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Perinatal BPA exposure alters body weight and composition in a dose specific and sex specific manner: The addition of peripubertal exposure exacerbates adverse effects in female mice

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Abstract

Body weight (BW) and body composition were examined in CD-1 mice exposed perinatally or perinatally and peripubertally to 0, 0.25, 2.5, 25, or 250 μg BPA/kg BW/day. Our goal was to identify the BPA dose (s) and the exposure window(s) that increased BW and adiposity, and to assess potential sex differences in this response. Both perinatal exposure alone and perinatal plus peripubertal exposure to environmentally relevant levels of BPA resulted in lasting effects on body weight and body composition. The effects were dose specific and sex specific and were influenced by the precise window of BPA exposure. The addition of peripubertal BPA exposure following the initial perinatal exposure exacerbated adverse effects in the females but appeared to reduce differences in body weight and body composition between control and BPA exposed males. Some effects of BPA on body weight and body composition showed a non-linear dose response.

Keywords

Bisphenol A (BPA); Obesity; Body composition; Sex differences; Non-monotonic dose response; Extreme hyperactivity; Developmental origins of adult disease

1. Introduction

Over the past few decades, obesity and associated elements of metabolic disease have reached epidemic proportions and have contributed substantially to increases in health care

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Conflict of interest

None.

costs in Western societies. The precipitous rise in body weight has stimulated much speculation regarding the factors responsible. The cause of obesity is clearly multifactorial and includes diet, exercise, and genetics; however the rapid rise in obesity levels within a relatively short period of time suggests potential involvement of additional environmental factors [1]. The idea that the exponential increase of chemicals in our environment may have played a role in the meteoric rise in body weight has been garnering increasing interest [2,3]. Blumberg and colleagues [4] coined the term “obesogens” to describe “chemical agents that inappropriately regulate and promote lipid accumulation and adipogenesis to promote obesity.” Bisphenol A (BPA) is just one chemical on a growing list of suspected obesogens that may have contributed to the current obesity epidemic and the upsurge in obesity – associated metabolic disease.

BPA is a ubiquitous industrial chemical that has been detected by the Centers for Disease Control and Prevention (CDC) in the urine of 92.6% of a cross section of the US population [5], and in the majority of individuals examined in numerous other research studies (for review see Ref. [6]). BPA is a component of polycarbonate plastics used in food and beverage containers and epoxy resins used to line food cans and for dental materials. High levels of BPA are also found in carbonless paper [7], and BPA has been measured in ground water, soil, dust and air (reviewed in Ref. [8]). Whereas ingestion is considered the main route of human exposure to BPA, questions have been raised about the potential importance of other exposure routes [9] particularly dermal absorption [10–13] and possibly inhalation. Recent data suggest that non dietary exposures may provide a significant source of exposure to BPA [14,15]. BPA is a xenoestrogen, and its estrogenic effects account for some of its reported actions; however, BPA is also known to interfere with the action of other hormones (reviewed in Ref. [16]), and therefore, the effects of BPA can reach beyond its estrogenic properties.

Some epidemiological studies have reported a positive correlation between urinary BPA levels and increased body weight or obesity, elevated waist circumference or body mass index (BMI) as well as elements of metabolic disease including altered glucose/insulin homeostasis, diabetes and cardiovascular disease [17–25]. These reported associations are provocative; however, additional data are needed to further explore causality in humans.

The current study examines the potential for developmental exposure to BPA to exert lasting influence on body weight and body composition in male and female CD-1 mice, a strain known to be sensitive to early BPA exposure [26–28]. Conflicting data from rodent studies indicate that developmental exposure to BPA can result in 1) increased BW [29–37], 2) decreased BW [37,38] or 3) no change in BW [39–41]. Among studies reporting increased body weight, some found that females were more likely to be influenced by early BPA exposure [29,34] and others reported increased body weights in males and not in females [37]. These differences in outcomes may relate to variations in study design including differences in species, strains, exposure windows, exposure doses, routes of administration, diet, and age at the time of study. The current study was undertaken to delineate the effects of BPA exposures during the perinatal or the combined perinatal plus peripubertal periods of development on body weight and body composition. Our goal was to identify the BPA dose (s) and the exposure window(s) that increased BW and adiposity, and to assess potential sex

differences in this response. Early review of body weight and body composition data prompted additional measurements to further examine the differences between perinatal and combined perinatal plus peripubertal BPA exposure windows in females.

2. Materials and methods

2.1. Animals

All animal protocols were reviewed and approved by the University Institutional Animal Care and Use Committee (IACUC), and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No 8023). The CD-1 mice for breeding were purchased from Charles River Breeding Labs (CRBL-Wilmington MA: all animals for these studies came from CRBL facilities in Kingston NY or Raleigh. NC). Animals were housed in an AALAC accredited facility at the Human Nutrition Center on Aging (HNRCA) at Tufts University where they were maintained in a temperature and humidity controlled room on a 14:10 light/dark cycle with lights on at 0400 h. Animals were fed Harlan Teklad 2018 rodent chow (Harlan Laboratories, Indianapolis, IN: 18.6% protein, 6.2% fat, energy density = 3.1 kcal/g.) ad libitum. To limit unplanned exposure to BPA, animals were housed in polysulfone cages (Ancare, Bellmore, NY) and filtered water (chlorine was removed) was provided in glass water bottles with rubber stoppers and metal sippers. Prior to purchase, food was assessed for overall estrogenic activity using the E-Screen assay [42]. The levels of estrogenic activity in the 3 batches of food purchased for this study contained the equivalent of 10.3, 15.9 and 18.2 pmol/g of food which is within the range used in our studies of BPA exposure for many years.

2.2. Generation of treatment groups for study

Males and females were placed together for mating. Females were checked daily for vaginal plugs, and once noted (gestational day 1-GD1), they were individually housed. Births were recorded at 4 PM daily (postnatal day 1- PND1), and on PND2, litters were culled to 8 animals (4 males and 4 females). Pups remained with their birth mothers and littermates until weaning on PND 21. A total of 11–14 l were obtained for each exposure group.

2.3. Pump preparation and implantation for perinatal exposure

Dams were weighed on GD6 and dilutions of BPA (in 50%DMSO/water, vehicle) were calculated to provide exposures of 0, 0.25, 2.5, 25, or 250 μg BPA/kg BW/day to the dams *via* osmotic minipumps (Alzet Osmotic Pumps, Cupertino CA) from GD 8 to lactational day 16 (Perinatal BPA exposure = P). From here on, exposures will be referred to as 0.25, 2.5, 25, or 250 μg BPA. Prior to implantation, pumps were equilibrated at 37C for 48 h as per manufacturer's specification. Dams were briefly anesthetized (isofluorane), and a small cut was made in the skin at the nape of the neck for subcutaneous pump placement. All BPA doses for study were well below the lowest observed adverse effect level (LOAEL) of 50 mg BPA/kg BW and the no observed adverse effect level (NOAEL) of 5 mg/kg BW based on oral exposure. Only the highest dose exceeded the "safe" or reference dose of 50 μg/kg BW/day, calculated by the Environmental Protection Agency [43].

2.4. Peripubertal BPA exposure

At weaning, 2 males and 2 females from each litter received additional BPA exposure *via* the drinking water at doses comparable to those delivered by the pumps from PND 21–35 (Perinatal + Peripubertal exposure = P + P). The remaining 2 male and 2 female siblings from each litter received unadulterated drinking water and therefore, their only planned exposure to BPA was during the perinatal period (P).

2.5. Planned measurements

The animals were weighed 4 times from birth to weaning, weekly on weeks 3–19, and then every two weeks through week 43. Body composition was assessed by MRI (Echo MRI, Echo Medical Systems LLC; Houston, Texas) in a single male and female from each litter at each exposure window at 5 time points during the study. Measurements of fat mass (g), lean mass (g) and total water content were collected, and percent fat, percent lean, and fat:lean ratio were calculated. At sacrifice, blood and tissues were collected and archived.

2.5.1. Elimination of extremely hyperactive females—It is important to note that, as in past studies in our animal model, we observed a subset of extremely hyperactive females. The extreme behavior became apparent only after 8 weeks of age and consisted primarily of excessive flipping behavior and/or constant running behavior. Rather than a simple increase in normal activity levels, these behaviors appear to be repetitive behaviors that serve no purpose and over which the animals appear to have little control. We visually documented extreme flipping/running behavior in females housed in cages that were in clear view during our daily visits to the mouse room. To identify additional extreme hyperactive females, we reviewed behavioral observations as well as body weight and composition data for the females that were randomly selected to be followed by MRI (1 female/litter/treatment group). For those animals, hyperactive behaviors were documented during handling at the time of MRI assessments, and the dramatic reductions in percent fat and in the fat:lean ratio observed were consistent with profound increases in activity. We graphed trajectories of body weights and body composition over time to define the range of all data points in documented flippers/runners, and these numbers were used to identify putative severe hyperactive animals. Animals were excluded from data analysis only if their percent fat at the time of expected heightened hyperactivity was greater than 2 standard deviations below the mean of the remainder of their treatment group.

2.6. Additional measurements

Upon noting differences in body weight and body composition profiles in the P + P females relative to the P females, the decision was made to examine additional parameters of obesity- associated metabolic disease in females in the two exposure windows. Examinations of serum, liver, and glucose/insulin homeostasis were performed in P and P + P females to further delineate differences resulting from the two exposure windows.

2.6.1. Serum measurements—Serum leptin was measured with ELISA kits for mouse Leptin (EMD Millipore, Billerica, MA). The sensitivity range was from 0.2 ng/ml to 30 ng/ml and the sensitivity limit was 0.05 ng/ml. Serum triglycerides (TG) were measured using a colorimetric assay kit (L-type TG M, Wako, Mountain View, CA). The range of the

standard curve was 0–107 mg/dL, and linearity continued through 2000 mg/dL. Serum was also analyzed for cholesterol (Pointe Scientific, Inc., Canton, MI), and nonesterified fatty acids (NEFA-HR (2), Wako, Mountain View, CA).

2.6.2. Lipid accumulation in liver—A sample of frozen liver (50–75 mg) was homogenized in 1 ml of phosphate-buffered saline at 4 °C and 200 μl of homogenate was placed into a borosilicate glass tube with 3.75 ml of chloroform/methanol solution (2:1; v/v). Tubes were vigorously mixed, 500 μl of distilled water added, and the suspension was vortexed and centrifuged at 3000 rpm for 5 min. The chloroform-methanol layer was removed, dried under nitrogen gas, and the lipid residue was suspended in 1%Triton x-100 (Fluka Chemicals, Switzerland) in isopropanol (Sigma- Aldrich, St Louis, MO). Levels of triglycerides and cholesterol were assessed using the Pointe Scientific kits, and nonesterified fatty acids were measured using the Wako kit.

Accumulation of neutral lipids was visualized with Oil Red O (Fisher Scientific, Fair Lawn, NJ). Frozen livers were sectioned at 5 μm on a Leica CM 1950 cryostat (Leica Microsystems, Germany), fixed in neutral buffered formalin (5 min), washed (cold tap water for 10 min), and dipped (5×) in 60% isopropanol prior to incubation with Oil Red 0 (15 min at room temperature). Slides were washed 2× in 60% isopropanol, counterstained with Mayer's hematoxylin, cover slipped with Vectamount aqueous mounting medium (Vector Laboratories Inc., Burlingame, CA) and examined by an observer blind to the treatment groups.

2.6.3. Measurements of insulin/glucose homeostasis in P and P + P females

2.6.3.1. Fasting glucose and insulin levels: Fasting glucose and insulin levels were assessed at 28 and 34 weeks. After a 6 h fast, blood glucose levels were determined using a One Touch Ultra glucometer (Lifescan, Milpitas, CA) and a drop of blood obtained by a tail nick. Additional drops of blood were collected and placed into a K2-EDTA tube (BD microtainer, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged for plasma separation. Plasma insulin levels were determined using Ultra Sensitive mouse insulin ELISA kits (Crystal Chem, Inc, Downers Grove, IL). The standard curve of the Low Range Assay was from 0.1 ng/ml–6.4 ng/ml and the sensitivity of the assay was 0.05 ng/ml.

2.6.3.2. Insulin tolerance tests: Insulin tolerance tests (ITT) were performed at 40 weeks. P and P + P Females were fasted for 6 h, beginning at 0800 h and insulin (Humulin R U-100, Lilly USA, LLC, Indianapolis, IN) was injected intraperitoneally (ip) at a dose of 0.75 U/kg. Blood was collected at 0, 15, 30, 45, 60, and 90 min, and glucose was measured as described above.

2.6.3.3. Glucose tolerance test: Because there was an indication of decreased insulin tolerance in P + P females, a glucose tolerance test was performed in this group at 48 weeks. Mice were fasted for 12 h, starting at 2130 h. Glucose was injected ip (1.5 g/kg BW) and blood glucose levels were measured at 0, 15, 30, 45, 60, 90 and 120 min as described above.

2.7. Measurements of serum BPA

A separate cohort of animals was generated (as described above) to provide blood for the measurement of internal BPA dose. A group of these animals were anesthetized with ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (AnaSed, Lloyd Laboratories Shenandoah, Iowa) on GD 18 (n = 5 litters/treatment group) and blood was collected from pregnant dams and their fetuses. Blood from litters of fetuses was pooled to obtain enough serum for analysis. Blood was also collected from dams and pups on lactational day 11 (n = 5 litters/treatment group) and from P + P animals on PND 32 (n = 5 litters/treatment group) during the peripubertal exposure to BPA *via* their drinking water. Materials for blood collection, serum separation, and sample storage for BPA measurements were provided by the CDC. Serum samples were frozen and sent to the CDC where they were analyzed for total and unconjugated BPA as described by Ye et al. [44] using on-line solid phase extraction coupled to high performance liquid chromatography.-isotope dilution tandem mass spectrometry. Serum was treated with beta glucuronidase/sulfatase to estimate the concentration of total BPA (conjugated plus unconjugated) or serum was processed without enzymatic treatment to estimate the concentration of unconjugated BPA. The limit of detection (LOD), the lowest amount of an analyte that can be detected with a defined probability, was 0.3 ng/ml. The LOQ or level that can be quantified with accuracy and precision was 0.9 ng/ml.

2.8. Statistical analysis

As per our *a priori* plans, comparisons of offspring BW (weeks 7–43) and body composition measurements over time were analyzed individually for each of the 4 experimental groups using a mixed model analysis of variance with repeated measures. Bonferroni *post hoc* tests were used for comparisons across all groups. In the case of a significant interaction, one or two individual time points were assessed by ANOVA, and planned pairwise comparisons with controls were assessed by Dunnett's *t*-test. Please note, animals with missing values were removed from repeated measures analysis and associated tests. These tests used XLSTAT 2016.

Data that did not include repeated measures [*eg* body weights of the dams, neonates and weanlings (PND2-42), number of pups per litter, hormone levels, triglyceride levels, fasting glucose/insulin levels] were analyzed by ANOVA for comparisons across treatment groups. The Dunnett's *t*-test was used to assess planned pairwise comparisons with controls. These tests were completed using SPSS software (IBM-SPSS Statistics version 22).

3. Results

3.1. Dams and litters

Body weights of the dams on GD 6 and GD 8 and at the time of weaning are shown in Table 1. No significant differences are noted in body weights at any time point examined and mean litter size did not differ across treatment groups.

3.2. Body weights in males and females from birth through week 6

Body weights did not differ by treatment groups in the male or female neonates (Fig. 1A, D). Post weaning, increased body weights relative to controls were noted in the P females (Fig. 1B) at PND 28 (ANOVA @ 0.019, Dunnett's t, C *vs* 2.5, p = 0.002) at PND 35 (ANOVA@0.022; Dunnett's t, C *vs* 0.25, p = 0.025; C *vs* 2.5, p = 0.055) and at PND 42 (ANOVA @ 0.041; Dunnett's t, C *vs* 2.5, p = 0.056). In the P + P females (Fig. 1C), increased body weights were noted at PND 28 (ANOVA @ 0.0005, Dunnett's t, C *vs* 2.5, p = 0.002; C *vs* 25, p = 0.05) and at PND 35 (ANOVA = 0.043; Dunnett's t, C *vs* 2.5, p = 0.037).

Body weights in P and P + P males were not increased in any exposure group relative to controls during the period from 28 to 42 days of age (Fig. 1E, F, n = 44–56).

3.2.1. Male body weights: weeks 7–43—Body weights over time in the P males are shown in Fig. 2A. The repeated measures ANOVA revealed significant differences by exposure dose (F = 855.794, p < 0.0001) and time (F = 906.177, p < 0.0001) with no interaction (F = 0.665 p = 0.996). A Bonferroni *post hoc* test failed to identify overall significance between any groups at the modified significance level of p = 0.005. However, as depicted in Fig. 2A, the overall mean body weights of all BPA exposed groups were above the body weights of control males throughout the time of data collection; mean body weights of the 0.25 μg BPA males were increased on average from 7.0% to 8.5% above the mean body weights of control males.

For the P + P males (Fig. 2B), overall significant effects of treatment (F = 119.377, P < 0.0001) and time (F = 993.558, p < 0.0001) were observed with no interaction (F = 0.602, p = 0.999), and *post hoc* testing failed to delineate differences between groups. Relative to body weights of their P male siblings, the mean body weights of the P + P males showed less separation of exposure groups. The overall body weights of the 25 μg P + P males appeared most elevated and those of the 250 μg P + P males appeared decreased relative to the other groups although, unlike the P males, there was considerable overlap between individual exposure groups.

3.2.2. Body composition in males

3.2.2.1. P Males: Parameters of body composition of P males are shown in Fig. 3, Panel A. Overall significance of treatment was observed for fat mass (F = 22, p < 0.0001), percent fat (F = 65.2, p < 0.0001), and percent lean mass (F = 72.9, p < 0.0001). The effect of time was also significant for each of these measurements (p < 0.0001) and there was no interaction in any parameter. Bonferroni *post hoc* tests failed to identify significant differences between groups at the modified p value of 0.005; however, examination of the data suggests the most marked differences from controls in all parameters were noted in the 25 μg males. As depicted in Fig. 3A, 25 μg males had a mean fat mass that was 19%, 20%, and 17.7% above the controls at PND 50, 90 and 130 respectively. The mean percent fat in 25 μg males was 15%, 17% and 13% above the controls at PND 50, PND 90, PND 130 respectively, and the mean fat:lean ratio of 25 μg P males was 17%, 24% and 19% above the mean levels calculated for the control males at those same timepoints.

3.2.2.2. P + P Males: The overall pattern of body composition measurements in the P + P males differed from the P males; they showed more overlap between exposure groups in all parameters assessed (Fig. 3B). Analysis of fat mass and fat to lean ratio did not differ significantly in the P + P males; however percent fat and percent lean showed differences by treatment (percent fat: $F = 7.2$, $p < 0.0001$, percent lean: $F = 8.2$, $p < 0.0001$) and time ($P < 0.0001$) without significant interaction. Bonferroni revealed no differences between groups (range, $p = 0.575$ to $p = 0.978$).

3.2.3. Female body weights: weeks 7–43 (without extreme hyperactive animals)—As discussed, a subset of BPA exposed females demonstrated extreme hyperactivity. Therefore, we analyzed BW data from the single female from each group (P and P + P) that was followed for body composition analysis. Fig. 4 contains the BW data from one P (A) and one P + P (B) female/litter after removal of the extreme runners and flippers according to the criteria described in the methods section.

3.2.3.1. P Females: Hyperactive P females were noted only in the 25 μg ($n = 4$) and 250 μg ($n = 3$) BPA exposure groups. Comparisons of mean BW and body composition data from these hyperactive P females with data from the remainder of the females in each of their BPA exposure groups is shown in Fig. 5. The dramatic differences in the trajectories of these measurements in the hyperactive 25 μg P females (5A and C) and the hyperactive 250 μg P females (5B and D) in relation to mean values for the rest of each of their treatment groups is depicted in this figure.

As shown in Fig. 4A, the mean body weights for the P females exposed to BPA were elevated above mean body weights of the controls at virtually every time point with the exception of weeks 7 and 10 when mean body weights of the 250 μg females were below the controls. There was an overall significance of treatment ($F = 520.706$, $p < 0.0001$) and time ($F = 741.237$, $p < 0.0001$) with no interaction ($F = 0.896$, $p = 0.751$). No overall differences between groups were identified at the modified significance level of $p = 0.005$ (Bonferroni). As depicted in Fig. 4A, the 25 μg females appear to have the highest overall body weights relative to controls.

3.2.3.2. P + P Females: Extreme hyperactive animals were identified in all 4 P + P exposure groups (in 0.25 and 2.5 μg, $n = 3$; in 25 and 250 μg, $n = 2$) and in no control females. Because members of all BPA groups were affected, after removal of the extreme hyperactives, overall BW profiles resembled those for all P + P animals on study although differences between exposure groups were more pronounced.

The BW data for the P + P females (Fig. 4B) revealed significant effects of treatment ($F = 2517.484$, $p< 0.0001$), and time ($F = 1068.318$, $p < 0.0001$) and a significant interaction ($F = 1.749$, $p < 0.0001$). Further analysis of differences of main effects are not reported due to the significant interaction; however, as shown in Fig. 4B, the highest mean body weights were observed in the 2.5 μg BPA P + P females and the next highest in the 25 μg P + P females. The highest and lowest BPA doses had mean body weights similar to controls. Two time points were chosen (week 15 and week 31) to determine if the BWs of 2.5 or 25 μg females were significantly elevated above controls. Significant differences in BWs across exposure

groups was confirmed (ANOVA, week 15, p = 0.013 and week 31, p = 0.020), and pairwise comparisons with controls (Dunnett's *t*-test) revealed significant elevation of BW in the 2.5 μg females relative to controls at 15 weeks (p = 0.015) and at 31 weeks (p = 0.037).

3.2.4. Body composition in females—In contrast to the males, the additional peripubertal exposure enhanced differences in body composition between specific exposure groups. Analysis of body composition in the P + P females (Fig. 6B) revealed overall differences in fat mass by treatment (F = 317.827, p < 0.0001) and time (F = 381.170, p< 0.0001); however, there was a significant interaction (F = 2.981, p = 0.001) and therefore, results of *post hoc* tests were not reported. An ANOVA performed at day 141 revealed a significant difference between exposure groups (p = 0.006), and the Dunnett's *t*-test confirmed increased fat mass in 2.5 μg BPA females relative to controls (p = 0.031). Percent fat and percent lean both showed significant differences by treatment (percent Fat: F = 525, percent Lean: F = 383, P < 0.0001) and time (p < 0.0001) without significant interaction (percent fat: p = 0.129, percent lean: p = 0.295). Bonferroni *post hoc* tests revealed overall differences in percent fat between 2.5 μg and 0.25 μg BPA (p < 0.001). Comparisons of 2.5 μg BPA with Controls and with 250 μg BPA were significant at p = 0.007 which was just above the modified significance level of 0.005. For percent lean, comparisons of 2.5 μg BPA with 0.25 μg BPA were significant (p = 0.001). The comparisons of 2.5 μg to Control or 250 μg BPA were significant at p = 0.013, which is above the modified significance level.

In P females (See Fig. 6A), overall differences in body composition were less pronounced relative to the P + P females. Differences were observed in fat mass by treatment (F = 39.6, p < 0.0001) and time (F = 40.3, p < 0.0001), percent fat by treatment (F = 124.096, p < 0.0001) and time (F = 158.7, p < 0.0001) and percent lean by treatment (F = 84.4, p < 0.0001) and time (F = 108.133, p < 0.0001). No significant interactions were noted in the P female body composition data, and the Bonferroni *post hoc* test failed to identify overall significant differences between groups.

3.3. P + P Females reveal evidence of altered glucose homeostasis in adulthood

Due to more pronounced effects on body weight and body composition in the 2.5 and 25 μg BPA P + P females relative to P females, we chose to compare some parameters of obesity associated metabolic disease in the two exposure windows.

Analysis of fasting glucose and insulin levels in P + P females at 28 weeks of age (Fig. 7A) revealed similar glucose levels across groups; however, differences in insulin levels were observed (ANOVA sig @0.034), with an increase in 2.5 μg BPA P + P females over controls (p = 0.011, Dunnett's). At 34 weeks (Fig. 7B), insulin levels remained elevated in 2.5 μg females (p = 0.024, Dunnett's), and glucose levels differed between groups (Fig. 7B, ANOVA, p = 0.015), with levels in 2.5 μg P + P females elevated above controls (Dunnett's, p = 0.004). Similar alterations were not observed in P females examined at similar time points (data not shown).

Insulin tolerance tests (ITT) were conducted at 10 months of age. In the P females (Fig. 8A), following insulin injection (ip, 0.75 U/kg BW) mean glucose levels showed a similar response pattern across treatment groups with little separation between groups. In P + P

females (8B), more separation was noted between treatment groups and repeated measures ANOVA revealed significant differences by treatment and time ($p < 0.0001$) with no interaction. The overall comparison of 2.5 μg *vs* control approached significance ($p = 0.07$, Bonferroni). Dunnett's Test performed at the 45 min time point revealed higher glucose levels in 2.5 μg females ($p = 0.042$) and in 25 μg females relative to controls ($p = 0.046$).

A glucose tolerance test (GTT) in the P + P females (Fig. 8C) failed to reveal significant differences in glucose levels across groups in response to injection of 1.5 g glucose/kg BW.

3.4. Data from tissues collected at the time of sacrifice

3.4.1. Serum measurements—Serum leptin levels were elevated in BPA exposed P (Fig. 9A, ANOVA $p = 0.028$; C *vs* 25 μg, $p = 0.039$, C *vs* 250 μg, $p = 0.008$, Dunnett's t) and P + P females (Fig. 9B, C *vs* 25 μg $p = 0.045$, Dunnett's t) relative to controls. It should be noted that in the P + P BPA exposed females, several values were above the maximum range of the standard curve and assigned the maximum value of assay detection, 75 ng/ml. Therefore, mean values of the 25 and 250 μg P + P females would be higher if actual leptin values were available for all animals.

Serum triglyceride levels were increased in P and P + P BPA exposed females relative to controls with the highest mean levels observed in the P + P females (Fig. 9C, D); however, there were no statistically significant differences across treatment groups. Serum cholesterol and NEFA levels did not differ across treatment groups in either exposure window (data not shown).

3.4.2. Fat accumulation in liver—Triglyceride levels in liver extracts (Fig. 10) did not differ by treatment group in P females (Fig. 10A), but were significantly elevated in P + P females (Fig. 10B) exposed to 25 and 250 μg BPA relative to controls (ANOVA $p = 0.003$; C *vs* 25 μg $p = 0.028$, C *vs* 250 $p = 0.011$, Dunnett's). Oil red O staining also suggested an increase in neutral lipids in P + P females exposed to 25 and 250 μg BPA females relative to controls (Fig. 10C). Cholesterol and NEFA levels did not differ significantly by treatment group in the P or P + P females (data not shown).

3.5. Measurements of total and unconjugated BPA in serum reveal low levels

As shown in Table 2, mean total serum BPA concentrations were detectable on GD 18 in the dams and in pooled blood from the fetuses of both the 25 μg and 250 μg treatment groups. Concentrations of total BPA were also measured in the PND 32 P + P offspring during the period of direct exposure to 25 or 250 μg BPA/kg BW *via* drinking water. At no time point were mean serum concentrations of unconjugated BPA above the level of detectability (0.3 ng BPA/ml). Mean serum concentrations of total and unconjugated BPA in the dams and their pups on PND 11 were below the detectability of the assay (<0.3 ng/ml); although two of the five dams exposed to 250 μg BPA had detectable total BPA concentrations, neither value was at or above the LOQ. It should be noted that BPA serum concentrations were measured only in animals exposed to the two highest BPA doses, 25 μg and 250 μg. Although mean total BPA serum concentrations measured in the 25 μg animals were detectable, they were below the LOQ, the level that can be quantified with accuracy and

precision. Therefore, the decision was made not to analyze serum samples from the two lower BPA exposure groups. Based on the linearity reported in BPA exposure dose and internal dose measurements [45], serum concentrations of BPA in the two lower BPA exposure groups would be expected to be below the method LOD.

4. Discussion

4.1. Assessments of BPA internal dose

First, it is important to state that the measurements of total and unconjugated BPA serum concentrations in our animals suggest that exposure levels in this study are environmentally relevant. Mean total BPA serum concentrations were detectable in BPA exposed dams and fetuses on GD 18 and in the pups during peripubertal BPA exposure on PND 32; however unconjugated BPA serum concentrations were below the level of detectability (0.3 ng/ml) at all time points examined. The BPA levels measured appear to be within the range reported in humans. A review of available data suggests that circulating levels of unconjugated BPA in the majority of human studies is in the range of 0–1 ng/ml [6,46,47], although there are reports of higher levels in some studies and a lack of detectability of unconjugated BPA in others [6].

4.2. Effects of BPA exposure on body weight and body composition

Debate continues regarding the ability of early BPA exposure to exert lasting effects on body weight and body composition in rodents. This controversy does not appear to arise from a lack of reproducibility of similar experiments, but rather from the diversity of animal models, dose, route and time of administration, diet, developmental stage and sex of the animals studied. All these factors can play an important role in health and disease and must be considered when integrating BPA exposures with the multiple phenotypic effects described. In the present study of outbred CD-1 mice, early exposure to environmentally relevant doses of BPA resulted in alterations in body weight and body composition in a dose specific and sex specific manner that varied with the precise window of BPA exposure.

4.2.1. In males: effects of BPA exposure on body weight and body composition—In P males, BPA exposure groups showed an overall elevation in body weight relative to controls, and some exposure groups, in particular 25 μg BPA, showed an increase in adiposity and a decline in percent lean mass when compared with controls. Of particular interest, extending the window of BPA exposure through the peripubertal period notably reduced the differences between exposure groups observed in their brothers exposed only perinatally to BPA. This observation illustrates the importance of the precise window of BPA exposure to body weight and composition, and it raises questions about the mechanisms involved in the reduced effect of the combined BPA exposure window in the males. A prior study reported that continuous exposure of male CD-1 mice to a high phytoestrogen diet from conception to adulthood resulted in reduced body weight and adiposity, and increased energy expenditure [48]. These effects were also observed with postnatal exposure to the high phytoestrogen diet but not with prenatal exposure alone [49]. Therefore, estrogenic properties of BPA during the peripubertal period in addition to the

perinatal period may have contributed to a positive change in body composition in the P + P males relative to their brothers exposed only perinatally.

In a recent study of C57BL/6J mice [50], BPA (5 μg to 5000 μg/kg BW/day) was provided orally to males and females for a period of 30 days beginning at 5 weeks of age, the precise time that our peripubertal or early adolescent exposure period ended. Both sexes revealed increased body weight and fat mass at the end of treatment (approximately 65 days of age). Therefore, BPA exposure spanning the mid to post adolescent period (weeks 5 through 9+) may exert a pronounced effect on adiposity and body weight in males that was not observed here in males exposed perinatally and then from 3 to 5 weeks of age suggesting the need for further delineation of potential critical exposure windows. It will be important to determine if the increases in body weight and adiposity observed by Yang and colleagues [50] at the end of the 30 day treatment persist through adulthood.

4.2.2. In females: effects of early BPA exposure on body weight and body composition—As mentioned previously, a subset of BPA exposed females showed repetitive flipping and/or constant running behavior which represented a critical confound to measurements of body weight and adiposity. Analysis of BW was therefore restricted to the single female/litter that constituted the MRI cohort so that detailed behavioral observations and extreme alterations in body composition could be used to remove severely hyperactive females according to the specific criteria described.

Although other studies have reported increased activity and decreased body weight in BPA exposed female mice [37,38], it is not clear whether the repetitive nature of the behaviors observed in our animals was present. Anderson and colleagues [38] reported increased horizontal and vertical activity in their females which could be consistent with flipping and running behavior. Also of interest, van Esterik and colleagues [37] reported a decrease in weight in their females that emerged after 8 weeks of age when repetitive behaviors begin in our females and when body weights and body composition measurements begin to diverge dramatically from the rest of the group. Sullivan and colleagues [51] reported hyperactivity in female prairie voles exposed to a low dose of BPA, and newborn mice exposed prenatally to BPA were reported to show hyperactivity and defective neocortical development [52].

It is interesting to note that data from a human prospective study revealed problem behaviors and hyperactivity in 2–3 year old girls that were exposed to higher levels of BPA in utero [53,54]. Whether the hyperactivity will persist as these children advance in age remains to be determined. Harley et al. [55] reported an association between urinary BPA concentrations and conduct problems in girls and increased inattention and hyperactivity in boys and girls at age 7.

Regardless of the inclusion or exclusion of the hyperactive animals, the female data differs from that of their male siblings. Significant increases in body weights were noted in BPA exposed females, but not males in the post weaning period (PND 28–42). In contrast to the males, the addition of peripubertal BPA exposure appeared to enhance body weight and body composition differences in some exposure levels in P + P females relative to the P

females, and the body weights of the P + P females revealed a distinct non-monotonic dose response to BPA exposure.

4.3. Differences between studies

As mentioned, various studies of body weight in rodents following developmental (prenatal and/or postnatal) exposure to BPA have reported increased [29–35,56], decreased [37,38], and no change in body weight [40,41]. Data from the current study demonstrate that BPA's effects on body weight and composition are markedly affected by sex, dose and exposure window- with each of these factors driving different responses in siblings from the same litters.

Differences in chow diets are also likely to contribute to the controversy; the relationship between phytoestrogen levels and obesity have been discussed in previous studies [57,58]. It is important to note that studies that fed soy free diets reported decreased body weights or no change in body weights in BPA exposed females [37,39,41,51]. Cao and colleagues [39] have suggested that it is the soy diet and not BPA that was responsible for increased body weight in their Wistar rat model. Clearly that is not the case in the current study as BPA exposed CD-1 mice differ significantly from controls on our soy based chow diet in a dose specific, sex specific and window of exposure specific manner. It is likely that there is an important interaction between BPA exposure and the soy diet that differs from that of BPA exposure in animals eating a soy free diet. Dolinoy and colleagues [59] reported that phytoestrogen supplementation was able to counteract the effects of BPA exposure on hypomethylation in the developing viable yellow agouti mouse.

4.4. Evidence of elements of metabolic disease is increased in P+P females

The P + P females appeared to be particularly affected by the added peripubertal exposure to BPA, showing signs linked with obesity associated metabolic disease. They showed an increase in triglyceride levels relative to the P females. In addition, the P + P females showed evidence of impaired glucose/insulin homeostasis consistent with hyperinsulinemia and the development of insulin resistance. These data add to a growing body of evidence demonstrating effects of developmental exposure to BPA on glucose homeostasis in rodents [35–37,60]. It is interesting to note that Alonso-Magdalena and colleagues reported alterations in male but not female offspring exposed to BPA in utero [40]. The data in the present study suggest that the peripubertal period may be a sensitive window of exposure for BPA with regard to disrupting glucose/insulin homeostasis in females.

4.5. Effects of peripubertal exposure alone?

In this study, we did not look exclusively at peripubertal exposure without perinatal exposure. Therefore, we do not know whether peripubertal exposure alone would cause the changes observed in the P + P females or whether they may result from a more prolonged exposure to BPA. Peripubertal exposure has been reported to alter microglia number in the prefrontal cortex in female rats [61] and to increase ER alpha levels in several brain nuclei in female but not male mice [62] and rats [63]. As mentioned previously, male and female C57BL/6J mice exposed to BPA solely during the mid through post adolescent period revealed marked increases in body weight and adiposity at the end of the exposure period

[50] suggesting that this period could be a critical window for the obesogenic properties of BPA.

Interestingly, there is evidence that the peripubertal period might be a sensitive window for effects of BPA exposure on body weight and adiposity in humans. Urinary BPA levels were associated with overweight in 9–12 year old (peripubertal) females, but not males [20]. BPA levels have also been positively associated with BMI, waist circumference, fat mass, and increased BW/obesity at 9 years in boys and girls [55] and was not noted at earlier ages.

The need to further explore the peripubertal period and other periods of marked hormonal change as potential sensitive windows for BPA exposure is fueled by a recent study that highlights pregnancy as an important window for the influence of BPA on body weight and parameters of metabolic disease [64]. When pregnant mice treated with BPA from GD 9–16 were followed for 6 months postpartum, they showed increased body weight and increased perigonadal fat pad weight as well as impaired glucose and insulin tolerance and changes in pancreatic beta cells. The same BPA treatment of non-pregnant females failed to affect these end points indicating that pregnancy is a sensitive window for persistent adverse effects of BPA.

4.6. Data from human studies

Data from some, but not all epidemiological studies suggest a positive association between urinary BPA levels and overweight or obesity, elevated waist circumference or BMI as well as altered glucose/insulin homeostasis, diabetes and cardiovascular disease in adults [17–19,24]. There are also data from preadolescent and adolescent ages [20,22,25,55], and children and teens from age 6–19 [23,25]. These cross sectional studies reveal positive correlations but cannot test causality.

Ongoing prospective studies should enhance our understanding of early life exposure to BPA and the development of obesity in humans. To date, there are a few reports of a correlation between prenatal/neonatal BPA levels and increased body weights in children. Valvi et al. [65] reported a correlation between maternal urinary BPA exposure during the 1st and 3rd trimester of pregnancy and BMI and waist circumference at age 4. Another recent study [66] revealed that increases in BPA levels at 4 years were associated with higher BMI and waist circumference at that age; however, in that same study prenatal BPA levels were negatively associated with adiposity measures in girls but positively correlated with those measures in boys. Braun et al. [67] reported that prenatal and early childhood BPA exposures were not associated with increased BMI at ages 2–5 years, but higher early childhood BPA exposure was associated with accelerated growth. Late pregnancy urinary BPA levels were reportedly associated with increased leptin levels in 9 year old boys and early pregnancy BPA levels were associated with altered adipokine levels in 9 year old girls [68]. Most recently, Hoepner et al. [69] reported positive correlations between prenatal urinary BPA concentrations and adiposity at age 7 years that were positively associated with fat mass index, and waist circumference in girls. More data will follow from these ongoing prospective studies although accurate assessment of prenatal BPA exposure in humans remains challenging [70].

5. In summary

Perinatal and perinatal plus peripubertal BPA exposure to environmentally relevant levels of BPA exert lasting effects on body weight and body composition in CD-1 mice. The effects were dose-specific and sex-specific and were clearly influenced by the precise window of BPA exposure. A sex difference was apparent in response to the addition of a second peripubertal exposure; the second exposure appeared to exacerbate adverse effects in the females but reduced differences in body weight and composition in the males. Although behavioral changes have been reported in BPA exposed animals (for review see Ref. [71]), to our knowledge the repetitive behaviors exhibited by a subset of our females have not been previously reported, and they represent a serious confound to studies of BW and body composition. If present in other labs, these extreme behaviors could contribute to the controversy in this area. In this study we chose to provide continuous exposure to low levels of BPA due to evidence that humans may be continuously exposed [9]. It is conceivable that the effects of continuous low level BPA exposure may differ from that of a single bolus that provides the same daily dose but at a single time point during the day.

Finally, BPA is only one chemical on a growing list of possible obesogens that could be contributing to the obesity epidemic in the developed world. The potential for additivity and synergistic interactions of these various chemicals is a topic just beginning to be explored.

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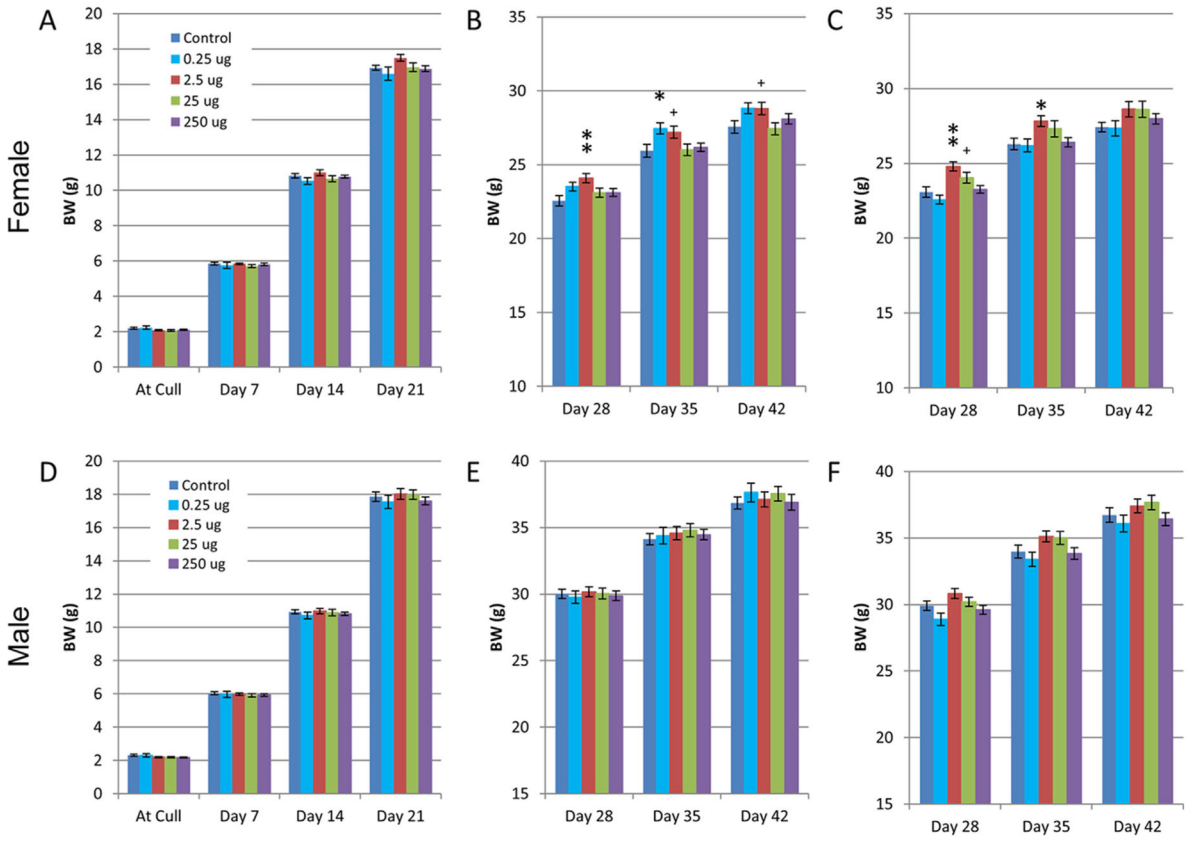

Fig. 1.

Body Weights from Postnatal Day 2–42. No differences in body weight by exposure level were noted prior to weaning (A, D); however, after weaning, P females (B) showed significant differences in body weight relative to controls. Differences between control and 2.5 μg BPA were significant at PND 28 ($p = 0.002$) and near significant at PND 35 and 42 ($p < 0.056$), and controls differed from 0.25 μg at PND 35 ($p = 0.025$). In P + P females, (C), overall differences were noted on PND 28 and 35 (ANOVA, $p < 0.05$) with controls differing from 2.5 μg on PND 28 ($p = 0.002$) and PND 35 ($p = 0.037$) and nearing significance in 25 μg females at PND 28 ($p = 0.05$). In males, BW was not increased above controls in any treatment group. Prior to weaning, n = 44–56 males and n = 44–56 females. After weaning n = 20–28 of each sex/group for each exposure window (P, P + P). ** $p < 0.005$, * $p < 0.05$ relative to controls, +p </ = 0.056.

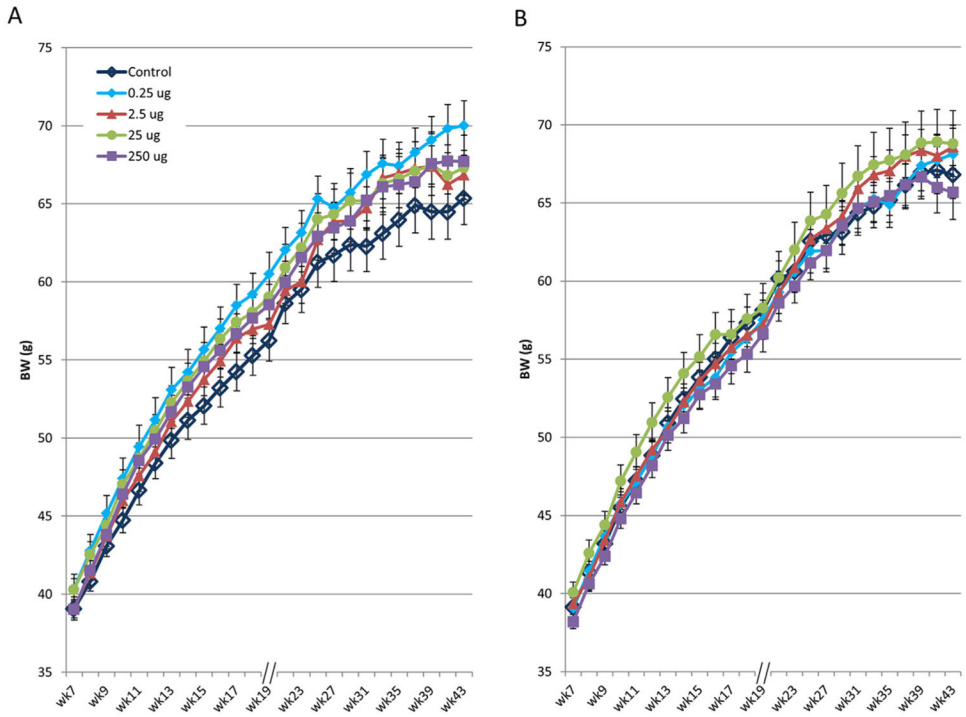

Fig. 2.

Body Weights of P and P + P males. (A) Body weights of P males showed overall significance by treatment and time ($p < 0.0001$). Although overall mean body weights of all exposure groups were higher than controls, the Bonferroni *post hoc* test failed to identify significant differences between any groups at the modified significance level of 0.005. (B) Comparison of the mean body weights of P + P males by treatment group showed overall significance by treatment and time ($p < 0.0001$) and the Bonferroni *post hoc* test failed to identify overall significance between groups. As depicted, differences between exposure groups were less distinct in the P + P males relative to their P brothers. (n = 18–27/group).

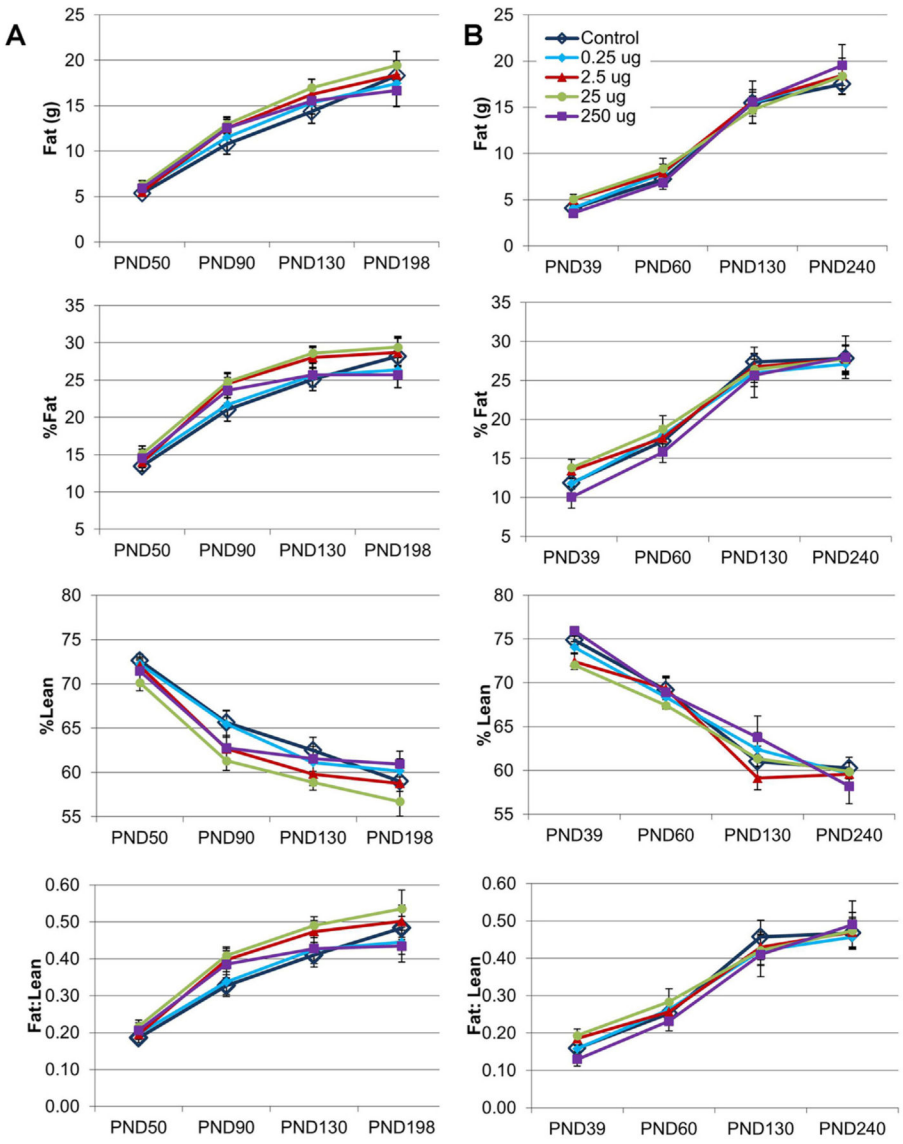

Fig. 3.

Body Composition (Echo MRI) of P males (Panel A) and P + P males (Panel B). In P males (A), overall significance of treatment was observed for fat mass, percent fat and percent lean ($p < 0.0001$). Bonferroni *post hoc* tests failed to reveal significant differences between groups at the modified p value of 0.005. As depicted, the 25 μg males were most different from controls. In the P + P males (Panel B), measurements were more overlapping. Overall differences in mean fat mass and in fat:lean ratio were not significant but percent fat and percent lean were ($p < 0.0001$). *Post hoc* tests failed to identify differences between groups which was not unexpected given the level of overlap in the data. (n = 7–13/group).

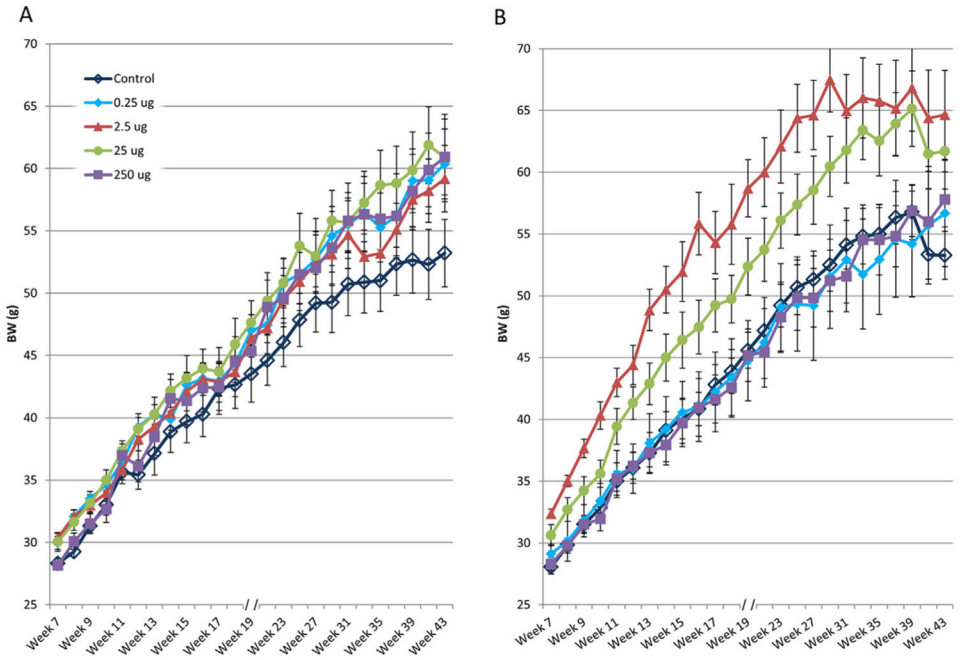

Fig. 4.

Body weights of P (A) and P + P (B) females over time. For P females (A), there was an overall statistical significance of treatment and time ($p < 0.0001$) with no interaction. As depicted, all treatment groups had higher mean body weights relative to controls; however, Bonferroni *post hoc* tests failed to identify differences between groups below the modified significance level of 0.005. For P + P females (B), analysis revealed significant effects of treatment and time ($P < 0.0001$) and a significant interaction ($p < 0.0001$) and therefore *post hoc* tests were not reported. As depicted the highest mean body weights were observed in 2.5 μg BPA females and then in 25 μg BPA females whereas the lowest and highest exposure groups had mean body weights similar to controls. (n = 6–13/group).

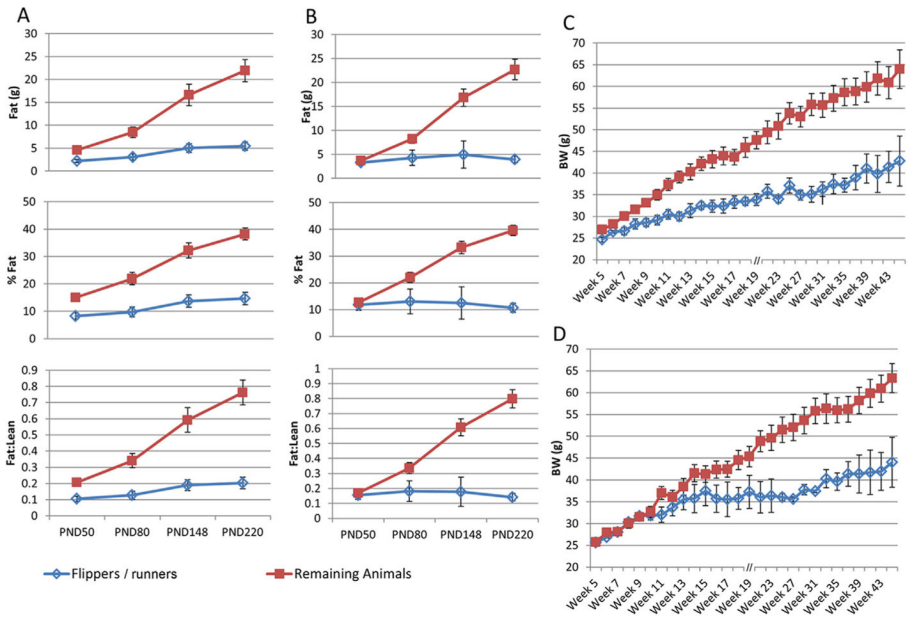

Fig. 5.

Comparisons of body weight and body composition in extreme hyperactive P females with the remainder of their exposure group. Data from extreme hyperactive P females that were excluded from analysis are shown in comparison with the compiled data from the remaining females from their respective groups, 25 μg BPA (A and C) and 250 μg BPA (B and D). As depicted, the extreme hyperactives weighed less than the remaining animals in their treatment groups despite similar bodyweights earlier in the study. As depicted, mean body composition measurements of the hyperactives remained constant over time and significantly lower than the mean measurements of their respective groups.

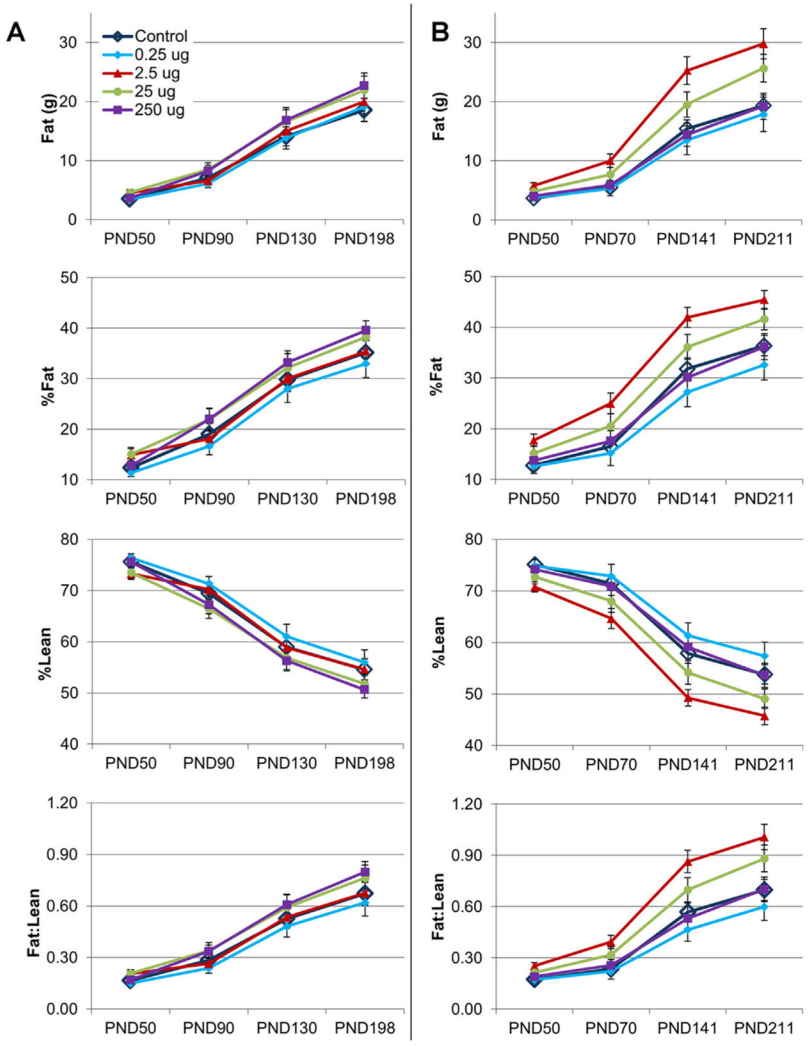

Fig. 6.

Body composition data (Echo MRI) of the P and P + P females without the extreme hyperactive mice. In P females (A), overall differences were observed in fat mass, percent fat, and percent lean by treatment and time ($p < 0.0001$) with no interaction. *Post hoc* tests (Bonferroni) failed to identify pairwise significant differences between groups. In P + P females (B), fat mass differed by exposure and time ($P < 0.0001$) with a significant interaction ($p < 0.0001$) precluding *post hoc* analysis. One way ANOVA for PND 141 revealed significant differences across groups ($p = 0.006$) with the 2.5 μg females differing from 0.25 μg and 250 μg females ($p = 0.022$, $p = 0.044$ respectively; Bonferroni) and from controls ($p = 0.031$; Dunnett's t). Percent fat and percent lean both showed overall differences by treatment and time ($P < 0.0001$). Percent fat of 2.5 μg females differed from 0.25 μg BPA ($p = 0.001$). Differences from controls and 250 μg BPA were significant @ $p = 0.007$, just above the modified significance level (Bonferroni). For percent lean, comparisons of 2.5 μg with 0.25 μg BPA was significant ($P = 0.001$) but for comparisons with control and 250 μg BPA, the significance was $p = 0.013$ which is above the modified significance level (Bonferroni). (n = 6–12/group).

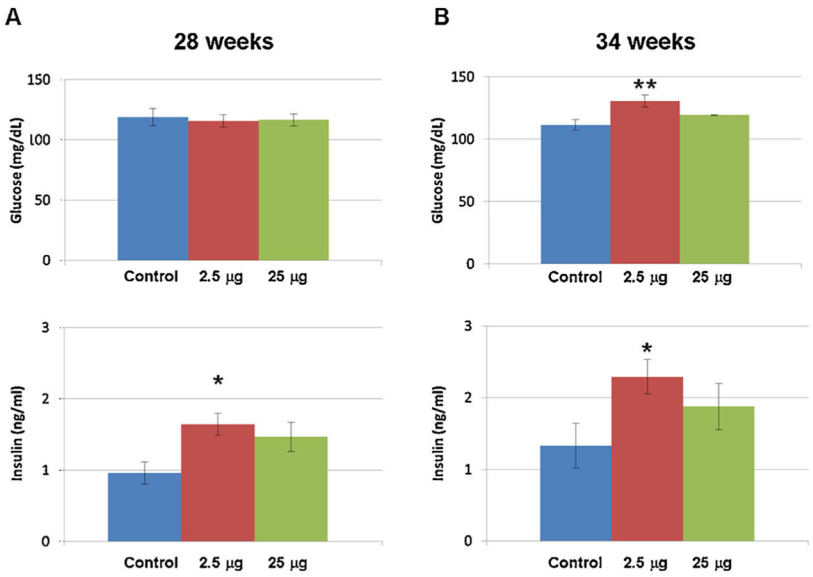

Fig. 7.

Glucose/Insulin Homeostasis. After a 6 h fast, glucose and insulin levels were measured at 28 weeks (A) and at 34 weeks (B) in control and P + P BPA exposed females. Insulin is increased at both time points in the 2.5 µg P + P females relative to controls ($p = 0.011$, $p = 0.024$; Dunnett's), and at 34 weeks glucose levels are also significantly elevated in the 2.5 µg females relative to controls ($p = 0.004$, Dunnett's t). ($n = 9–11$/group), $^{**}p < 0.01$ $^{*}p < 0.05$.

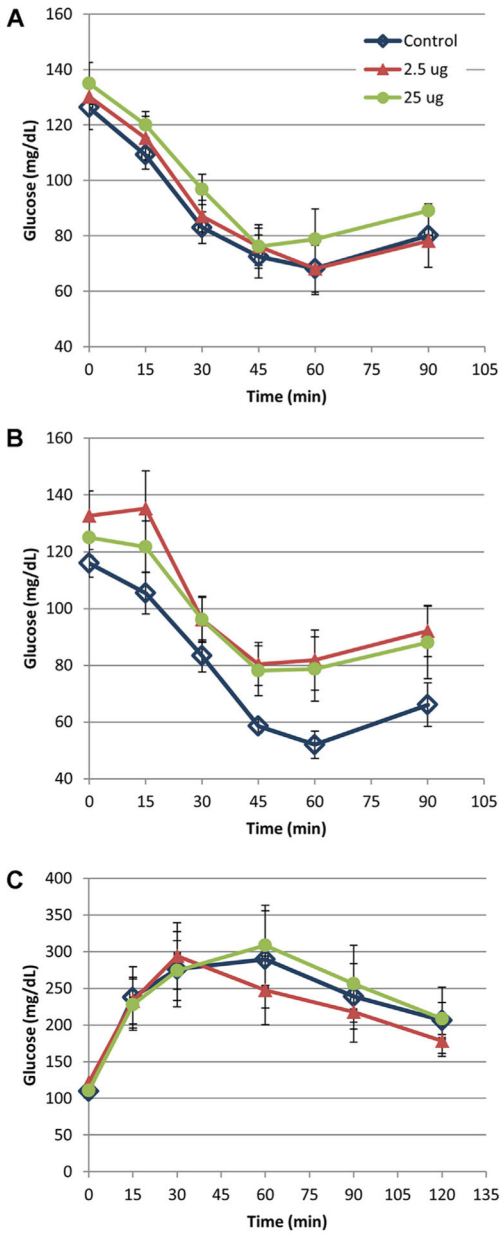

Fig. 8.

Insulin Tolerance Test (ITT) and Glucose Tolerance Test (GTT) data. ITT data was collected at 40 weeks in P (A) and P + P (B) females. Animals were fasted for 6 h prior to administration of insulin (ip, 0.75 IU/kg Insulin). A) P females in all exposure groups showed a similar pattern of mean glucose levels in response to insulin. B) P + P females revealed significant effects of treatment and time ($P < 0.0001$). Overall comparisons of 2.5 μg BPA *vs* control approached significance (0.07, Bonferroni). Dunnett's *t*-test revealed higher glucose levels in 2.5 μg females ($p = 0.042$) and in 25 μg females ($P = 0.046$) relative to controls at 45 min. C) GTT data from the P + P females did not reveal significant differences in response to an ip injection of 1.5 g/kg BW of glucose following a 12 h fast. (n = 9–11/group).

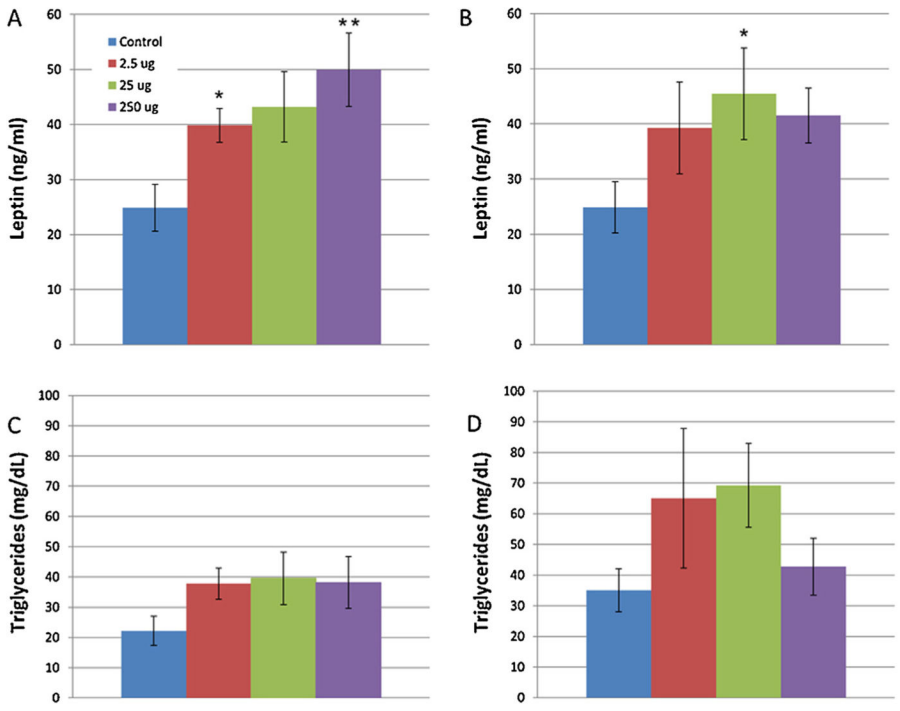

Fig. 9.

Serum leptin and serum triglyceride levels in P (A) and P + P (B) females. A. Serum leptin levels were elevated in 25 μg and 250 μg P females relative to controls (C *vs* 25 μg, p = 0.039, C *vs* 250 μg, p = 0.008, Dunnett's t). In P + P females (B), mean leptin levels were significantly elevated in the 25 μg females relative to controls (p = 0.045, Dunnett's t). Serum tryiglyceride levels for P (C) and P + P (D) females are shown. In both groups, BPA exposed females reveal higher mean levels of triglycerides relative to controls however the differences are not statistically significant. (n = 8–10/group), **p < 0.01, *p < 0.05.

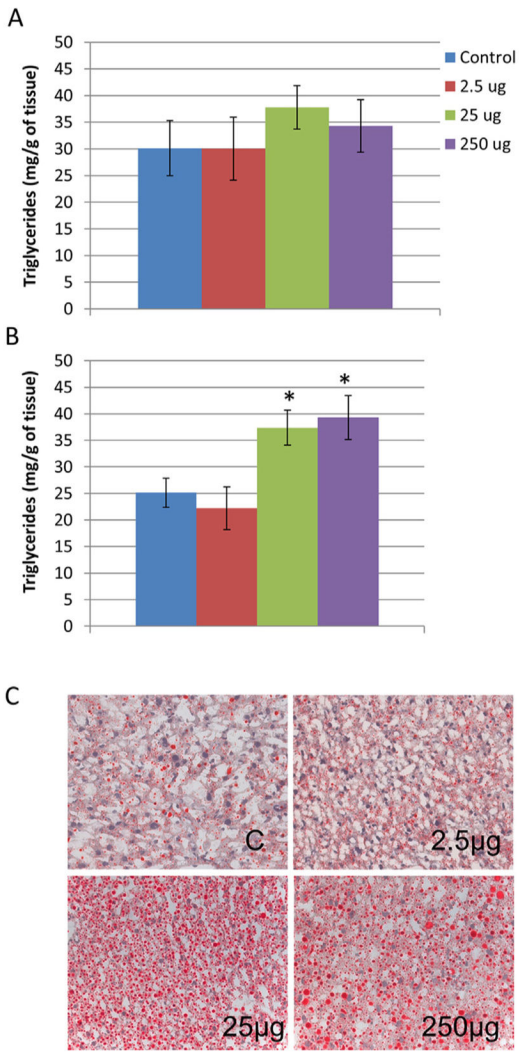

Fig. 10.

Assessments of Liver triglycerides in P (A) and P + P females (B). As depicted Liver triglycerides were significantly elevated in 25 μg and 250 μg P + P females (B) relative to controls ($p = 0.02$). (C) Oil Red O staining of 5 μm liver sections from P + P females suggested increased neutral lipid content in the 25 μg and 250 μg P + P females. C = control, 2.5 μg BPA, 25 μg BPA, 250 μg BPA. (n = 8–9/group), *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1

Body Weight (grams) of Dams at Gestational Day 6, 8, and at Weaning, and the Number of Pups Delivered.

Treatment	GD 6	GD 8	At Weaning (PND 21)	Mean Number of Pups Born
Control	29.44 ± 0.41	31.0 ± 0.51	39.55 ± 0.61	11.92 ± 0.37
0.25 μg	29.0 ± 0.41	31.4 ± 0.72	39.6 ± 0.92	12.5 ± 0.82
2.5 μg	29.34 ± 0.41	30.71 ± 0.40	40.58 ± 1.06	13.18 ± 0.40
25 μg	29.10 ± 0.39	30.33 ± 0.43	40.03 ± 0.67	12.57 ± 0.55
250 μg	29.18 ± 0.35	30.3 ± 0.47	39.82 ± 0.66	12.23 ± 0.41

Mean body weights (+/− SEM) of the dams did not differ by treatment groups at the time points assessed and mean litter size was comparable in all treatment groups.

Table 2

Mean Circulating Levels of Total and Unconjugated BPA.

Time Point	BPA Dose (ug/kg BW/day)	Animals	Incidence at or above LOD	Total BPA (ng/ml) Mean ± SEM	Range (ng/ml)	Unconjugated BPA
GD18	0	Dams	2/5	<LOD	0–0.3[a]	<LOD
	0	Fetuses-Pooled	N/A	<LOD	N/A	<LOD
	25	Dams	3/5	0.7 ± 1.14	0–2.7	<LOD
	25	Fetuses-Pooled	N/A	0.6	N/A	<LOD
	250	Dams	3/5	1.48 ± 2.33	0–5.5	<LOD
	250	Fetuses-Pooled	N/A	4.0	N/A	<LOD
PND32	0	Pups	2/5	<LOD	0–0.3[a]	<LOD
	25 (in water)	Pups	5/5	0.74 ± 0.09	0.5–1	<LOD
	250 (in water)	Pups	4/4	5.875 ± 1.09	4.5–8.6	<LOD

N/A = not applicable. Free BPA = Unconjugated BPA. Level of Detectability (LOD) = 0.3 ng BPA/ml. LOQ (level of BPA that can be quantified with accuracy and precision) = 0.9 ng/ml.

[a]All assessments are below the LOQ. Measurements of circulating BPA levels in dams and pups at PND 11 failed to reveal mean levels of Total or Unconjugated BPA above the LOD and no individual measurements were at or above the LOQ.