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ORIGINAL ARTICLE

Sex-dependent effects of developmental exposure to bisphenol A and ethinyl estradiol on metabolic parameters and voluntary physical activity

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Endocrine disrupting chemicals (EDC) have received considerable attention as potential obesogens. Past studies examining obesogenic potential of one widespread EDC, bisphenol A (BPA), have generally focused on metabolic and adipose tissue effects. However, physical inactivity has been proposed to be a leading cause of obesity. A paucity of studies has considered whether EDC, including BPA, affects this behavior. To test whether early exposure to BPA and ethinyl estradiol (EE, estrogen present in birth control pills) results in metabolic and such behavioral disruptions, California mice developmentally exposed to BPA and EE were tested as adults for energy expenditure (indirect calorimetry), body composition (echoMRI) and physical activity (measured by beam breaks and voluntary wheel running). Serum glucose and metabolic hormones were measured. No differences in body weight or food consumption were detected. BPA-exposed females exhibited greater variation in weight than females in control and EE groups. During the dark and light cycles, BPA females exhibited a higher average respiratory quotient than control females, indicative of metabolizing carbohydrates rather than fats. Various assessments of voluntary physical activity in the home cage confirmed that during the dark cycle, BPA and EE-exposed females were significantly less active in this setting than control females. Similar effects were not observed in BPA or EE-exposed males. No significant differences were detected in serum glucose, insulin, adiponectin and leptin concentrations. Results suggest that females developmentally exposed to BPA exhibit decreased motivation to engage in voluntary physical activity and altered metabolism of carbohydrates *v.* fats, which could have important health implications.

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Key words: BPA, California mouse, DOHaD, EDC, exercise

Introduction

At least 35% of the US population is obese, with costs for treating ailments relating to this condition in 2008 estimated at $147 billion.[1] More than one-third of children are obese with a predisposition to type 2 diabetes mellitus (T2DM) and most of them will remain so as adults. The World Health Organization has estimated that close to 350 million people world-wide already have T2DM and that this number is increasing annually, particularly in developing countries as populations gain greater access to a so-called Western-style diet and become more sedentary.[2] A 2014 report by the Institute of Medicine Roundtable on Obesity and Solutions suggested that obesity has been curbed in some US states, likely due to increased physical activity and improved nutrition.[3] Yet, this summary warned that obesity rates continue to rise in other states, and importantly, the incidence of severe obesity has escalated dramatically. Further reports indicate that children are more physically inactive now than they were in past decades, which may be due to a convenient lifestyle with automated transportation, reduced accessibility to parks and other areas to play, and increasing amount of time engaged in sedentary activities.[4–6] However, the dramatic rise in physical inactivity and obesity also suggests that environmental chemicals may be contributing to these disorders.[7]

There has been mounting interest as to whether developmental exposure to endocrine disrupting chemicals (EDC), including bisphenol A (BPA) may be significantly contributing to the epidemic. Most EDC are manufactured chemicals.[8] BPA is one of the most ubiquitous,[9–11] with production reported to

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be 6.8 billion kilograms in 2013.[12] Its stability and pervasiveness[13] ensure continued exposure.[14] BPA is detectable in the urine of 93% of the US population,[15] as well as in fetal plasma, placenta[16] and breast milk.[17] In 2012, the FDA banned the production of baby bottles and sippy cups containing BPA.[18] This restriction though fails to address the transfer of BPA across the placenta and through the milk.[19–23] Moreover, fetuses and neonates lack many enzymes needed to metabolize BPA and may experience greater levels of active BPA than the mother.[20–22]

Human epidemiological and animal model data provide evidence that early exposure to BPA is associated with later obesity[24–46] with the potential for transgenerational transmission.[32] However, other conflicting rodent and human data suggest either decreased body weight or no response to BPA exposure.[47–49]

The majority of the studies examining underlying mechanisms as to the obesogenic effects of BPA have focused on how this chemical affects cellular adipogenesis and differentiation,[37,50–54] metabolism[28,31,33,40,42,44,55] and hunger/satiety.[31,56] There is scant information on how BPA exposure affects *in-vivo* metabolic function and voluntary (spontaneous) physical activity. Notably, the amount of time of rodents spend engaged in spontaneous activity within their home cage is a strong predictor of later adiposity and weight gain.[57–59]

Physical inactivity has risen to be the leading cause of many chronic, non-communicable diseases.[60–67] A few examples of the 35 chronic conditions linked with physical inactivity include obesity, other metabolic disorders (including T2DM), coronary heart disease, other cardiovascular disorders (hypertension, stroke, and congestive heart failure), depression, anxiety, cognitive dysfunction, osteoporosis and cancer. Current research has largely focused on understanding the underpinning internal causes of physical inactivity, such as genetic-predisposition, sex, overall health status, self-assessment and motivation with some interest in how the surrounding social and physical environment impacts this behavior.[68–71] There is a major deficit in our understanding of how the *in utero* and postnatal environment shapes later motivation to engage in voluntary physical activity.

To address how BPA affects metabolism, adipose deposition and voluntary physical activity, we tested these parameters in California mice (*Peromyscus californicus*) developmentally exposed to BPA or ethinyl estradiol (EE) (estrogen present in birth control pills). Our prior studies indicate that early contact to these EDC in California mice can lead to other behavioral disruptions.[72] In addition, we sought to determine whether developmental exposure to BPA and EE leads to sex-dependent differences in these parameters, as we have observed in prior studies with this animal model[72] and their related deer mice (*Peromyscus maniculatus bairdii*) cousins.[73,74] This outbred animal model was also chosen as they may better mirror the genetic diversity of most human populations, and they have been proposed to be a good animal model for human metabolic disorders, including T2DM.[75]

Materials and methods

Animal husbandry

Founder outbred adult (60–90 days of age) California mouse females and males, free of common rodent pathogens, were originally purchased from the Peromyscus Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC, USA), and placed in quarantine for a minimum of 8 weeks to ensure that they did not carry any transmittable and zoonotic diseases. From the time the animals had been captured between 1979 and 1987, *P. californicus* captive stocks have been bred by the PGSC to maintain their outbred status. We have since established our own breeding colony at the University of Missouri. Additional animals are purchased, as needed, from the PGSC to maintain the outbred line. All experiments were approved by University of Missouri Animal Care and Use Committee (protocol #7753) and performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Two weeks before breeding, virgin females, 8–12 weeks of age were randomly assigned to receive one of the three diets: (1) a low phytoestrogen AIN93G diet supplemented with 7% by weight corn oil to minimize potential phytoestrogenic contamination that would otherwise be present with inclusion of soybean oil in the diet, (2) the same diet supplemented with 50 mg BPA/kg feed weight, which we have documented to lead to internal serum concentrations close to those measured in pregnant women unknowingly exposed to this chemical[73,76] and (3) AIN93G diet supplemented with 0.1 parts per billion of EE, as the US Food and Drug Administration (FDA) required estrogen positive control for BPA studies.[77] The FDA has requested EE be included in BPA studies that may guide policy decisions based on the premise that BPA acts primarily as a weak estrogen.[14] The diets were started 2 weeks before breeding to span the periconceptional period. Females were maintained on these diets throughout gestation and lactation, as described previously.[72–74] The F_1 generation sons and daughters were weaned at 30 days of age and placed on the AIN control diet. To avoid any potential litter effects, where possible, we only examined one male and one female offspring per litter.

Neonatal to post-weaning body weight measurements

At postnatal day (PND) 2, F_1 pups from the three groups above were weighed (OHAUS CS200, Parsippany, NJ, USA) every other day from PND 2 to 20 and then again before and after weaning at PND 30. In litters, where there was more than a single pup, individual pups were demarcated with a distinguishing tattoo on either their front or back paws (Fine Science Tools, Foster City, CA, USA). The number of pups and litters tested is indicated in Supplementary Table S1. These F_1 pups were not used for any other tests.

Indirect calorimetric testing

A separate group of F_1 adult (90 days of age) animals were tested in the Promethion continuous measurement indirect calorimetry

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system (Sable Systems International, Las Vegas, NV, USA) for 3 days. As recommended by the company, the 1st day was considered the habituation period, and thus, measurements are only based on the last 2 days. Data were broken down into 12-h light and 12-h dark cycles. The system continuously measured each individual cage for every second of the day as opposed to most multiplexed systems, which only measure one cage at a time and thus only measure an animal's metabolism for a certain percentage of the day. Continuous measurement systems thus dramatically increase the resolution of the system and its ability to track how spontaneous activity drives energy expenditure and respiratory quotient (RQ). In addition to measuring energy expenditure and RQ from oxygen consumption and CO_2 production, the system also continuously measures activity by beam breaks (X – vertical, Y – horizontal and Z – rearing), food and water intake including monitoring the times the hoppers are touched and the amount consumed at each touch. This equipment measures all of the parameters listed above and additional ones detailed in Supplementary Table S2. The total number of F_1 offspring and litters examined in the indirect calorimetric unit is listed in Supplementary Table S1. All attempts were made to test these same animals for the other tests described below. Exceptions are discussed in the relevant sections.

Voluntary wheel running

Voluntary wheel running was measured after indirect calorimetry was performed. BPA-free exercise wheels (Kaytee, Chilton, WI, USA) with a diameter of 5.75 inches were used for these studies. The wheels were connected to a bicycle computer (Sigma Sport BC12.12; Sigma Sport USA LLC, St. Charles, IL, USA) to measure total distance traveled, average speed, maximum speed and total time spent running on the wheels (Supplementary Fig. S1). The animals were tested with the exercise wheels for 5 days in a row. The total number of F_1 offspring and litters examined with voluntary wheel running is listed in Supplementary Table S1. Voluntary wheel running was not tested in the first group of animals measured in the indirect calorimetry because it was only after we analyzed their data that we realized that they engaged in less voluntary physical activity.

EchoMRI

After the voluntary wheel running experiments were completed, the animals were placed in the EchoMRI-1100 (EchoMRI LLC, http://www.echomri.com/Body_Composition_Rats_2MHz.aspx, Houston, TX, USA) to measure body composition. In seconds, this equipment measured total body fat, free water (that is present in the stomach and urinary bladder), and total water mass in a non-invasive manner and without the use of anesthesia. This system is highly accurate for animals weighing between 7 and 1100 g. The total number of F_1 offspring and litters examined with echoMRI is listed in Supplementary Table S1. EchoMRI was not performed in the first group of animals tested in the indirect calorimetry as the instrument was purchased and installed after this group was euthanized.

Serum hormone analyses

Food was removed at ~ 17:00 h the day before serum collection. The next day, fasted animals were humanely euthanized in accordance with our approved animal protocol (#7753) at ~ 09:00 h, and cardiac blood was collected by using a 25 gauge needle attached to a 1 ml syringe (Fisher Scientific, St. Louis, MO, USA), then placed in a 1.5 ml microcentrifuge tube, and stored on ice. After the serum clotted (~20 min after collection), the blood was centrifuged at 7500 g for 20 min, and the serum fraction pipetted and transferred to a new sterile 1.5 ml centrifuge tube. Samples were stored at −20°C until they were analyzed.

Serum glucose was determined by using a commercial clinical chemistry analyzer (Beckman-Coulter AU680, Brea, CA, USA) and automated, commercially available assay (Beckman-Coulter, Brea, CA, USA). Plasma insulin (Crystal Chem, Downers, Grove, IL, USA. Catalog # 90080), adiponectin (Crystal Chem, Catalog # 80569) and leptin (Crystal Chem, Catalog # 90030) concentrations were analyzed in general according to the manufacturer's instructions for each of these ELISA kits. However, for adiponectin, plasma was diluted 1:2 to 1:6 before analyses. Serum insulin and leptin were analyzed with undiluted samples. The number of replicates tested for each group is listed in Supplementary Table S1. Because of insufficient serum, hormone and metabolite analyses could not be measured in some of the adult animals tested in the indirect calorimetry unit.

Statistical analyses

SAS version 9.2 software analyses software (SAS Institute, Cary, NC, USA) was employed for these analyses. Unless otherwise stated, the reported data are based on mean ± standard error of the mean.

F_1 pup body weight measurements

These data were analyzed as a randomized complete block design in which the model contained the effects of parents (combination of dam and sire), day, sex and the interaction of day X sex. All mean differences were determined by using Fisher's least significance difference (LSD). PROC MIXED procedure in SAS 9.2 was used in this analysis.

Indirect calorimetric testing

The data were analyzed as a repeated measurement analysis in which the main plot contained the effects of the three maternal diets and two offspring sexes. The denominator of F for the main plot was litter within maternal diet and offspring sex. The subplot contained the time series of both in day and cycle. The day and cycle were factorial arranged in which the cycle contained two cycles (dark and light) and day contained the 2 days in which animals were measured in this unit. The subplot effect of day and cycle and day × cycle and the

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interactions of day and cycle with the main plot effect were tested using litter within maternal diet, offspring sex, day and cycle as the denominator of F. Fisher's protected LSD was tested if the overall of F was significant.

EchoMRI

The data were analyzed as a complete randomized design (CRD) in which treatments were arranged as a three by two factorial (three maternal diets and two offspring sexes). Since some pups came from the same litter, dam within maternal diet and offspring sex was used as the denominator of F. If the overall F was significant, then differences were determined using Fisher's protected LSD.

Voluntary wheel running

The data were analyzed as a repeated measurement design in which the main plot contained maternal diet and offspring sex and maternal diet × offspring sex in a three by two factorial design. The subplot contained day and all possible interactions with the main plot effect. The denominator of F for the main plot was dam within maternal diet and offspring sex. The denominator of F for the subplot effects was dam within maternal diet, offspring sex and day. Mean differences were determined using Fisher's protected LSD when the overall *F* test was significant.

Serum hormone data

For these data, the litter was considered as the experimental unit. The data were analyzed as a simple 3 × 2 factorial arrangement (three treatments and two sexes) considering the design as a CRD. If the overall F was significant, a Fisher's protected LSD was performed.

Results

Eating and drinking

There were no differences based on diet, cycle and sex for total amount of food consumed (Fig. 1a and 1b). In the AIN and EE groups, males consumed more food during the dark compared with the light cycle (*P* value range = 0.05). No such difference though was detected in BPA males. BPA females ate more during the light than the dark cycle ($P = 0.04$). EE females ate more during the light cycle than EE males ($P = 0.05$). Total feeding time varied based on sex, diet and cycle (Fig. 1c and 1d). AIN and EE females spent more time eating during the dark than light cycle (*P* value range ⩽0.0001–0.001). During the dark cycle, EE females spent more time eating than BPA females (Fig. 2c, $P < 0.04$). Males in the EE group spent more time eating in the dark cycle ($P = 0.0001$).

Total water uptake and drinking episodes varied according to maternal diet, sex and cycle (Supplementary Fig. S2a and b). BPA males drank less water than AIN males during the dark and light cycles (Supplementary Fig. S2b, *P* value range = 0.01). During the dark cycle, BPA males also drank less water than BPA females ($P = 0.01$). In the EE group, females drank more water during the light cycle ($P = 0.03$). BPA females exhibited decreased drinking episodes during the dark cycle than AIN females (Supplementary Fig. S2c, $P = 0.02$). For all maternal treatment groups and sexes, the bouts of drinking was more

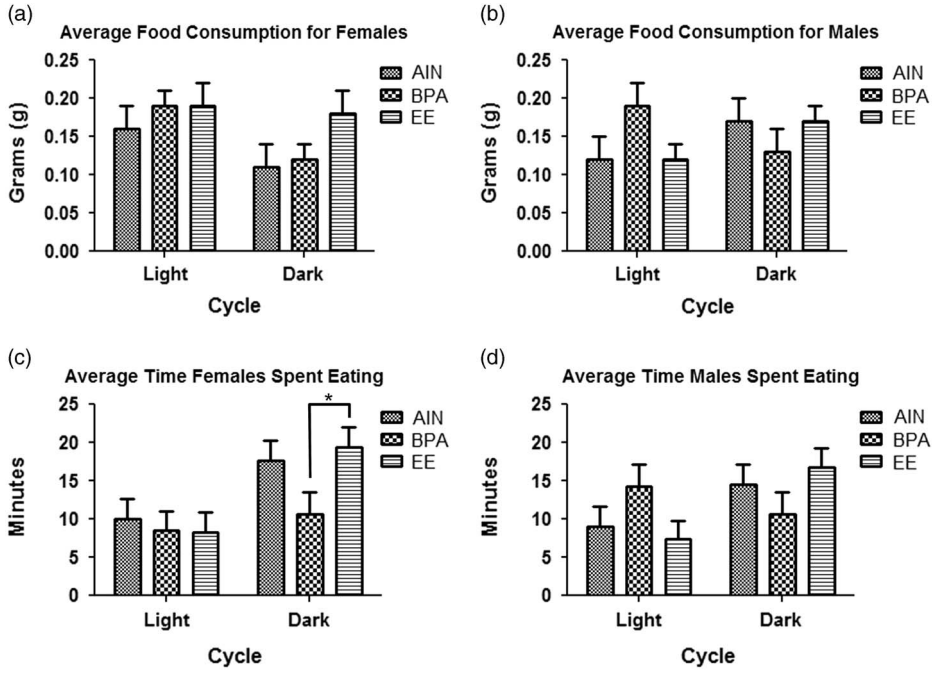

Fig. 1. Total amount eaten and eating episodes. (*a*) Total amount eaten for females. (*b*) Total amount eaten for males. (*c*) Feeding episodes for females. (*d*) Feeding episodes for males. *$P = 0.04$. BPA, bisphenol A; EE, ethinyl estradiol.

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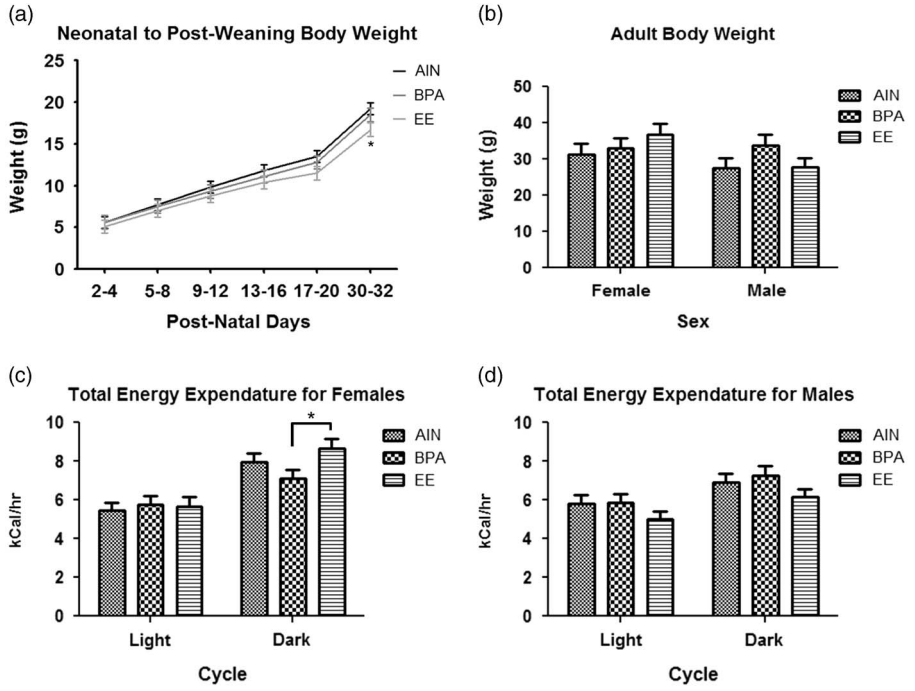

Fig. 2. Body weight and energy expenditure. (*a*) Body weight for females and males. (*b*) Total energy expenditure for females. (*c*) Total energy expenditure for males. *$P = 0.02$. BPA, bisphenol A; EE, ethinyl estradiol.

during the dark cycle (Supplementary Fig. S2c and d, P value range <0.0001–0.0007). During this time period, females in the AIN group also engaged in more drinking episodes compared with AIN males ($P = 0.02$).

Body weight, energy expenditure and RQ

For the neonatal to peri-weaning body weight assessments, no differences were detected based on sex of the F_1 pups. Therefore, male and female pup data within each litter were combined. From birth to 2 days post-weaning, there were no differences in body weight in BPA compared with control pups (Fig. 2a). At the peri-weaning stage, EE pups weighed less than controls ($P = 0.02$). There were, however, no differences in body weight at 90 days of age for females or males in any of the treatment groups (Fig. 2b).

Total energy expenditure indicates how many calories are burned or, in other words, it is sum of internal heat produced and external work. There were differences in this category based on maternal diet, sex and cycle. BPA females expended less total energy during the dark cycle than EE females (Fig. 2c, $P = 0.02$). In all maternal diet groups and sexes, more calories were burned during the dark compared with the light cycle (Fig. 2c and 2d, P value $\leqslant 0.0001$). EE females had greater total energy expenditure than EE males during the dark cycle (P value $\leqslant 0.0001$).

A greater RQ indicates that an animal is burning more carbohydrates relative to fats (Fig. 3a and 3b). During the light and dark cycles, BPA females exhibited a higher average RQ than AIN females (P value range = 0.02–0.03). The resting RQ for the 30 min (R_RQ_30) of lowest activity was greater in BPA females during the dark and light cycles compared with AIN females (Fig. 3c, $P = 0.02$–0.01). BPA females also showed an elevated R_RQ_30 compared with BPA males during both cycles (Fig. 3c and 3d, $P = 0.03$). The average RQ during 15 min of peak energy expenditure (A_RQ_15) was greater in BPA females than AIN females (Figure S3, $P = 0.03$). No differences in A_RQ_15 were detected for the male groups.

Voluntary physical activity

The indirect calorimetry unit allows several assessments of voluntary physical activity in a home cage system, including the total number of times the animals breaks beams on the X, Y and Z axis, which allows for calculations of total meters traveled for both running and voluntary locomotion, total meters walked or pedestrian locomotion, walking or pedestrian speed, percentage of time spent walking, percentage of time remaining still in the home cage, percentage of time spent sleeping and total hours spent sleeping.

During the dark cycle, AIN females broke the XYZ beams more times than BPA and EE females, suggestive of decreased activity in these latter treatment groups (Fig. 4a, P value range = 0.03–0.05). For all groups and sex, the combined beams were broken more times during the dark than the light cycle (Fig. 4a and 4b, P value range = 0.0001). In the AIN group, there was also a trend for females to demonstrate this behavior more than males during the dark cycle ($P = 0.06$).

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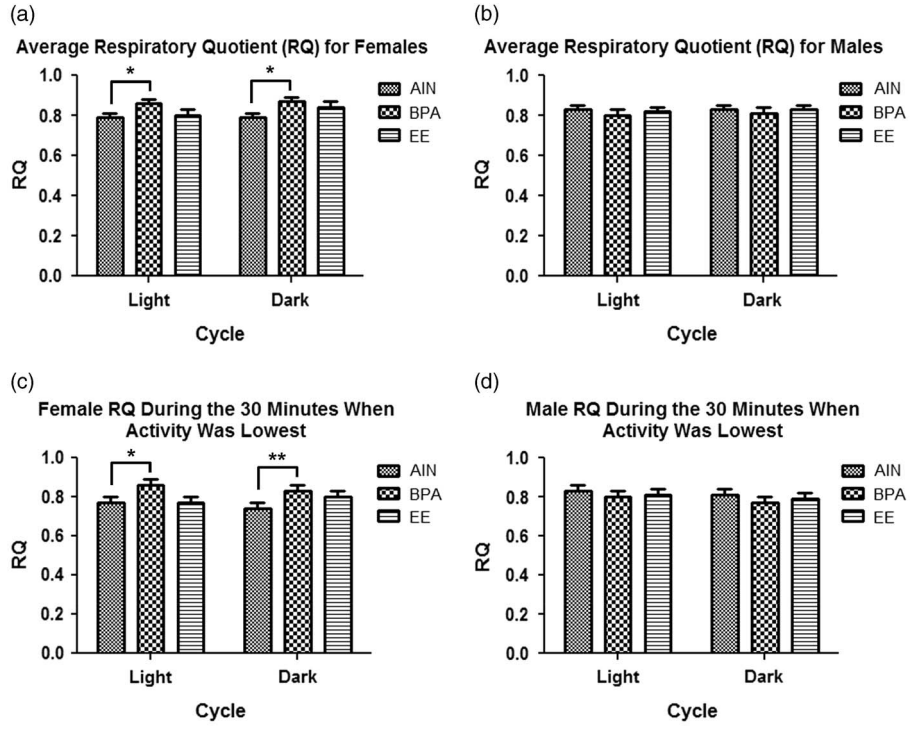

Fig. 3. Respiratory quotient (RQ). (*a*) Average RQ for females. (*b*) Average RQ for males. (*c*) Resting RQ for 30 min of lowest activity (R_RQ_30) for females. (*d*) R_RQ_30 for males. $^{*}P \leqslant 0.05$, $^{**}P = 0.01$. BPA, bisphenol A; EE, ethinyl estradiol.

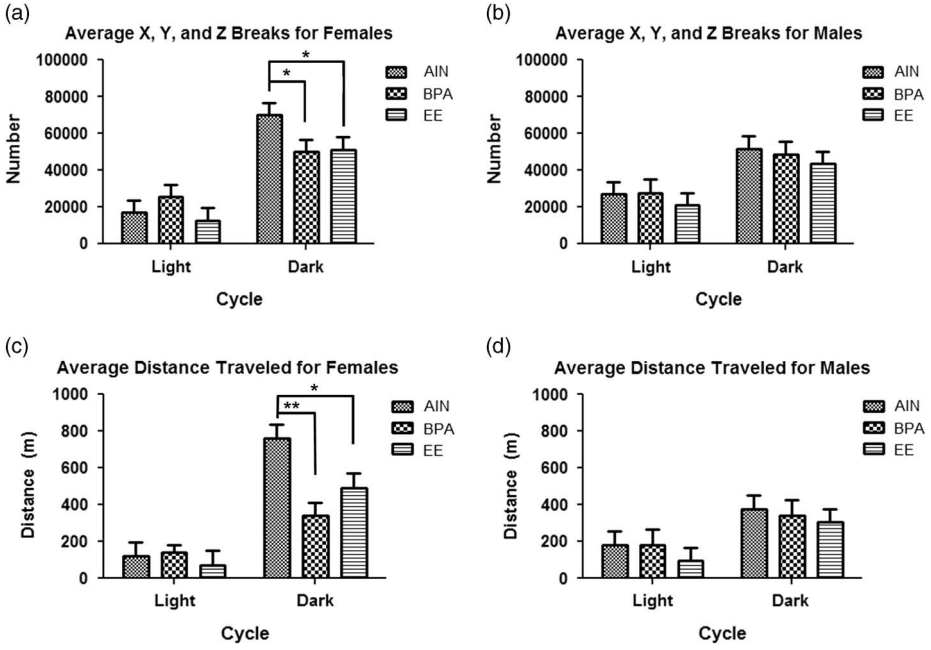

Fig. 4. XYZ beam breaks and total distance traveled. (*a*) XYZ beam breaks for females. (*b*) XYZ beam breaks for males. (*c*) Total distance traveled for females. (*d*) Total distance traveled for males. $^{*}P \leqslant 0.05$, $^{**}P = 0.0003$. BPA, bisphenol A; EE, ethinyl estradiol.

Consistent with the XYZ beam break data, AIN females traveled more distance during the dark cycle than BPA and EE females (Fig. 4c, *P* value range = 0.0003–0.02). For all groups and sex, the combined distanced traveled was greater during the dark than the light cycle (Fig. 4c and 4d, *P* value range = 0.0001–0.002). AIN females traveled more distance than AIN males during the dark cycle (*P* value = 0.0007).

The walking speed also varied according to maternal treatment, sex and cycle. AIN females were quicker than BPA females during the dark cycle (Fig. 5a, $P = 0.004$). Females in all three

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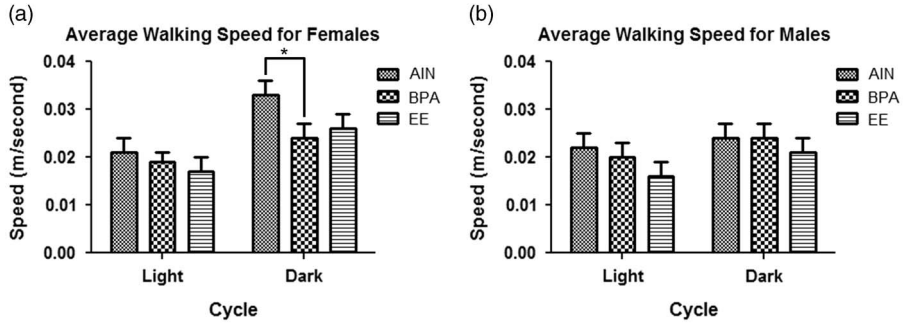

Fig. 5. Average walking speed. (*a*) Walking speed for females. (*b*) Walking speed for males. *$P = 0.004$. BPA, bisphenol A; EE, ethinyl estradiol.

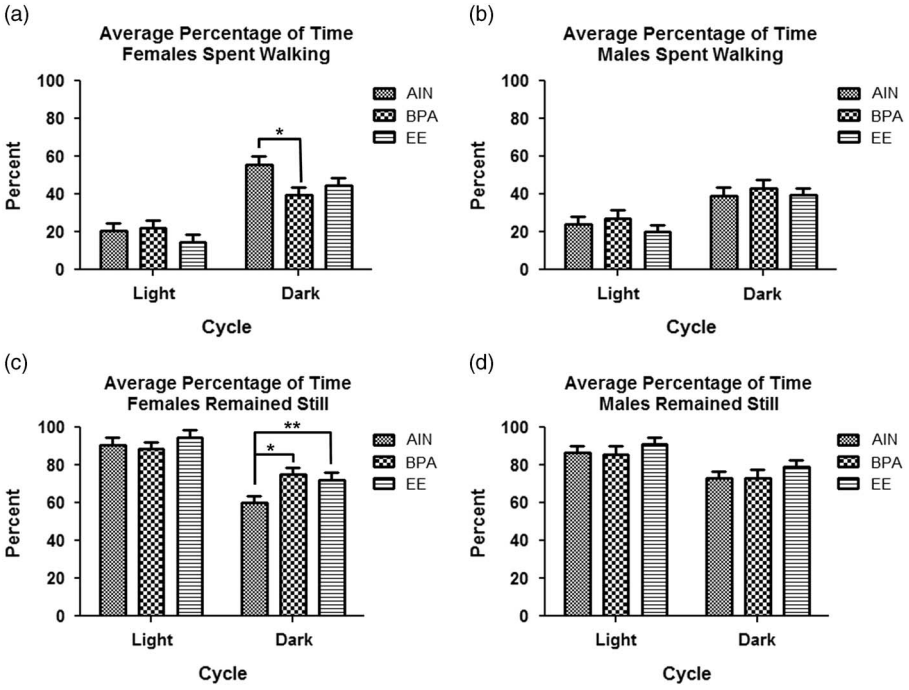

Fig. 6. Percentage of time spent walking and remaining still. (*a*) Percentage of time spent walking for females. (*b*) Percentage of time spent walking for males. (*c*) Percentage of time spent remaining still for females. (*d*) Percentage of time spent remaining still for males. *$P = 0.009$, **$P = 0.03$. BPA, bisphenol A; EE, ethinyl estradiol.

treatment groups were speedier during the dark compared with the light cycle (Fig. 5a and 5b, *P* value range = 0.0001–0.006). In contrast, only males in the EE group demonstrated any difference in speed based on the dark compared with light cycle (*P* value = 0.007). AIN females walked faster than AIN males during the dark cycle (*P* value = 0.04).

The percentage of time spent walking and remaining still also differed based on all three variables. AIN females spent greater percentage of the dark cycle walking around the home cage than BPA females (Fig. 6a, *P* value = 0.009). The percentage of time spent walking during the dark cycle was greater for all treatments and sex than the light cycle (Fig. 6a and 6b, *P* value < 0.0001). In the AIN group, females spent greater percentage of time walking around during the dark cycle than AIN males (*P* value = 0.009). The percentage of time remaining still during the dark compared with the light cycle showed the opposite results as percentage of time spent walking. During the dark cycle, AIN females spent less time remaining still compared with BPA and EE females (Fig. 6c, *P* value range = 0.004–0.03). All treatments and sexes spent less percentage of time remaining still in the dark *v.* the light cycle (Fig. 6c and 6d, *P* value < 0.0001). AIN males spent greater percentage of time remaining still during the dark cycle than AIN females (*P* = 0.02).

In line with the above results, time spent sleeping and total hours asleep varied according to maternal diet, sex and cycle. AIN females spent less percentage of time and total hours sleeping compared with BPA females (Fig. 7a and 7c, *P* value range = 0.04–0.004). Percentage of time spent sleeping and total hours asleep for all treatments and sexes was predictably

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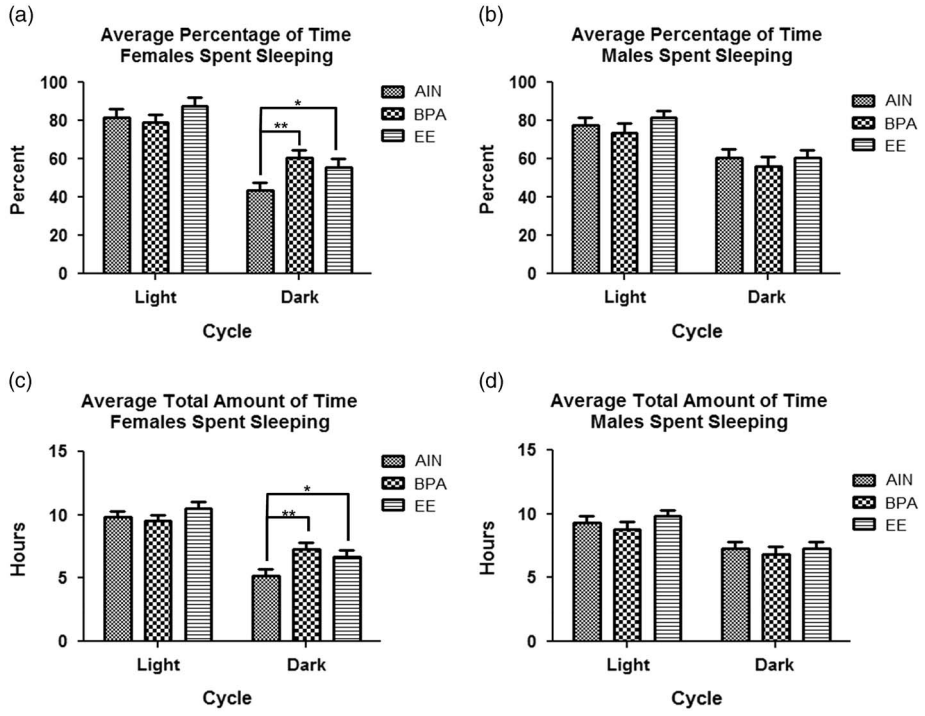

Fig. 7. Percentage of time and total hours spent sleeping. (*a*) Percentage of time spent sleeping for females. (*b*) Percentage of time spent sleeping for males. (*c*) Total hours spent asleep for females. (*d*) Total hours spent asleep for males. $^{*}P = 0.04$, $^{**}P = 0.004$. BPA, bisphenol A; EE, ethinyl estradiol.

less during the dark compared with the light cycle (Fig. 7, P value < 0.0001). In contrast to the above behaviors, there were no sex differences in sleeping percentage or total hours slept for the AIN group.

echoMRI results

There were no differences in body fat or total and free water for AIN males or females (Supplementary Figs S4, S5a and b). The only difference in this experiment was that BPA males averaged more free water than EE males (Supplementary Fig. S5b, $P = 0.03$). It is not clear though the significance of this finding.

Voluntary wheel running

There were no differences in distance traveled or average speed based on maternal diet or offspring sex (Supplementary Fig. S6).

Serum glucose and metabolic hormone analyses

There were no differences in glucose, insulin, leptin or adiponectin based on maternal diet or offspring sex (Supplementary Fig. S7).

Discussion

There were three main goals of this work. The first was to determine whether developmental exposure to BPA or EE in our rodent model, California mice, altered whole body outcome measures, including food intake, systemic energy metabolism and correspondingly increased body weight. The second attendant goal was to determine if exposed animals exhibited alterations in home cage activity and voluntary physical inactivity. Finally, we sought to determine whether BPA and EE-exposed animals demonstrated alterations in serum metabolites and metabolic hormones that could potentiate their risk for obesity and T2DM.

In terms of the first goal, we did not detect any difference in body weight from PND 2 to peri-weaning in BPA compared with control pups. For reasons that are not clear, EE pups weighed less than control pups at peri-weaning. However, we did not detect any differences in body weight for male and female offspring in the BPA and EE groups when they were tested as adults.

Several prior animal model and human studies have examined for an association between developmental exposure to BPA and increased body weight later in life. Our current findings are consistent with an earlier report showing no differences at adulthood for CD-1 mice exposed during gestation and lactation to one part per billion BPA in the diet compared with controls.[49] Another study with the same strain of mice perinatally exposed to BPA showed increased body weight later in life.[31] Body composition was not different throughout the lifespan for female or male C57Bl6J mice perinatally exposed to BPA.[47] A cross-sectional study in humans suggested that 9 year old daughters whose mothers

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who had greater BPA concentrations while pregnant were more likely to exhibit decreased body mass index, body fat and overweight/obesity; whereas, children with higher urinary BPA concentrations at this age showed greater adiposity.[48] Other rodent and human studies suggest that early exposure to BPA is associated with increased body weight later in life.[24–46] The conflicting animal model data may be due to the species examined, varying windows of exposure (periconceptional, gestational and/or lactational), dose tested and assement period (weanling, pubertal or adulthood). It is possible that differences based on BPA/EE exposure and sex might emerge with age, although 90 days of age, when the animals were examined herein, is routinely used for adult testing.

In many inbred rodent models, such as C57Bl6J, males weigh 10–30% more than females.[78,79] However, such sex differences were not observed in California mice on the AIN control diet. Adult California mice have been reported to weigh around 33.2–54.4 g,[80] which is similar to our current findings. Other studies with California mice indicate that males and females demonstrate comparable body weight (table 2 in[81]) and (table 6 in[82]). *Peromyscus* are evolutionarily distinct from *Mus* and *Rattus* with over 25 million years of separation from a common ancestor.[83] It is thus not surprising that sexually dimorphic differences in body weight are absent in California mice. In addition, the life history and sexual selection pressures in this species are distinct from most laboratory rodent models. Unlike mice and rats, California mice are monogamous, biparental, and both males and females defend the nest and surrounding territory.[84–86]

While a difference in total amount of food consumed was evident based on light cycle, there was no difference in food consumption for females or males exposed to BPA or EE relative to control counterparts. One study with CD-1 mice indicated that females perinatally exposed to BPA consumed more food and were heavier.[31]

While the treatments did not effect energy expenditure, BPA-exposed females demonstrated a greater RQ than AIN females during both cycles. It is uncertain why similar effects were not observed in EE-exposed females. However, the results suggest that these metabolic effects of BPA might be independent of estrogen receptor binding and activation, and instead, involve other steroid or non-steroid receptor pathways.[87–89] Small but statistical differences in RQ have been repeatedly noted between lean and obese human subjects. The most prominent hypothesis is that elevated RQ leads to reduced fat oxidation and greater fat deposition over time. In addition, the metabolic inflexibility concept that has gained favor in the obesity field suggests that obese individuals have an elevated RQ during both fasting and fed conditions, while lean individuals have low RQ during fasting and high RQ during fed conditions, respectively.[90,91] Again, the concept is that this leads to differences in substrate storage patterns (fat *v*. muscle), which affect body composition. Therefore, the greater RQ detected in BPA females may predispose them to other metabolic disorders, including obesity.

In relation to goal two, the most striking finding in the current study was that females developmentally exposed to BPA, and to a lesser extent EE, were less active, especially during the night time hours. This manifested in several ways. These females voluntarily moved around the cage less during this time, were slower moving, drank less water and instead spent more time asleep compared with control counterparts. It is uncertain why early exposure to BPA and EE decreased voluntary physical activity in females but not males. Observed sex differences might be due to gonadal steroid hormones or sex-chromosomal dependent. Another possibility is that the brain regions (discussed below) governing voluntary physical activity may be more vulnerable to EDC-induced programming disturbances in females compared with males. These chemicals may also lead to underpinning sex-dependent epimutations in these brain areas.[92]

The combination of decreased home cage activity and increased RQ value suggests that females exposed to BPA, and possibly EE, may be prone to later metabolic disorders. It is also possible that no differences in body weight are detected when the animals are maintained on regular chow diet. However, placement of such females on a high fat diet might potentiate increased body weight gain and ensuing metabolic diseases. Studies are currently underway to test this hypothesis.

We presumed that differences would also be observed in the voluntary wheel running. It may be that in the typical home cage setting, BPA-exposed females are not stimulated to the same extent as controls to engage in voluntary physical activity. Provisioning though with an external stimulus such as a running wheel may mitigate these differences. If the findings translate to humans, it suggests that studies should be designed to examine whether children exposed to BPA are at risk for metabolic disorders due to physical inactivity. If so, they may benefit from various exercise promoting activities. An additional consideration is that the wheels were only placed in the cages for a short period. Differences between treatment groups might have emerged if the animals had continual access to the running wheel from weaning through adulthood. In essence, those in the BPA and EE groups may have become habituated and less interested in using the wheels compared with those in the control group. This possibility warrants further exploration.

Suppression of voluntary physical activity in the home cage setting may be due to BPA/EE disruptions in various brain regions, including the hypothalamus, hippocampus, amygdala, pre-frontal cortical region, nucleus accumbens, caudate-putamen, mid-brain, locus coeruleus and pons. These regions all govern this trait.[71,93–99] Further, these areas have already been shown to be affected by developmental exposure to BPA or EE.[100–119] Specific neural transcripts associated with voluntary physical activity whose expression pattern might be altered by EDC exposure include *DeltaFosb*,[120] dopamine receptor and transporter (*Drd1-5* and *SlcA3)*,[121–127] *Bdnf*,[98,128,129] orexin and orexin receptor (*Oxa* and *Oxr*).[57,59,99,130–132] Future studies will examine how early exposure to BPA and EE affects these brain regions and candidate genes, as such experiments might

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reveal the underlying mechanisms of how BPA/EE suppresses this behavior in female California mice.

The few other studies examining how developmental exposures to BPA and the *in utero* environment as a whole affects spontaneous activity in animal models have yielded mixed results. In other rodent models, BPA-exposed males were reported to be hyperactive.[40,47,133,134] One study that simultaneously examined energy expenditure and physical inactivity reported that BPA-exposed females were more hyperactive than control females and both BPA-exposed males and females demonstrated elevated energy expenditure.[47] However, closer examination of this work reveals that the reported *P* values failed to reach the generally accepted significance cut-off of $P \leqslant 0.05$. Other work supports our current findings. Developmental exposure to BPA has been reported to decrease voluntary physical activity in female rats[135] and zebrafish.[50] Fetal growth restriction of *a*/*a* (wild-type) female mice gestated by viable yellow (A^{vy}/*a*) mice mothers [who possess an intracisternal A particle with its own cryptic promoter site that drives constitutive and ubiquitous expression of the agouti (*A*) gene] results in them engaging in less physical activity, expending less energy expenditure, and becoming obese at adulthood compared with control *a*/*a* females.[136] The above contradictory findings might be due to variation in the *in utero* environmental insult, dosage in the case of BPA, windows of exposure and animal model examined.

Our prediction at the outset was that there would be differences in serum metabolites and hormones due to early BPA/EE exposure. However, no differences in glucose, insulin, leptin or adiponectin were detected in response to maternal diet or offspring sex. Another study with C57 females developmentally exposed to the same dose tested herein (50 mg/kg feed weight) did not find significant changes in glucose, insulin and leptin but increased adiponectin concentrations were reported.[47] Other results indicate that BPA exposure results in hyperglycemia, disruptions in glucose-stimulated insulin release, and elevates leptin concentrations.[31,44] Besides some of the above confounding factors, diet, pregnancy status, exposure to other environmental chemicals and genetic background may also be important considerations. For instance, dams exposed during gestation to BPA demonstrated glucose intolerance and insulin insensitivity. However, treatment of non-pregnant female mice with BPA failed to elicit these same changes.[45] Long-term treatment of non-obese diabetic mice with BPA exacerbated spontaneous insulitis and diabetes development.[137] Male mice maintained on a high fat diet and concurrently exposed to BPA became glucose intolerant and insulin resistant but did not show any differences in white adipose tissue and percentage of body fat relative to those on a high fat diet but not exposed to this chemical.[138] The animal model examined, dose and timing of BPA exposure, and serum hormone or metabolites measured may explain some of the conflicting findings, including our own with BPA-exposed California mice.

In conclusion, developmental exposure of California mice to BPA or EE did not increase body weight in early adulthood, food intake or glucose and serum metabolite hormones when they were maintained on a control diet. The most notable finding though was that BPA and EE-exposed females were significantly less active, and the former group exhibited a greater RQ value than control females, indicative of altered metabolism of carbohydrates *v.* fats. Follow-up studies are underway to determine if such lifestyle and metabolic changes predisposes these females to later obesity and other metabolic disorders, especially when maintained on a high fat diet.

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Conflicts of Interest

None.

Ethical Standards

The authors confirm that all procedures were performed in accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and have been approved by the University of Missouri Animal Care and Use Committee.

Supplementary material

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