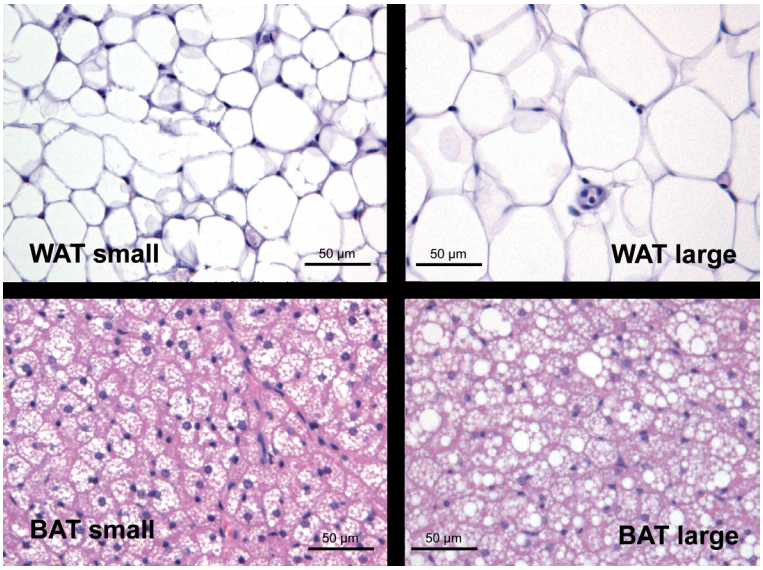

Figure 5. Photomicrographs of adipose tissue in males. These photomicrographs illustrate differences of adipocyte size in WAT *(top panels)* and lipid accumulation in BAT *(bottom). (left)* Control male, *(right)*, top dose male (3000 µg/kg bw/d). For magnification, see scale bars.

Serum chemistry

In males, there were no effects of perinatal BPA on serum lipid parameters (Table 1, Supplementary Table 1). Endocrine profiling in males showed a dose-dependent decrease of circulating glucagon (Figure 6) with a BMDL of 0.26 µg/kg bw/d, although the BMDU/BMDL ratio of 270 did not fully meet the acceptance criterion (Table 1). Litter size did not affect glucagon. No effects were seen in adiponectin, insulin and leptin, while ghrelin and PYY-36 were below the detection limit of the assay.

In females, there was a dose-dependent decrease in the serum free fatty acids and triglycerides, as well as in the hormones adiponectin and leptin. Insulin and glucagon were not affected (Table 1).

Because leptin is known to be proportional to total body fat mass, correlations between this parameter and sum weight of all fat pads were also calculated, and showed high values (r = 0.85 and 0.90 for males and females, respectively). Correlation coefficients between leptin and body weight were in the same range (r = 0.70 and 0.79 for males and females, respectively).

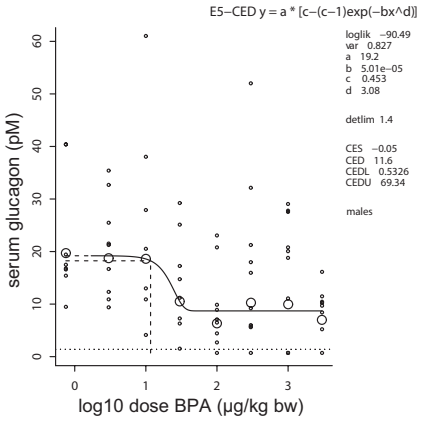

Figure 6. Dose-response of serum glucagon in males. Serum glucagon as measured after necropsy at w 23. Explanation of the dose-response graph is in Figure 2 legend. detlim = detection limit; five out of 68 glucagon values are below detection limit of the assay and replaced with 0.9 x lowest detected value.

Discussion

In this study, we investigated the hypothesis that exposure to BPA early in life can program an organism for higher susceptibility to develop obesity and related metabolic impairment later in life. The study was initiated to provide further clarity to the contradictory evidence in literature of the obesogenic effects of BPA, and designed to mimic the human situation by applying continuous oral maternal exposure during gestation and lactation. The applied doses of BPA were below the BMDL of systemic effects in adults and offspring in a reproductive study in mice (Tyl et al., 2008), and the low doses approximated human oral exposure. The concentration of BPA was confirmed in selected samples of the feed and in serum of dams and pups at the time of weaning, though only higher doses exceeded the limit of detection. Although internal concentrations during gestation are missing, fetal exposure is probable because we confirmed maternal uptake from the feed, and placental transfer is known to occur, including deglucuronidation (and thus reactivation) of glucuronidated BPA in the placenta (Ginsberg and Rice, 2009; Nishikawa et al., 2010). The metabolic phenotype of offspring appeared to be affected after perinatal BPA exposure, although differently between sexes.

Body, organ and fat pad metrics, metabolic homeostasis

In males, a dose-dependent increase in body weight was observed, that was already present closely after weaning (6 weeks of age) and persisted during adulthood. When considering confounding variables, litter size is known to affect body weight (Epstein, 1978). This was confirmed in the total F1 study population, which showed a negative effect of litter size on body weight, detectable closely after weaning, and persisting until the end of the study, in both sexes. Because small litters were overrepresented at the highest doses in the male, but not female, population, it cannot be excluded that litter size is a determining factor for the increased body and liver weight in males. The absence of an effect in fat pads and in body weight relative to femur length suggests the increased body weight in males is not solely due to an increased fat mass, but an increased overall body size also plays a role. Impaired energy balance of BPA exposed males was suggested by the dose-dependent decrease of circulating glucagon, which could be explained as a compensatory mechanism to balance blood glucose levels. While litter size could be an alternative explanation for the effect on body weight and related parameters in males, data did not indicate this for the decreased glucagon (not shown). In contrast, in rats, glucagon has been shown to be negatively related

to litter size (Noack et al., 1982). On the side of energy expenditure, the decreased total cage activity in top dose BPA exposed males compared to controls could be interpreted as a cause of increased body weight, where as absence of effect in expression of thermoregulatory *Ucp1* in BAT does not contribute to an explanation of increased body weight. Food consumption recordings were not reliable, and effects on this key parameter for energy balance could therefore not be established (same in females).

The effect of BPA on body and liver weight was sex-dependent since adult females showed a decrease in body weight, emerging at 8 weeks of age and onwards, and in liver weight. Dose-responses of relative liver weight in both males and females were not apparent, suggesting that the effects on liver weight were not independent of effects on body weight. The same was true for muscle weight and weight of some fat pads in females. However, weight decreases of perirenal and caudal subcutaneous fat pads in females remained statistically significant even when expressed relative to body weight. This suggests that these fat pads had a relatively high contribution to the overall decrease of weight of these female animals, which is in line with the dose-dependent decrease of body weight relative to femur length, supporting decreased body mass rather than decreased body size underlays the decreased body weight.

Energy balance and metabolic homeostasis were affected differently in females, although comparison between males and females is not fully justified in view of the application of a high fat diet only in females. Females in this study received a high fat diet in the final four weeks to test whether BPA exposure changed the sensitivity to develop an overweight phenotype under high energy intake, which was not the case. Also, a BPA induced resistance to a high fat diet in female mice, as reported by Ryan et al. (2010b), was not confirmed. Explanatory for the decrease in body weight, females showed a BPA induced increase of energy expenditure, as increased physical activity (trend), and as an increase in *Ucp1* expression, indicating higher energy expenditure in thermoregulation. The observed dose-dependent decrease of leptin is in line with the decrease in fat mass, because the concentration of circulating leptin is known to reflect the total body fat mass (Frederich et al., 1995). This was confirmed by the high correlation coefficients between leptin on the one hand and either sum fat pad weight or body weight on the other hand. The BPA dose-related decreases of free fatty acids and triglycerides in females could be in line with low fat mass, assuming that the complex multi-hormonal regulation of these serum lipids, also involving insulin, glucagon, leptin and adiponectin, are directed at balancing adipocyte and serum lipid contents.

Programming

The observed effects of BPA exposure during early life were expressed soon after termination of the actual exposure, and persisted thereafter. This suggests that BPA initiated permanent functional changes, affecting energy homeostasis, through the exposure during early development. Such permanent functional changes, which persist or appear after removal of the initiating agent, can be understood as programming. A possible explanation for programming is permanent epigenetic modifications such as changes in DNA methylation (Jirtle and Skinner, 2007). Epigenetic programming is presumed to lead to numerous developmental, metabolic, and behavioral disorders (Bernal and Jirtle, 2010) and therefore, could be the explanation for the observed altered metabolic phenotype in females. Although epigenetics is a speculative explanation in the present study, the potential of BPA to modify the epigenome has been shown for *in vitro* models (Bastos Sales et al., 2013) and was shown elegantly in the Agouti mouse model (Dolinoy et al., 2007).

Sexual dimorphism

The sexual dimorphism of body weight and other effects suggests that BPA interfered in a sex-dependent way. This may relate to a differential sensitivity between sexes for the estrogenic activity of BPA, e.g. related to gender specific expression patterns of estrogen receptors (Wilson et al., 2011). Alternatively, gender differences not specifically related to steroid hormone pathways may play a role, including sex-dependent metabolism (Mugford and Kedderis, 1998), which is even known to vary widely among strains of mice and also among species, and may at least in part explain the variability of sex-dependency (as well as other variations) of BPA effects among studies (see below).

Is BPA an obesogen?

As discussed above, BPA affected body weight in animals in this study, but the effects contrasted between sexes. Moreover, the body weight effect in males may be mainly determined by a changed overall body size, whereas a change in fat mass is more likely to underlay the body weight effect in females. As such, these observations are in line with the variation in effects reported by other *in vivo* studies. Even when only considering studies with similar early life low dose exposure as in this study, the variation in outcomes is noticeable (Table 4). Table 4, which includes results of the present study, shows that increased body weight is reported mostly, and that predominance of increased body weight is somewhat more obvious in males (10

studies with upregulated body weight versus 5 no effect 1 down and 2 with variable results in males, and 6 up versus 4 no effect, 5 down in females). Seven studies report equal responses between sexes (Howdeshell et al., 2008; Ryan et al., 2010a), and 4 report different responses between sexes. One study reports opposite effects in one sex, males, depending on the window of exposure (Liu et al., 2013). The variation of experimental conditions in terms of animal species and strains, exposure dose, route and window, contents of background diet (e.g. phytoestrogens and methyl donors), microbiome and litter or individual animal as the statistical unit is too diverse to derive a pattern of conditions and outcomes. This is also true for studies with an outcome comparable to the present study, that is decreased body weight in females and a different outcome in males (Alonso-Magdalena et al., 2010; Anderson et al., 2013), where the use of mice is the only unifying factor. It thus appears that, without considering chance findings within limits of normality, effects of BPA on body weight are not at all robust and reproducible. The best explanations are that either BPA interacts differently in the organism among studies, or that similar early events lead to a different downstream phenotype, depending on other experimental conditions.

This reasoning is further supported by the limited magnitude (below 10%) of effects of BPA on body weight observed in this study, and the wide confidence interval of the body weight effects, indicating a high variation of individual responses. These limitations do not suggest that BPA primarily and strongly affects body weight, but rather affects underlying metabolic processes, which eventually, and depending on confounding factors (such as sex), may or may not be expressed in a change of body weight. The descriptive design of the present study does not allow deducing a single effect as primary in the complex of observations, should such a key effect exist. In any case, based on the sexual dimorphism of effects and diversity of outcomes between studies, BPA cannot be marked as a specific obesogen.

Implications for risk evaluation

When extrapolating results from mice to humans the difference in toxicokinetics should be considered since BPA demonstrates a higher bioavailability due to enterohepatic recirculation in rodents (Doerge et al., 2010). We modeled human exposure as close as possible, in contrast to many studies that used experimental conditions which are not relevant for the human situation (i.e. short exposure window, high doses of BPA, non-enteric exposure or oral peak exposure). The range of continuous oral low dose exposures that we applied was below the BMDL of 3633 μg/kg bw/d for systemic

effects in adults and offspring in a reproduction study in mice (Tyl et al., 2008) and down to a level that is approaching highest estimated human exposure. This approach should facilitate the evaluation of the risk associated with BPA exposure, for which only robustly affected parameters are suitable, i.e. with a low variation (BMDU/BMDL ratio < 100; Table 1). The only parameter in males that fully met this criterion is increased liver weight, which however is invalidated by a possible confounding by litter size. Therefore, only effects in females remain as a robust effect, with a cluster of effects for various weight related parameters showing BMDLs in the close range of 233–781 μg/kg bw/d, with decreased weight of interscapular fat determining the lowest BMDL. The informative low BMDL for white adipocyte size should only be considered as an alert because it deviates largely from the weight related parameters. The BMDL of 233 μg/kg bw/d for interscapular fat pad weight is a factor 16 below the BMDL referred above, but above the highest estimated human exposure level of up to 1.5 μg/kg bw/d (EFSA, 2014) even when considering safety factors for interspecies and interindividual differences.

In conclusion, findings of the present study confirmed a phenotype of metabolic effects in offspring with persistence into adulthood after termination of BPA exposure at weaning, with an associated body weight increase in males and body weight decrease in females. Based on the sexual dimorphism of effects, the probability that the increased body weight in males is not due to increased fat mass, and the diversity of outcomes among published studies, BPA cannot be marked as a specific obesogen. Altogether, this indicates that if programming is accepted as a mechanism underlying the temporal distance between exposure and effect, this occurs without apparent linearity of cause and effect, i.e. circumstantial cofactors very much determine the apical adult outcome of the exposure early in life. Results of this study suggest that as of yet unidentified upstream key elements in energy homeostasis are affected, and sex-dependent factors contribute to the final phenotypic outcome. None of the observed changes can be marked as adverse in itself, but they can be considered as marks of undesirable effects on metabolic regulation. BMDLs associated with the effects cannot support reliably that BPA is active at the low levels relevant for human exposure.

Table 4. Body weight effects of early life exposure to BPA in rodent studies

Strain species	Dose (µg/kg bw/d unless stated otherwise)	Diet	Exposure route	Exposure window	Endpoint bw (age)		Time of effect [1]	Other relevant effects of BPA	Reference
					M	F			
CF-1 mouse	2–20	RM1 maintenance diet (Special Diet Services; 6.5% soy)	Oral, micro-pipettor	GD11–17	↑ PND185	– PND23–310	E		Ashby et al. 1999
CF-1 mouse	0.2–2–20–200	Certified Rodent Chow #5002 (PMI feeds)	Oral, micro-pipettor	GD11–17	↑ PND36–90 [2]	nd	T		Cagen et al. 1999
CF-1 mouse	2.4	not stated	Oral gavage	GD11–17	↑ PND22	↑ PND22	E	F: advanced vaginal opening and first vaginal estrus	Howdeshell and vom Saal 2000
SD rat	1–10 mg/L water (equals 100; 1200 µg/kg bw/d)	Purina Rodent Chow	Drinking water	GD6–PND21	↑ PND4–110	↑ [3] PND4–110	T	F: altered patterns of estrous cyclicity	Rubin et al. 2001
ICR/Jcl mouse	2–20	CE-2 (CLEA)	s.c.	GD11–17	↓ PND0–60	↓ PND0–60	D [4] T	F: advanced vaginal opening and first vaginal estrus	Honma et al. 2002
C57BL/6N mouse	2–20–200	PLD (Oriental Japan; phytoestrogen-low)	Oral gavage	GD11–17	– w12	nd	–		Nagao et al. 2002
LE rat	2.4	Purina Rodent Chow	Oral gavage	GD12–PND21	↑ PND90	nd	E	↓ Reproductive tissue weights	Akingbemi et al. 2004
CD-1 (ICR) mouse	500–10,000	NIH-07 PLD (oriental Yeast; low-phytoestrogen)	s.c.	GD15–18	nd	↑ w16	E	Altered patterns of estrous cyclicity; lack of corpora lutea	Nikaido et al. 2004
ICR mouse	1–10 µg/mL water (equals 260–2700 µg/kg bw/d)	HFD	Drinking water	GD10–PND31	↑ PND30	↑ PND30	E	M+F: ↑ adipose tissue weight	Miyawaki et al. 2007
CD-1 (ICR) mouse	10–100–1000	NIH-31 (46 µg/g genistein)	s.c.	PND1–5	nd	– m18	–	Pathology of the reproductive tissues	Newbold et al. 2007

Table 4. Continued

Strain species	Dose (µg/kg bw/d unless stated otherwise)	Diet	Exposure route	Exposure window	Endpoint bw (age)		Time of effect [1]	Other relevant effects of BPA	Reference
					M	F			
LE rat	2–20–200	Purina Rat Chow 5008, perinatally; Purina Rat Chow 5001, post-weaning	Oral gavage	GD7–PND18	– PND150 nd	nd – PND23	– –		Howdeshell et al. 2008 Ryan et al. 2010a
LE rat	50	AIN-93G; Purina 5K96, PND58 onwards (phytoestrogen free)	s.c.	PND0–3	↑ PND68	nd	E	Anxiety behavior affected	Patisaul and Bateman 2008
SD rat	1 mg/L water (equals 70 µg/kg bw/d)	KLIBA NAFAG 3250 low phytoestrogen, perinatally; standard chow diet or highfat diet hfd, after weaning	Drinking water	GD6–PND21	↑ w9–14; only HFD	↑ PND1–w14; both diets	T	Adipose tissue weight F	Somm et al. 2009
OF-1 mouse	10–100	2014 Teklad Global 14% Protein Rodent Maintenance Diet, no alfalfa or soy	s.c.	GD9–16	– PND22–180	↓ [3] PND22–180	P	Glucose homeostasis affected (only males, only low dose)	Alonso-Magdalena et al. 2010
CD-1 mouse	1 µg/kg diet (equals 0.25 µg/kg bw/d)	AIN93G, perinatally; low fat diet, w3–9; HFD, 9w onwards	Diet	GD0–PND21	– w14	– w14	–	F: hypophagia, ↓ body fat	Ryan et al. 2010b
Wistar rat	50–250–1250	Normal, perinatally; normal or HFD, PND21 onwards	Oral gavage	GD0–PND21	↑ [3] w3–26	↑ [3] w3–26	D [5]	M+F: glucose homeostasis affected; ↑ body fat percentage; ↑ adipocyte size	Wei et al. 2011
Agouti *a/a* mouse	0.05–50–50.000 µg/kg diet (equals 0.01–10–10.000 µg/kg bw/d [6])	Phytoestrogen-free AIN93G (diet 95092 with 7% corn oil substituted for 7% soybean oil)	Diet	2w premating–PND22	– m3–9	↓ [3] m3–9	T	M+F: ↑ energy expenditure; F: ↓ body fat; ↑activity; improved endocrine serum profile	Anderson et al. 2013

Table 4. Continued

Strain species	Dose (µg/kg bw/d unless stated otherwise)	Diet	Exposure route	Exposure window	Endpoint bw (age) M	Endpoint bw (age) F	Time of effect [1]	Other relevant effects of BPA	Reference
CD-1 mouse	5–50–500–5000–50.000	Soy-based Purina Rodent Chow 5008, perinatally; Purina Rodent Chow 5001, post-weaning	Oral, micro-pipettor	GD9–16	↓[8] ↑[9] PND0–w19	nd	T	Glucose homeostasis, food intake and adiposity affected; changed endocrine serum profile	Angle et al. 2013
C57BL6 mouse	100	No alfalfa or soy	s.c.	GD6–PND21	↓–↑[7] w3–35	↓ w3–35	–	M: glucose homeostasis affected	Liu et al. 2013
C57BL/6JxFVB mouse	3–10–30–100–300–1000–3000	NIH-07; HFD (D12451) in F w17–21	Diet	2w premating–PND21	↑[7] w6–21	↓ w8–21	D	M: liver weight ↑, glucagon ↓ F: liver weight ↓, fat pad weight ↓, adipocyte size ↓, serum lipids ↓, leptin ↓, adiponectin ↓	This study

bw, body weight; m, month; w, week(s); GD/PND, gestation/postnatal day; HFD, high fat diet; s.c. subcutaneous; nd, no data; M/F, male/female

[1] P, permanent effect on body weight throughout study; D, delayed (late onset) of effect; T, transient effect; E, only determined at the end of the study

[2] No body weight dose-response, body weight outcomes variable over dose groups and time of observation

[3] Low dose more effective

[4] Onset of effect differs between sexes

[5] High fat diet decreased delay in onset of effect

[6] Conversion of dose based on Ryan et al. 2010b

[7] Depending on exposure window

[8] Incidental observation

[9] Tendency

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Supplementary data

Supplementary data associated with this article can be found in Appendix A.

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Supplementary Table 1A. Arithmetic mean ± SD of perinatally BPA exposed F1 males for organ and body metrics and serum parameters

	BPA dose group (µg/kg bw/d)							
	0	3	10	30	100	300	1000	3000
Organ weights (mg)								
Adrenal glands	5.1 ± 1.5	5.1 ± 2.7	5.6 ± 2.4	7.1 ± 3.0	7.2 ± 3.8	6.3 ± 1.6	6.1 ± 2.8	5.5 ± 1.1
Brain	475 ± 8.5	488 ± 21	478 ± 12	486 ± 13	475 ± 6.3	476 ± 21	486 ± 13	479 ± 13
Femur	99.1 ± 12	108 ± 19	121 ± 47	117 ± 17	120 ± 15	111 ± 19	112 ± 17	101 ± 11
Liver	1602 ± 128	1483 ± 266	1786 ± 140	1853 ± 228	1871 ± 166	1662 ± 101	1722 ± 112	1772 ± 119
Quadriceps femoris muscle	256 ± 23	268 ± 39	295 ± 62	288 ± 34	263 ± 47	241 ± 71	322 ± 55	272 ± 52
Pancreas	306 ± 60	329 ± 64	316 ± 76	328 ± 51	277 ± 79	331 ± 53	368 ± 55	337 ± 77
Fat pad weights (mg)								
Interscapular	146 ± 36	230 ± 152	191 ± 61	204 ± 58	172 ± 51	148 ± 46	234 ± 101	212 ± 66
Mesenterial	45.7 ± 35	118 ± 74	87.5 ± 73	160 ± 233	116 ± 95	114 ± 108	109 ± 129	134 ± 114
Perigonadal	160 ± 56	297 ± 181	281 ± 168	301 ± 234	237 ± 103	167 ± 54	282 ± 204	313 ± 141
Perirenal	50.8 ± 25	118 ± 68	103 ± 76	132 ± 126	71.6 ± 44	50.5 ± 18	118 ± 103	110 ± 75
Subcutaneous - caudal mammary gland	100 ± 32	149 ± 73	120 ± 58	174 ± 110	237 ± 240	110 ± 29	142 ± 64	134 ± 50
Subcutaneous - rostral mammary gland	98.0 ± 47	170 ± 144	136 ± 52	191 ± 164	206 ± 190	109 ± 20	160 ± 53	137 ± 73
Sum fat pads[1]	1048 ± 308	1814 ± 1126	1559 ± 757	2085 ± 1749	1926 ± 945	1135 ± 230	1747 ± 1021	1733 ± 741
White adipocyte size (µm)[2]	51.6 ± 7.6	66.2 ± 8.5	63.7 ± 10	68.3 ± 11	59.8 ± 8.2	56.5 ± 14	59.6 ± 9.7	60.8 ± 11
Body size (mm)								
Body length	97.6 ± 2.4	99.3 ± 2.5	99.9 ± 3.2	100 ± 2.2	100 ± 4.5	99.2 ± 2.2	102 ± 2.1	100 ± 4.0
Femur length	16.1 ± 0.64	15.9 ± 0.34	16.4 ± 0.72	16.0 ± 0.41	16.2 ± 0.48	16.1 ± 0.46	16.2 ± 0.55	15.9 ± 0.41
Lipid profile (mmol/L)								
Cholesterol	2.69 ± 0.20	2.52 ± 0.19	2.71 ± 0.26	2.70 ± 0.24	2.53 ± 0.29	2.39 ± 0.16	2.60 ± 0.52	2.66 ± 0.32
Free fatty acids	0.83 ± 0.16	1.00 ± 0.14	1.22 ± 0.22	1.10 ± 0.26	1.05 ± 0.30	0.91 ± 0.18	1.06 ± 0.24	1.12 ± 0.26

Supplementary Table 1A. Continued

	BPA dose group (µg/kg bw/d)							
	0	3	10	30	100	300	1000	3000
High-density lipoproteins	2.20 ± 0.20	2.10 ± 0.18	2.21 ± 0.35	2.16 ± 0.23	1.99 ± 0.29	1.87 ± 0.17	2.11 ± 0.47	2.14 ± 0.33
Triglycerides	0.81 ± 0.24	0.77 ± 0.28	1.38 ± 0.30	1.15 ± 0.60	1.14 ± 0.42	0.80 ± 0.22	0.94 ± 0.28	1.05 ± 0.44
Endocrine profile								
Adiponectin (µg/mL)	9.0 ± 0.84	11.3 ± 1.8	8.7 ± 1.3	8.7 ± 1.4	9.0 ± 3.1	9.4 ± 1.4	9.8 ± 1.6	9.2 ± 1.5
Glucagon (pM)[3]	22.0 ± 12	20.6 ± 9.3	25.1 ± 19.5	14.1 ± 9.5	9.4 ± 7.6	16.7 ± 15	17.5 ± 11	9.1 ± 4.4
Insulin (ng/mL)	0.74 ± 0.46	0.63 ± 0.51	0.70 ± 0.27	0.91 ± 0.58	1.08 ± 0.72	0.54 ± 0.25	0.93 ± 0.49	0.78 ± 0.24
Leptin (ng/mL)	0.63 ± 0.55	1.82 ± 3.18	2.41 ± 2.23	2.03 ± 3.16	1.48 ± 1.08	0.63 ± 0.29	1.63 ± 1.43	2.24 ± 2.02

[1] Sum fat pads was calculated with weights of all fat pads collected, including a doubling of the weight for paired fat pads.
[2] White adipocyte size was measured in sections of perirenal fat.
[3] Five out of 68 glucagon values in males were below detection limit of the assay and replaced with 0.9 x lowest detected value.

Supplementary Table 1B. Arithmetic mean ± SD of perinatally BPA exposed F1 females for organ and body metrics and serum parameters

	BPA dose group (µg/kg bw/d)							
	0	3	10	30	100	300	1000	3000
Organ weights (mg)								
Adrenal glands	13.2 ± 2.8	12.5 ± 2.1	14.4 ± 4.0	14.5 ± 4.0	14.3 ± 1.9	12.1 ± 3.2	13.4 ± 1.1	13.1 ± 2.0
Brain	499 ± 22	479 ± 26	514 ± 16	502 ± 18	505 ± 22	494 ± 25	487 ± 31	485 ± 13
Femur	92.1 ± 7.5	90.9 ± 9.0	90.3 ± 13	94.1 ± 13	89.1 ± 7.7	95.2 ± 14	92.1 ± 7.2	89.0 ± 17
Liver	1315 ± 169	1229 ± 164	1299 ± 104	1345 ± 71	1250 ± 146	1179 ± 87	1237 ± 108	1073 ± 115
Quadriceps femoris muscle	198 ± 52	188 ± 23	200 ± 32	198 ± 23	196 ± 41	178 ± 17	197 ± 26	170 ± 20
Pancreas	323 ± 70	276 ± 55	325 ± 35	257 ± 47	320 ± 55	305 ± 51	297 ± 39	285 ± 40
Fat pad weights (mg)								
Interscapular	171 ± 56	142 ± 91	143 ± 94	152 ± 51	135 ± 45	126 ± 22	157 ± 30	99.5 ± 15
Mesenterial	92.6 ± 70	126 ± 167	66.0 ± 38	150 ± 131	68.6 ± 39	81.9 ± 123	45.2 ± 16	65.9 ± 51

Supplementary Table 1B. Continued

	BPA dose group (µg/kg bw/d)							
	0	3	10	30	100	300	1000	3000
Perigonadal	311 ± 316	207 ± 276	216 ± 279	228 ± 221	140 ± 127	136 ± 111	168 ± 97	94.8 ± 24
Perirenal	107 ± 135	80.7 ± 114	39.9 ± 54	50.0 ± 35	25.9 ± 21	31.5 ± 37	28.3 ± 15	22.1 ± 8.0
Subcutaneous - caudal mammary gland	164 ± 111	144 ± 144	106 ± 58	148 ± 50	93.2 ± 37	90.9 ± 31	116 ± 25	74.8 ± 11
Subcutaneous - rostral mammary gland	203 ± 157	146 ± 106	138 ± 108	128 ± 54	119 ± 59	131 ± 58	125 ± 43	116 ± 48
Sum fat pads[1]	1836 ± 1483	1425 ± 1497	1207 ± 1119	1409 ± 809	959 ± 530	986 ± 589	1162 ± 399	781 ± 147
White adipocyte size (µm)[2]	54.2 ± 12	53.3 ± 12	49.8 ± 12	47.9 ± 7.1	52.1 ± 12	42.5 ± 8.3	46.3 ± 3.6	41.2 ± 5.2
Body size (mm)								
Body length	96.4 ± 2.6	94.4 ± 3.7	96.3 ± 1.3	96.1 ± 1.7	95.0 ± 1.8	94.8 ± 2.5	97.0 ± 1.8	94.3 ± 3.7
Femur length	15.8 ± 1.2	15.8 ± 0.42	16.0 ± 0.34	16.2 ± 0.51	16.1 ± 0.45	16.0 ± 0.49	16.2 ± 0.34	15.4 ± 0.71
Lipid profile (mmol/L)								
Cholesterol	2.02 ± 0.28	1.97 ± 0.28	2.21 ± 0.17	2.13 ± 0.19	2.11 ± 0.21	2.04 ± 0.19	2.32 ± 0.35	2.15 ± 0.37
Free fatty acids	1.78 ± 0.70	1.18 ± 0.31	1.08 ± 0.32	1.02 ± 0.25	0.91 ± 0.17	0.83 ± 0.25	1.06 ± 0.26	0.76 ± 0.29
High-density lipoproteins	1.69 ± 0.31	1.58 ± 0.21	1.85 ± 0.18	1.78 ± 0.23	1.72 ± 0.17	1.62 ± 0.21	2.01 ± 0.30	1.78 ± 0.42
Triglycerides	2.16 ± 1.37	1.19 ± 0.44	1.16 ± 0.48	1.15 ± 0.42	0.87 ± 0.19	0.92 ± 0.27	1.16 ± 0.38	0.54 ± 0.28
Endocrine profile								
Adiponectin (µg/mL)	26.7 ± 7.4	20.8 ± 4.5	18.1 ± 3.0	19.5 ± 3.1	18.9 ± 1.3	17.4 ± 4.5	18.3 ± 4.2	19.0 ± 1.8
Glucagon (pM)	62.8 ± 33	41.3 ± 17	92.0 ± 50	68.6 ± 24	67.7 ± 35	56.5 ± 26	93.4 ± 59	55.1 ± 20
Insulin (ng/mL)	0.94 ± 0.54	0.61 ± 0.41	0.85 ± 0.43	1.12 ± 0.61	0.82 ± 0.29	0.64 ± 0.10	0.80 ± 0.39	0.77 ± 0.27
Leptin (ng/mL)	3.38 ± 3.36	1.65 ± 2.34	1.40 ± 1.95	1.54 ± 0.70	0.91 ± 0.69	0.83 ± 0.56	1.44 ± 0.41	0.39 ± 0.13

[1] Sum fat pads was calculated with weights of all fat pads collected, including a doubling of the weight for paired fat pads.
[2] White adipocyte size was measured in sections of perirenal fat.