



## Gestational and lactational exposure to BPA, but not BPS, negatively impacts trabecular microarchitecture and cortical geometry in adult male offspring

Rebecca K. Dirkes<sup>a</sup>, Rebecca J. Welly<sup>a</sup>, Jiude Mao<sup>b</sup>, Jessica Kinkade<sup>b</sup>, Victoria J. Vieira-Potter<sup>a</sup>, Cheryl S. Rosenfeld<sup>b</sup>, Pamela S. Bruzina<sup>a,\*</sup>

<sup>a</sup> Nutrition and Exercise Physiology, University of Missouri, Columbia, MO, United States of America

<sup>b</sup> Biomedical Sciences, Christopher S. Bond Life Sciences Center, MU Institute for Data Science and Informatics, Thompson Center for Autism and Behavioral Disorders, Genetics Area Program, University of Missouri, Columbia, MO, United States of America

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### ABSTRACT

Bisphenol-A (BPA) and bisphenol-S (BPS) are endocrine disrupting chemicals (EDCs) found primarily in plastics. Estrogen is a primary hormonal regulator of skeletal growth and development; however, the impact of gestational BPA or BPS exposure on skeletal health of offspring remains relatively unknown. Here, adult female mice were randomized into three treatment groups: 200 µg BPA/kg BW (BPA), 200 µg BPS/kg BW (BPS) or control (CON). Animals were then further randomized to exercising (EX) or sedentary (SED) groups. Treatment continued through mating, gestation, and lactation. One male offspring from each dam ( $n = 6-8$ /group) was assessed at 16 weeks of age to evaluate effects of EDC exposure on the adult skeleton. Cortical geometry of the mid-diaphysis and trabecular microarchitecture of the distal femur were assessed via micro-CT. Biomechanical strength and mineral apposition rate of the femoral diaphysis were assessed via three-point bending and dynamic histomorphometry, respectively. Two-factor ANOVA or ANCOVA were used to determine the effects of maternal EX and BPA or BPS on trabecular and cortical bone outcomes. Maternal EX led to a significant decrease in body fat percentage and bone stiffness, independent of EDC exposure. Offspring exposed to BPA had significantly lower trabecular bone volume, trabecular number, connectivity density, cortical thickness, and greater trabecular spacing compared to BPS or CON animals. In conclusion, gestational BPA, but not BPS, exposure negatively impacted trabecular microarchitecture and cortical geometry in adult male offspring. If these findings translate to humans, this could have significant public health impacts on expecting women or those seeking to become pregnant.

### 1. Introduction

Bisphenols are endocrine disrupting chemicals (EDCs) found in plastics, which can significantly impact human health (Rochester, 2013; Rochester and Bolden, 2015). The initial bisphenol used in mass production was bisphenol-A (BPA), but with the current health concerns surrounding BPA, more manufacturers have switched to using bisphenol-S (BPS) and bisphenol-F (BPF) (Usman and Ahmad, 2016). Bisphenols predominantly act through binding estrogen receptors (ERs) (Wetherill et al., 2007; Vom Saal and Vandenberg, 2021). Human studies associate increased BPA exposure, especially during the

perinatal period, with several endocrine disorders, from low circulating sex hormones and decreased birth weight to metabolic diseases, such as obesity and cardiovascular disease (Rochester, 2013). The most common route of BPA or BPS exposure in humans is food contamination, usually from epoxy resins used to line metal cans (Geens et al., 2012; Qiu et al., 2019) or from BPA leaching into food from plastic storage containers (Halden, 2010). While less impactful to human exposure, BPA and its analogues can also be found in thermal paper, dust, and certain dental and medical equipment (Geens et al., 2012; Qiu et al., 2019). BPA exposure is essentially ubiquitous in humans, with BPA being detectable in almost 100% of the blood or urine samples tested (Rochester, 2013).

\* Corresponding author: Department of Nutrition and Exercise Physiology, University of Missouri – Columbia, 204 Gwynn Hall, Columbia, MO 65211, United States of America.

E-mail address: [BruzinaP@missouri.edu](mailto:BruzinaP@missouri.edu) (P.S. Bruzina).

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Many industrialized countries, such as the United States, China, Germany, and Australia have exposure rates above the current tolerable daily intake (TDI) (Huang et al., 2017). In addition, the majority of epidemiological studies show that BPA exposure is associated with adverse health effects even at intakes below the current TDI (Rochester, 2013). However, despite the known anti-estrogenic effects of BPA and its ubiquitous exposure, little is known about the effects that exposure to BPA or its analogs can have on the skeletal system (Turan, 2021), especially compared to other EDCs. This is surprising given the key role of estrogen in skeletal health (Cauley, 2015).

Estrogen is one of the most influential hormones in growth and maintenance of the skeleton across the entire lifespan in both males and females due to its actions on each bone cell type (Cauley, 2015; Cutler, 1997; Fortes et al., 2014; Yilmaz et al., 2005; Khosla et al., 2001). Estrogen prevents osteocyte and osteoblast apoptosis, induces osteoblast differentiation, increases osteoclast apoptosis, and reduces osteoclast differentiation (Khosla et al., 2012; Syed and Khosla, 2005; Shevde et al., 2000; Bord et al., 2003; Khalid and Krum, 2016). Estrogen also downregulates osteocyte expression of sclerostin (Drake and Khosla, 2017; Delgado-Calle et al., 2012; Dirkes et al., 2020a), which acts locally to inhibit bone growth. In older men, estrogen treatment lowered circulating sclerostin (Il Mödder et al., 2011), implying there is an inverse relationship between estrogen and sclerostin in males.

Estrogen actions are mediated primarily by estrogen binding with the ER, which is found in two isoforms, ESR1 (i.e., ER $\alpha$ ) and ESR2 (i.e., ER $\beta$ ). Bone expresses both forms; however, ESR1 tends to be more prevalent in cortical bone, whereas ESR2 is more widely distributed in cancellous bone (Onoe et al., 1997). BPA and its analogs have structural features that allow ER-binding, although at a lower affinity than estradiol, the most active form of estrogen (Acconcia et al., 2015). Competitive binding assays suggest that both BPA and BPS can inhibit estrogen binding (Molina-Molina et al., 2013). After binding with ER, BPA and BPS stimulate gene transcription that is altered from the gene transcription produced with traditional estrogen binding (Li et al., 2018), although BPS binding usually leads to a weaker response than BPA binding (Rochester and Bolden, 2015; Molina-Molina et al., 2013; Le Fol et al., 2017; Maćczak et al., 2017).

In humans, few studies have looked at relationships between BPA exposure and skeletal health. In school-aged boys, there was a significant negative correlation between urinary BPA levels and height, which remained when adjusted for pubertal status and at a follow-up visit 19 months later (Wang et al., 2018); this correlation was not seen in school-aged girls. In another study, maternal urinary BPA levels in the first trimester were negatively correlated with offspring BMD at age 10, regardless of sex (van Zwol et al., 2020). While these studies suggest BPA exposure negatively impact skeletal growth, the impact of BPA exposure across the lifespan on BMD or fracture incidence has not been investigated, nor have any studies looked at skeletal effects of bisphenols other than BPA. While BPA exposure could have effects on either sex, this study will be focusing on male offspring from this point forward. Little is known about the effects of other bisphenol analogs, such as BPS, and there are still many unanswered questions about the mechanisms behind the morphological impairments seen in response to BPA exposure.

In addition to effects of gestational BPA or BPS exposure, little is known about the effects of maternal exercise on bone. One study shows that maternal exercise can increase the production of osteogenic genes in female offspring (Gaeini et al., 2017); however, another study indicated that maternal exercise could lead to decreases in BMD of the tibia in both male and female offspring (Rosa et al., 2013). Indirectly, maternal exercise has been shown to be protective against obesity in both male and female offspring (Harris et al., 2018; Wasinski et al., 2015), and obesity is considered detrimental to bone health, particularly for men (Nielson et al., 2011). Gestational BPA exposure is also associated with increased body weight in male mice (Van Esterik et al., 2014), so it is possible that maternal exercise could have a protective effect on skeletal health in the context of BPA exposure indirectly through

improvements in body weight.

In this study, we use a murine model to explore the effects of BPA or BPS exposure and maternal exercise throughout gestation and lactation on skeletal outcomes in adult male offspring. Female offspring were also studied but will be reported in a separate paper, as they were studied at a different age than male offspring. Based on previous findings, we hypothesized that male offspring exposed to BPA or BPS during gestation and lactation would have decrease cortical thickness and trabecular bone volume relative to controls, and that these morphological differences could coincide with impairments in biomechanical strength. We also hypothesized that these changes would be associated with reduced bone formation. Based on the often-weaker anti-estrogenic activity of BPS, we hypothesized that BPS exposure would lead to less orthopedic changes than BPA exposure.

## 2. Methods

### 2.1. Experimental design

Female, sexually mature, C57B6/J (Jackson Labs, Bar Harbor, ME) mice were randomly assigned to three treatment groups: BPA exposure (BPA), BPS exposure (BPS), or control (CON). Within those treatment groups, they were further randomized into two groups – an exercising treatment (EX) that had an unlocked running wheel in the cage, or a sedentary control (SED) that had a locked running wheel in the cage, resulting in six treatment groups. All animals were given one small vanilla wafer (Nilla Wafer, Nabisco, East Hanover, NJ) daily. The animals in the BPA group had the equivalent of 200  $\mu$ g BPA/kg body weight dissolved in 70% ethanol and pipetted onto the wafer that was provided daily to the mice. The animals in the BPS group had the equivalent of 200  $\mu$ g BPS/kg body weight dissolved in 70% ethanol and pipetted onto the wafer that was provided daily to the mice. The control animals had 70% ethanol alone placed on the wafer. Cookies from all groups were air dried before feeding to animals. Dams were weighed weekly and chemical doses were adjusted according to body weight. The BPA dose falls below the diet-administered maximum nontoxic dose for rodents (200 mg/kg body weight per day), is within the presumptive no-observed-adverse-effect level, and yields serum concentrations comparable with those identified in human populations unknowingly exposed to this chemical (Mao et al., 2020). Two weeks after treatment started, all mice were mated to untreated C57B6/J male mice which had not been exposed to BPA or BPS. BPA/BPS/CON and EX/SED treatment continued during gestation and lactation. Once weaned, one male offspring ( $n = 6-8$ /group) was randomly selected from each litter and included in the bone studies detailed below (Fig. 1). Dams were singly housed and kept on a 12-h light-dark cycle in a temperature-controlled room.

After weaning, male offspring ( $n = 6-8$ /group) were placed on a high-fat diet (18.6% protein, 37.7% carbohydrate, and 43.8% fat by kcal; Envigo, Madison WI). This study was part of a larger study designed to evaluate the protective effects of maternal exercise on offspring weight and metabolism in the context of an obesogenic diet and environmental stress, which is why a high-fat diet was selected. Mice were pair-housed and maintained on a 12-h light-dark cycle in a temperature-controlled room until 16 weeks of age. One week before sacrifice, body composition was measured via EchoMRI and daily activity and energy expenditure was measured via metabolic chamber, with a 12-h light-dark cycle. As mice are nocturnal creatures, data from the metabolic chamber was separated and presented as two distinct time frames – light (sleeping/sedentary) and dark (active). At sacrifice, body weight was measured, and hind limbs were collected, flash frozen, and stored at  $-80^{\circ}\text{C}$  for further analysis. All procedures were approved in advance by the University of Missouri Institutional Animal Care and Use Committee (Protocol #8693).

# Experimental Design

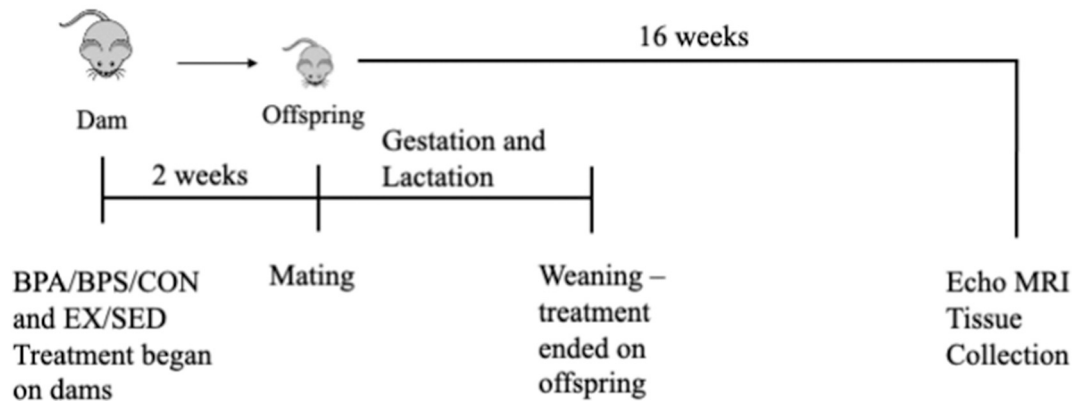


Fig. 1. Experimental design.

## 2.2. Femoral cortical geometry and trabecular microarchitecture

Micro-computed tomographic ( $\mu$ CT) imaging of the right femur was performed using a high-resolution imaging system (Xradia 520 Versa, ZEISS, Oberkochen, Germany), as previously described (Dirkes et al., 2020b). The methods used were in accordance with guidelines for the use of  $\mu$ CT in rodents (Bouxsein et al., 2010). Scans were acquired using an isotropic voxel size of 0.012 mm, a peak X-ray tube potential of 60 kV, and a 2-s exposure time. Trabecular bone microarchitecture was evaluated in a 0.5-mm region of interest directly above the growth plate of the distal femur, as previously described (Ortinou et al., 2017a; Ortinou et al., 2017b). Cortical bone cross-sectional geometry was evaluated at a 1-mm region of interest at the mid-diaphysis of the femur as previously described (Ortinou et al., 2017a; Ortinou et al., 2017b). The optimized threshold function was used to delineate mineralized bone from soft tissue. Scans were analyzed using BoneJ software (Doube et al., 2010) (NIH public domain), and measures of cortical geometry and trabecular microarchitecture were collected. Outcomes for cortical geometry included: tibia length (Le), total cross-sectional area inside the periosteal envelope (Tt.Ar), marrow area (Ma.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), mean cortical thickness (Ct.Th), and robustness (R, total bone area over length calculated as  $R = Tt.Ar/Le$ ). Voxel gray-scale intensity of the cortical bone was measured as a proxy for tissue mineral density. Outcomes for trabecular microarchitecture included: bone volume fraction (BV/TV), connectivity density (Conn.D, degree of trabeculae connectivity normalized to total bone volume), mean trabecular thickness (Tb.Th), trabecular separation (Tb.Sp, distance between trabeculae), trabecular number (Tb.N, average number of trabeculae per unit length calculated as  $1/(Tb.Th + Tb.Sp)$  (Bruker-microCT, 2012)), structural model index (SMI), and degree of anisotropy (DA).

## 2.3. Femoral biomechanical strength

Biomechanical strength testing of the right femur was performed using three-point bending (Jepsen et al., 2015). Briefly, frozen femurs were thawed, cleaned of all soft tissue, kept hydrated in PBS, and placed in the three-point bending apparatus one at a time with a span of 6-mm. Femora were loaded via a material testing machine (Instron 5942; Instron, Inc., Norwood, MA) at a rate of 10 mm/min at the midpoint of the femur until fracture. Outputs from the Instron machine were used to produce a load-displacement curve. The slope of the load-displacement curve was used to estimate material stiffness, and the area under the

load-displacement curve was used to estimate work-to-fracture (Turner and Burr, 2001). Maximal load was measured as the highest force applied to the bone before fracture (Turner and Burr, 2001). Load-displacement data were converted into stress and strain to produce a stress-strain curve using the geometric measurements of the bone and following the equations of Turner and Burr (Turner and Burr, 2001). The slope of the stress-strain curve was used to estimate Young's modulus of elasticity, and the area under the curve was used to estimate the modulus of toughness (Turner and Burr, 2001). Ultimate stress was measured as the highest stress applied to the bone before fracture (Turner and Burr, 2001).

## 2.4. Tibial osteocyte sclerostin expression

Sclerostin expression was evaluated using immunohistochemistry as previously described (Dirkes et al., 2020b). Briefly, right tibiae were fixed in 10% formalin at sacrifice for 48 h at 4 °C, and then decalcified in 14% EDTA at 4 °C. Decalcified tibiae were embedded in paraffin wax blocks, and 5- $\mu$ m sections were taken transversely at the mid-diaphysis of the tibia for measures of cortical bone. The sections were deparaffinized and underwent heat-induced epitope retrieval overnight at 60 °C using a 10 mM sodium citrate buffer, followed by blocking of endogenous avidin and biotin expression (Avidin Biotin Blocking Solution, Thermo Scientific, Waltham, MA). Sections were then incubated in anti-sclerostin primary antibodies (Abcam, Cambridge, UK) overnight at 4 °C, followed by blocking of endogenous peroxidase activity (3%  $H_2O_2$ , Ricca Chemical, Arlington, TX) and secondary antibody application. Secondary antibody binding and detection were accomplished using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), with diaminobenzidine (ImmPACT DAB, Vector Laboratories, Burlingame, CA) as the chromogen. Sections were counterstained with hematoxylin (Fisher Scientific, Hampton, NH), dried, and mounted. Sections were analyzed at 20 $\times$  for sclerostin expression by a blinded researcher. Sclerostin positive (Sost+) osteocytes were defined as osteocytes exhibiting brown staining, and sclerostin negative (Sost-) osteocytes were defined as osteocytes exhibiting blue (hematoxylin) staining. Data are reported as percent Sost+ osteocytes. In addition to Sost+ and Sost- osteocytes, empty osteocytic lacunae revealed by hematoxylin staining were counted, and data are reported as percent empty lacunae, as described previously (Pereira et al., 2017).

## 2.5. Dynamic histomorphometry of the femur

Dynamic histomorphometry of the left femur was analyzed by using calcein and alizarin fluorescent labeling. Calcein (15 mg/kg BW) and alizarin (20 mg/kg BW) were administered via intraperitoneal injection 7 and 3 days before sacrifice, respectively. Femora were cleaned of all soft tissue, fixed in 10% formalin overnight at 4 °C, dehydrated in graded alcohols, and dried overnight. Samples were then embedded in low-viscosity epoxy (Epo-Thin, Beuhler Ltd., Lake Bluff, IL) under a vacuum and allowed to cure overnight. A 1-mm slice was taken from the mid-diaphysis using a low-speed saw. Sections were mounted on slides and polished to smooth the bone surface. Slides were imaged using a fluorescent confocal microscope (Leica GSD 3D, Leica Biosystems, Buffalo Grove, IL) with excitations at 560 nm and 642 nm for calcein and alizarin, respectively. Images were analyzed in ImageJ. Mineral apposition rate (MAR) was calculated as the distance between the corresponding edges of the two consecutive labels divided by the time between injections (um/day), as recommended by ASBMR (Dempster et al., 2013).

## 2.6. Statistical analysis

Two-way ANOVA was used to assess the main and interactive effects of gestational BPA or BPS exposure and exercise status of the dam on metabolic outcomes, trabecular microarchitecture, and percentage of sclerostin+ osteocytes. Body weight is a strong predictor of cortical bone size and strength, so cortical geometry and biomechanical strength outcomes were assessed by two-way ANCOVA with final body weight included as a covariate (Jepsen et al., 2015). If an interaction was present, one-way ANOVA or ANCOVA based on dam group was used as necessary to determine the location of the interaction. Data are presented as means  $\pm$  SEM or adjusted means  $\pm$  SEM. Statistical significance was set at  $p < 0.05$ . All analyses were performed by using SPSS software (SPSS/25.0, SPSS, Chicago, IL, USA).

## 3. Results

### 3.1. Whole animal phenotypes

There were no main or interactive effects of gestational BPA or BPS exposure or exercise status on final body weight of the offspring. There was a main effect of maternal exercise status on offspring body fat percentage ( $p = 0.017$ ), with offspring of exercising dams having lower body fat percentage than those from sedentary dams, regardless of gestational exposure. (Fig. 2) During the light cycle, there were no main

or interactive effects of BPA or BPS exposure or exercise on average or resting energy expenditure, respiratory quotient, or spontaneous physical activity. During the dark cycle, there were no main or interactive effects of BPA or BPS exposure or exercise on resting energy expenditure, respiratory quotient, or spontaneous physical activity, but there was a main effect of exercise on average energy expenditure, with offspring from exercising dams having a lower average energy expenditure than offspring of sedentary dams (Fig. 3).

### 3.2. Femoral cortical geometry

There were no main effects of gestational BPA or BPS exposure or maternal exercise status on Tt.Ar, Ma.Ar, Ct.Ar, Ct.Ar/Tt.Ar, voxel intensity, or Imax/Imin ratio. There was a significant interaction ( $p = 0.022$ ) between gestational exposure and exercise status on Ct.Ar/Tt.Ar, in that exercise decreased cortical area fraction in BPA and BPS exposed offspring but increased it in the CON offspring. There was a main effect of gestational exposure on Ct.Th ( $p = 0.025$ ), with BPA offspring having significantly lower Ct.Th than BPS or CON offspring (Fig. 4).

### 3.3. Femoral biomechanical strength

There were no main effects of gestational BPA or BPS exposure or maternal exercise status on maximal force, Young's modulus of elasticity, ultimate stress, work-to-fracture, or modulus of toughness. There was a main effect of maternal exercise status on stiffness ( $p = 0.031$ ), with offspring from exercising dams having significantly higher stiffness than offspring from sedentary dams. There was a significant interaction between gestational BPA or BPS exposure and maternal exercise status on work-to-fracture ( $p = 0.044$ ) and modulus of toughness ( $p = 0.018$ ), in that exercise increased both work-to fracture and modulus of toughness in BPS and CON offspring, but not BPA offspring (Fig. 5).

### 3.4. Femoral trabecular microarchitecture

There was a main effect of gestational BPA or BPS exposure on BV/TV ( $p = 0.001$ ), Tb.Sp ( $p = 0.001$ ), Tb.N ( $p = 0.001$ ), Conn.D ( $p = 0.001$ ). BPA exposure significantly decreased BV/TV, Tb.N, and Conn.D compared to BPS and CON offspring. BPA exposure significantly increased Tb.Sp compared to BPS and CON offspring. There was a significant interaction between gestational exposure and maternal exercise status on Conn.D ( $p = 0.036$ ), in that exercise increased Conn.D in BPA and CON animals, but not BPS animals. There was a significant interaction between gestational exposure and exercise status on SMI ( $p = 0.001$ ), in that exercise decreased SMI in BPA and BPS exposed offspring

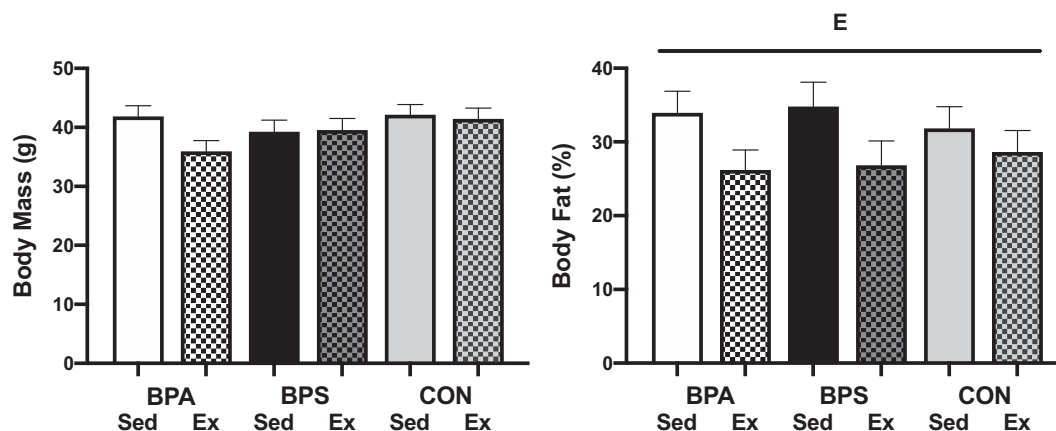
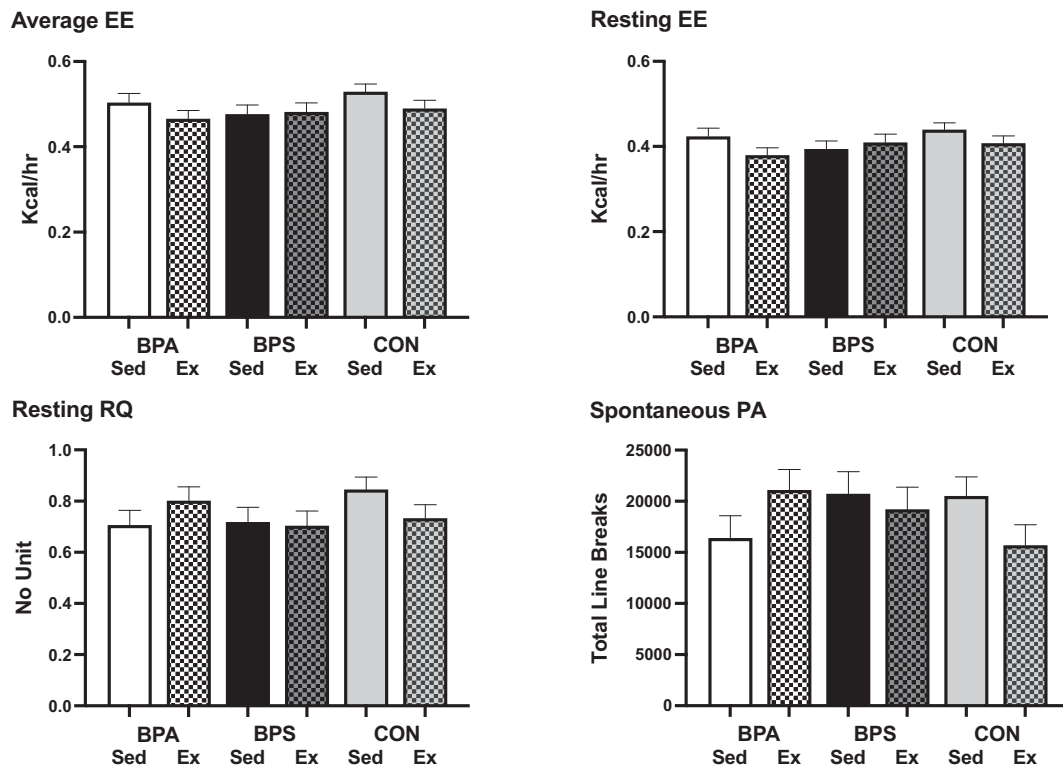
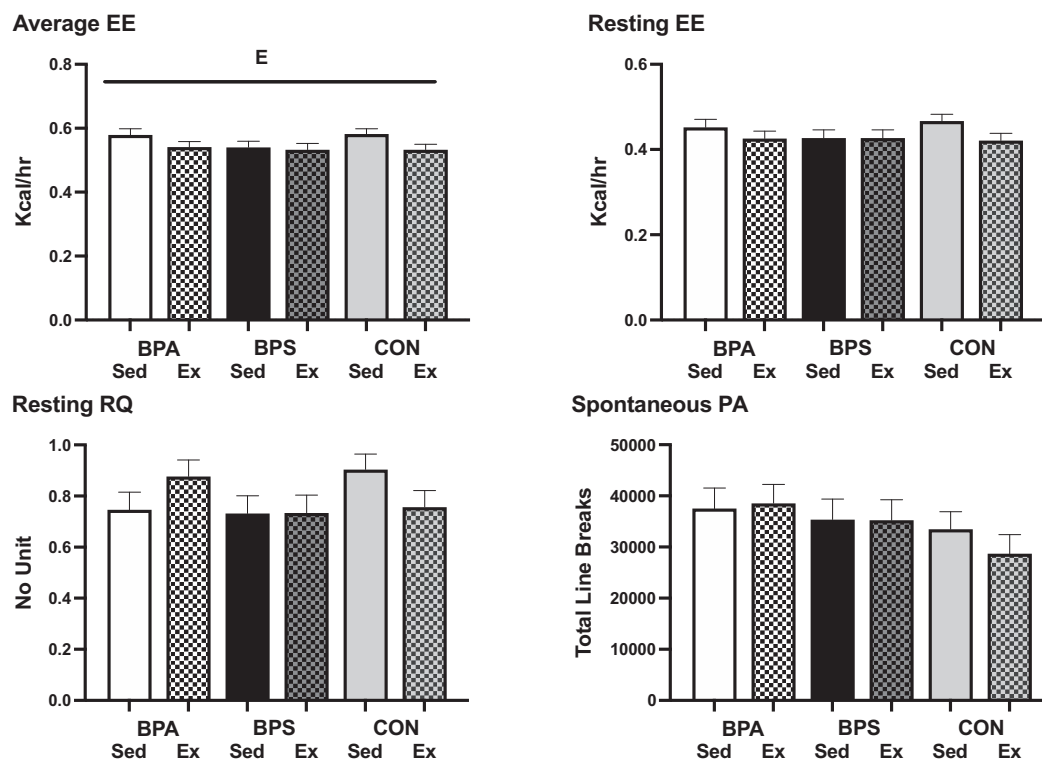


Fig. 2. Animal characteristics. Data are means  $\pm$  SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control group; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel. E: main effect of exercise status ( $p < 0.05$ ). There was a main effect of exercise on body fat percentage [(%) Ex:  $27.23 \pm 1.72$ ; Sed:  $33.53 \pm 1.76$ ;  $p = 0.017$ ].

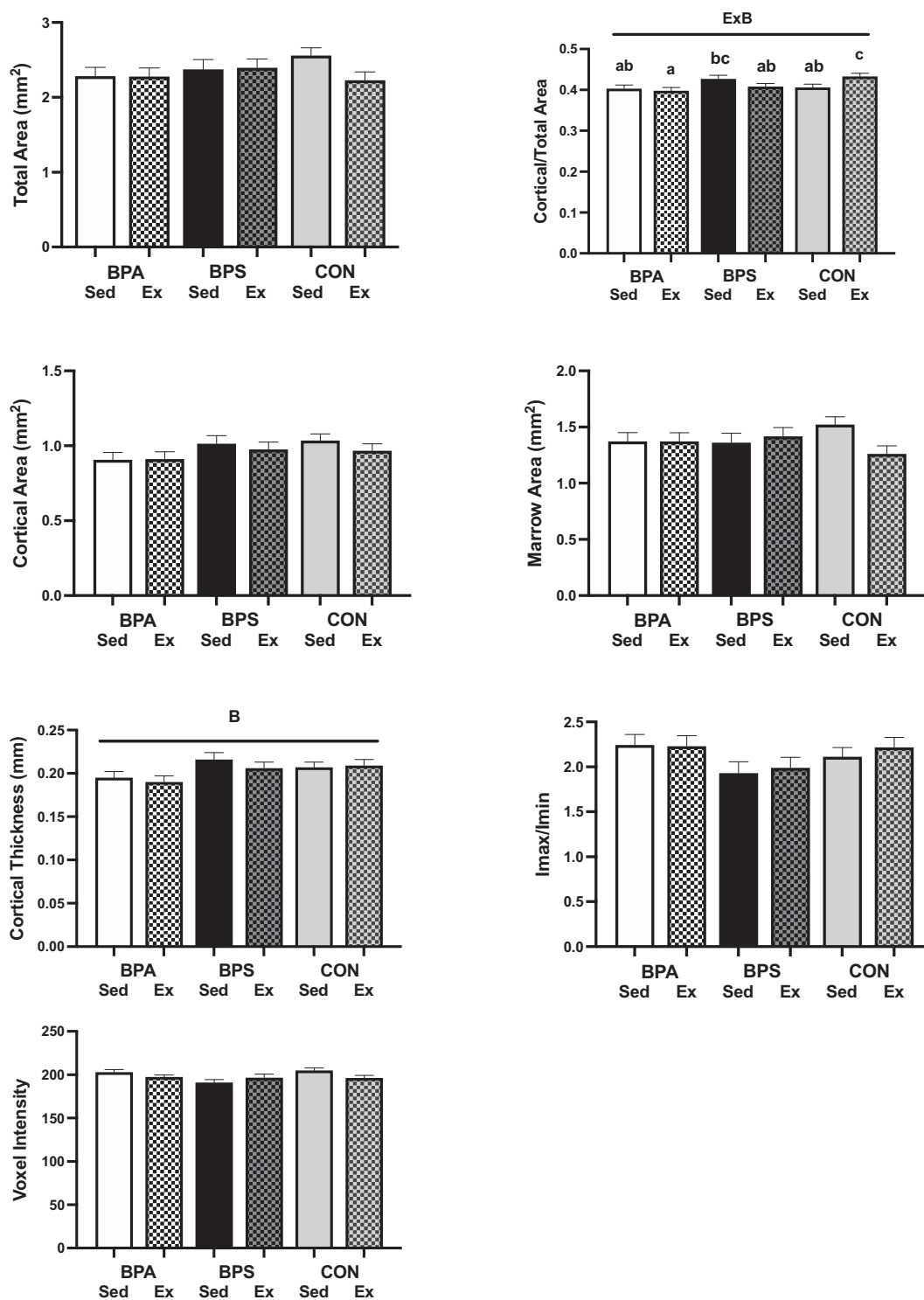
### Light Cycle



### Dark Cycle



**Fig. 3.** Metabolic chamber data. Data are means ± SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control group; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel; EE: energy expenditure; RQ: respiratory quotient, PA: physical activity. E: main effect of exercise status ( $p < 0.05$ ). There was a main effect of exercise on average energy expenditure [(kcal/h) Ex:  $0.54 \pm 0.01$ ; Sed:  $0.57 \pm 0.01$ ;  $p = 0.037$ ].



**Fig. 4.** Cortical geometry of the femur. Data are adjusted means  $\pm$  adjusted SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel. B: main effect of gestational bisphenol exposure ( $p < 0.05$ ); E: main effect of exercise status ( $p < 0.05$ ). Different letters denote significance if a B\*E interaction was present. There was a main effect of gestational exposure on cortical thickness [(mm): BPA:  $0.193 \pm 0.005$ ; BPS:  $0.211 \pm 0.005$ ; CON:  $0.208 \pm 0.004$ ;  $p = 0.025$ ].

but increased it in the CON offspring. There were no main or interactive effects of gestational exposure or exercise status on Tb.Th, DA, or ellipsoid factor (Fig. 6).

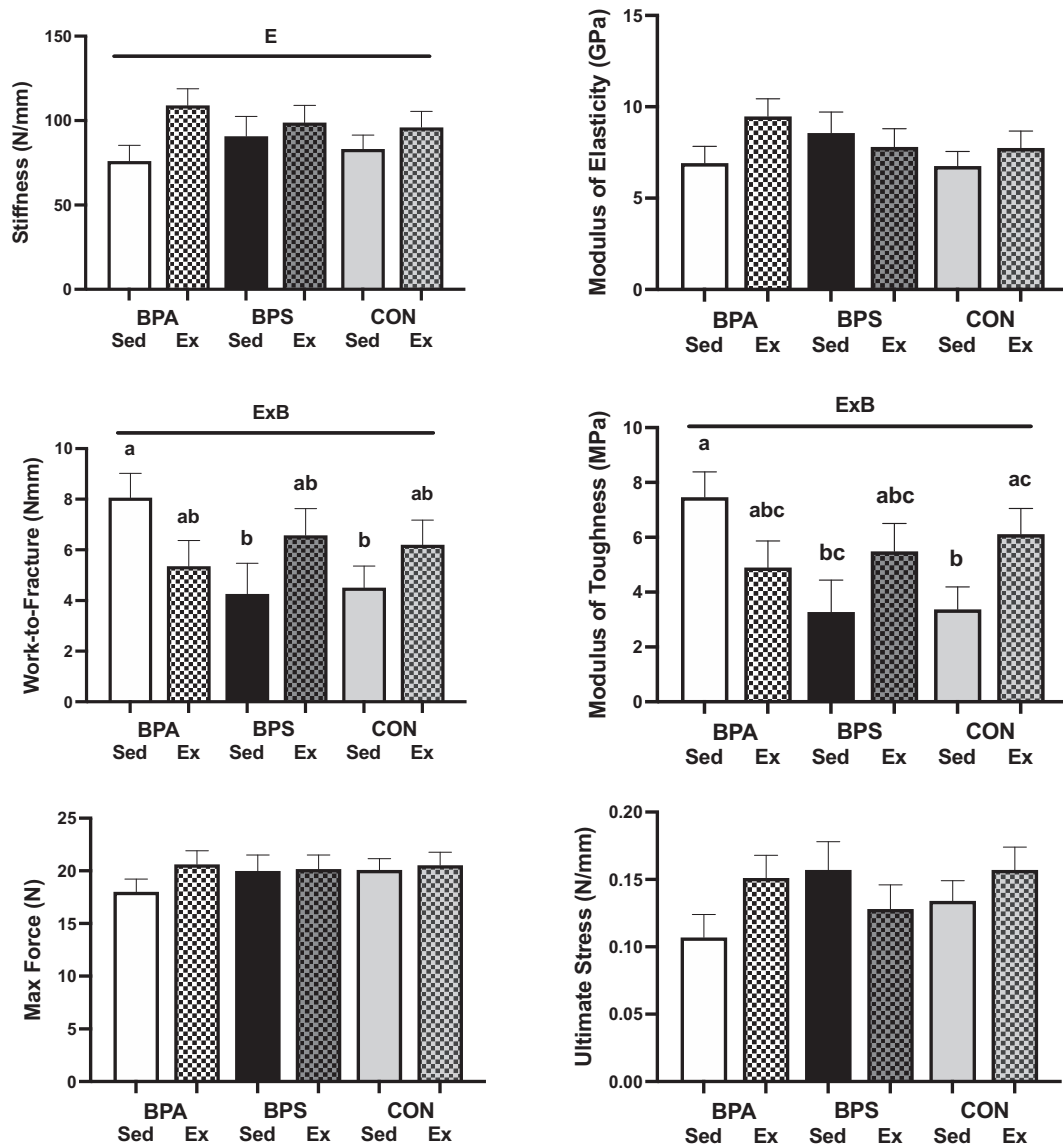
### 3.5. Cortical osteocyte sclerostin expression of the tibia

There were no main or interactive effects of gestational exposure or

exercise status on percent empty lacunae or percent sclerostin positive osteocytes in the mid-diaphysis of the tibia (Fig. 7).

### 3.6. Dynamic histomorphometry of the femur

There were no main or interactive effects of gestational exposure or exercise status on mineral apposition rate of the mid-diaphysis of the



**Fig. 5.** Biomechanical strength of cortical bone of the femur. Data are adjusted means ± adjusted SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel. B: main effect of gestational bisphenol exposure ( $p < 0.05$ ); E: main effect of exercise status ( $p < 0.05$ ). Different letters denote significance if a B\*E interaction was present. There was a main effect of exercise on stiffness [(N/mm): Ex:  $101.29 \pm 5.58$ ; Sed:  $83.33 \pm 5.61$ ;  $p = 0.031$ ].

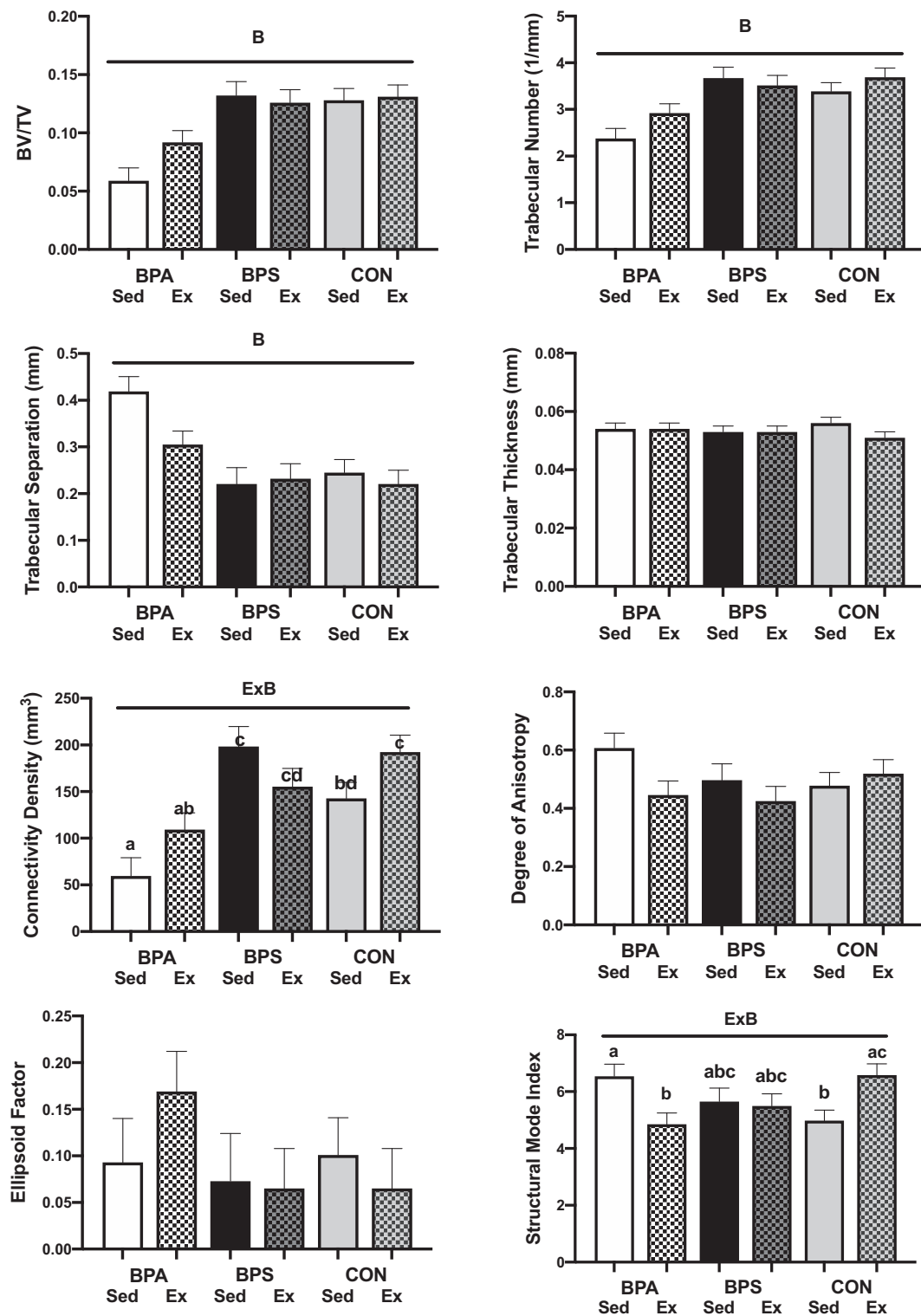
femur (Fig. 8).

#### 4. Discussion

Here, we explored the effects of BPA or BPS exposure and maternal exercise throughout gestation and lactation on skeletal outcomes in adult male offspring. We showed that gestational and lactational exposure to BPA, but not BPS, negatively impacted multiple measures of skeletal health in adult male offspring. We also showed that maternal exercise during gestation and lactation can impact bone material properties of adult male offspring. More specifically, offspring of dams exposed to BPA during gestation and lactation had significantly lower indices of cancellous and cortical bone quantity and quality. Interestingly, these reductions did not correlate with decreases in biomechanical strength, measured via maximal load or ultimate strength, although there was an interaction between maternal BPA exposure and exercise on biomechanical strength, such that maternal exercise increased work-to-fracture and toughness in control and BPS-exposed offspring, but not in BPA-exposed offspring. Maternal exercise also decreased the stiffness

of cortical bone. Finally, we showed that neither gestational exposure to bisphenols nor maternal exercise impacted sclerostin expression or mineral apposition rate of the adult offspring.

In the current study, gestational BPA exposure decreased cortical thickness in adult male offspring. Previous studies showed that responses in cortical thickness are dose-dependent, with cortical thickness increasing at  $25 \mu\text{g}/\text{kg}$  bw/day, but decreasing at  $250 \mu\text{g}/\text{kg}$  bw/day (Lejonklou et al., 2016). The exposure rate in our study was  $200 \mu\text{g}/\text{kg}$  bw/day, which provides further evidence that higher doses lead to decrease in cortical thickness. These alterations in cortical thickness seen in our study and others are most likely due to interrupted estrogen signaling through ESR1, which plays a significant role in periosteal expansion in male mice (Almeida et al., 2013). These alterations could also be due to decreased osteoblast or endosteal activity. Previous studies have shown that in vitro treatment with BPA blocks both osteoblastic and osteoclastic differentiation and correspondingly increases markers of apoptosis (Strong et al., 2016; Hwang et al., 2013; Fic et al., 2015). While in this study, we did not detect differences in osteoblastic activity as measured via dynamic histomorphometry, this could be



**Fig. 6.** Trabecular microarchitecture of the femur. Data are means ± SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel. B: main effect of gestational bisphenol exposure ( $p < 0.05$ ); E: main effect of exercise status ( $p < 0.05$ ). Different letters denote significance if a B\*E interaction was present. There was a main effect of gestational exposure on bone volume fraction [(no unit): BPA:  $0.076 \pm 0.008$ ; BPS:  $0.129 \pm 0.008$ ; CON:  $0.129 \pm 0.007$ ;  $p = 0.001$ ], trabecular number [(1/mm): BPA:  $2.65 \pm 0.15$ ; BPS:  $3.60 \pm 0.16$ ; CON:  $3.54 \pm 0.14$ ;  $p = 0.001$ ], trabecular separation [(mm): BPA:  $0.362 \pm 0.022$ ; BPS:  $0.226 \pm 0.024$ ; CON:  $0.233 \pm 0.020$ ;  $p = 0.001$ ], and connectivity density [(mm<sup>3</sup>): BPA:  $84.43 \pm 13.27$ ; BPS:  $176.86 \pm 14.45$ ; CON:  $167.58 \pm 12.35$ ;  $p = 0.001$ ].



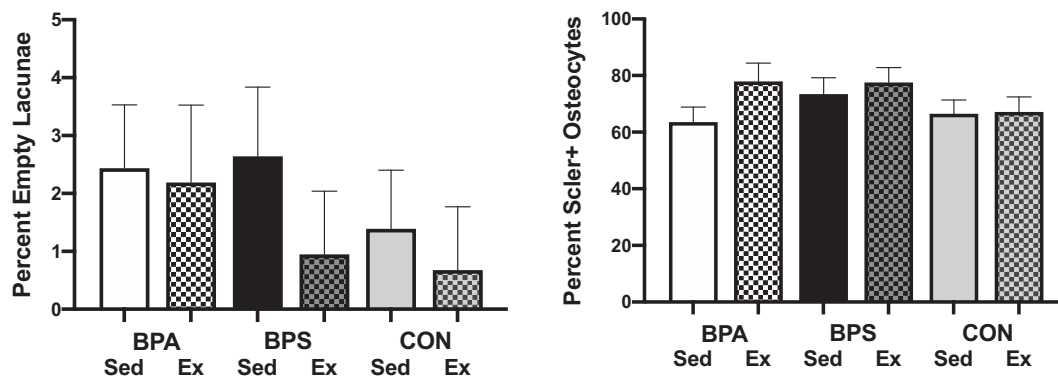


Fig. 7. Sclerostin expression of the tibia. Data are means ± SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel.

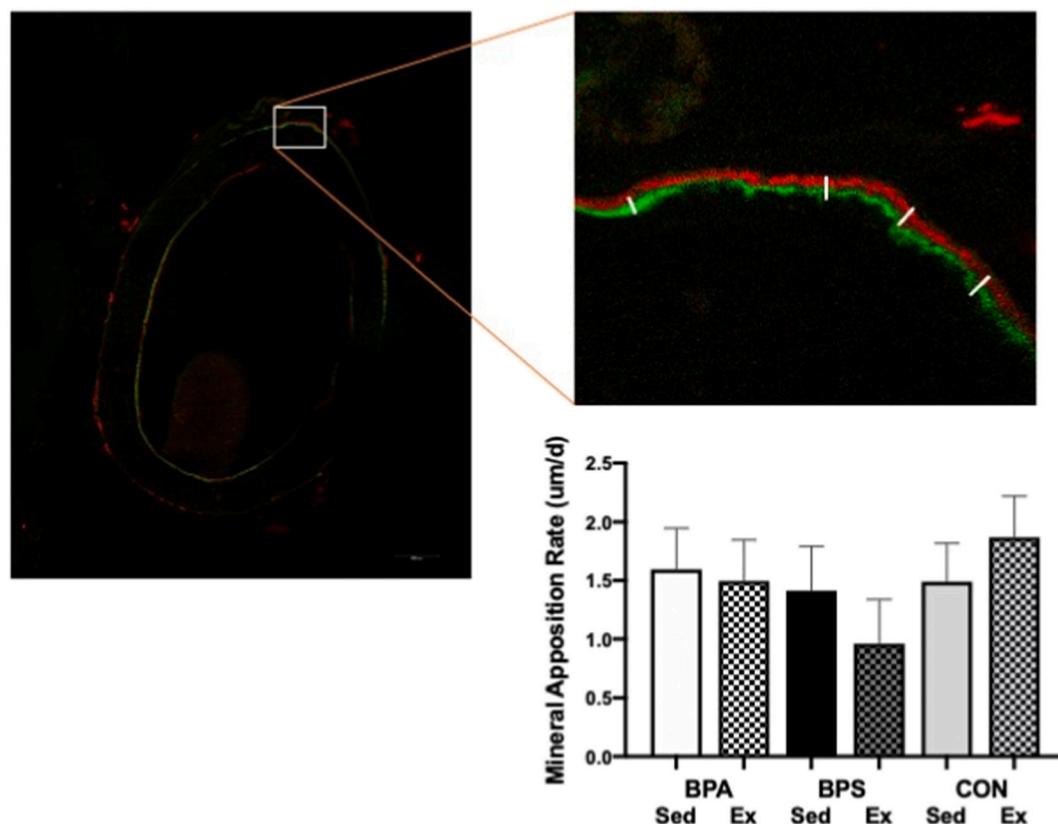


Fig. 8. Mineral apposition rate of the femur. Data are means ± SEM. BPA: gestational bisphenol-A exposure; BPS: gestational bisphenol-S exposure; CON: control; Ex: dam had access to unlocked running wheel; Sed: dam had locked running wheel.

related to the age of the animals as fluorescent markers were not administered until after skeletal maturity. In addition, a recent study showed that morphological alterations seen at 13 weeks in male mice exposed to BPA during gestation and lactation only were no longer present at 52 weeks (Lind et al., 2019), implying that the changes could be reversible. Further exploration into the mechanisms behind the morphological alterations seen in younger but not older animals is warranted.

In addition to BPA, we were the first to explore the effects of other bisphenol analogs by including BPS exposure. We found that the negative alterations associated with BPA exposure were not seen in the BPS exposure animals, indicating a significant difference in the effects of these two analogs. This could partially be explained by differences in binding kinetics, recruitment of coregulators, or post-binding gene

expression modifications between BPA and BPS when bound to ERs (Li et al., 2018). BPA can bind with both ESR1 and ESR2, but there is conflicting evidence on whether BPS can bind with both ESR1 and ESR2 or just ESR1 (Molina-Molina et al., 2013; Li et al., 2018; Le Fol et al., 2017). In addition, there are differences between the response after binding to ESR1 between BPA and BPS. For example, when BPA bound to ESR1 it had the capability of recruiting seven of the 32 coregulators recruited by estradiol, whereas BPS recruited 14, implying post-binding gene modification of BPS is more similar to that of estradiol (Li et al., 2018). In addition, some studies suggest BPS may be a weaker ER antagonist than BPA (Molina-Molina et al., 2013). These key molecular differences together could explain the null effects in BPS-exposed offspring.

While gestational and lactational exposure to BPA led to significant

morphological impairments, they did not correlate with any alterations in material properties or biomechanical strength. It was surprising that decreases in quantity did not correlate with decreases in strength; however two other studies also showed morphological changes without differences in biomechanical strength after gestational BPA exposure (Pelch et al., 2012; Lind et al., 2017). This could partially be due to the way that strength was tested. There are limitations to *ex vivo* tests, since they do not exactly recreate the load that a bone would experience *in vivo*. Additionally, the equations used to estimate material properties from bending tests are based off engineering beam theory, which assumes that the beam has standardized geometry. Because bone does not have a uniform shape, this can lead to overestimations of certain material properties (Jepsen et al., 2015). This also could be partially related to compensation by other intrinsic properties of bone strength, such as collagen content or mineral crystallization (Fonseca et al., 2014), which we were unable to assess at this time. However, as bones were all tested in the same manner, group differences should still be apparent if present, and thus more studies on the cellular and structural differences of the bone are warranted to explore the effects of BPA and BPS on bone size but not bone strength.

We were most surprised by the interactive effects of maternal BPA or BPS exposure and maternal exercise on biomechanical outcomes. There was a significant interaction between maternal exercise and BPA or BPS exposure on work-to-fracture and modulus of toughness, which are both measures of the ability of the bone to absorb energy before deformation or fracture. Maternal exercise increased toughness and work-to-fracture in BPS and CON animals, but not BPA animals, implying that the presence of BPA could interrupt protective effects of maternal exercise. Maternal exercise also increased stiffness, regardless of BPA or BPS exposure. Stiffness and toughness of the bone material are controlled by different micro-properties of the bone, such as mineral or collagen distribution (Bala and Seeman, 2015; Zioupos and Currey, 1998), and this data would imply that maternal exercise altered both mineral and collagen distribution. Further, the stiffness, or elasticity, and toughness of a material work together to determine the ultimate force and stress a structure can withstand (Zioupos and Currey, 1998). Here, maternal exercise altered both stiffness and toughness, but there was no significant impact on maximal load sustained before fracture. This could imply maternal exercise altered stiffness and toughness in such a way as to balance out the changes, but further analysis of the microstructure of bone is needed to confirm this hypothesis. There are very few other studies on the effects of maternal exercise on bone outcomes in offspring, especially in adult offspring. One other study showed that maternal exercise significantly increased the OPG/RANKL ratio (Gaeini et al., 2017), an indicator of osteoclast formation and activity (Chen et al., 2018). Another study showed that moderate maternal resistance exercise resulted in lower cortical BMD of the tibia, regardless of the sex of the offspring (Rosa et al., 2013). These data are intriguing and suggest that future studies that systematically examine the effects of maternal exercise, including different types of exercise, on skeletal health of the offspring are warranted.

Despite the morphological changes in cortical bone in the BPA-exposed animals, no effect of BPA or BPS exposure on mineral apposition rate or sclerostin expression was noted. While we have previously shown that loss of ER $\alpha$  signaling from genetic knockout could increase sclerostin expression in aged animals (Dirkes et al., 2020b), these results would indicate that disruption of ER signaling through exogenous estrogens does not impact sclerostin expression. This discrepancy could suggest that ER is still present and functioning, and thus signaling from endogenous estrogens or ligand-independent actions are sufficient to maintain the effects of estrogen and ERs on sclerostin expression (Drake and Khosla, 2017). The lack of effect on cortical mineral apposition rate was most surprising, considering the significant deficit in cortical thickness. However, the fluorescent labels were not given until around 15 weeks of age, and thus the animals were past skeletal maturity and growth rates had significantly slowed (Somerville et al., 2004). The

decreases in cortical thickness were most likely established at a younger age, during a time of quick growth, and then remained into adulthood.

In conclusion, gestational exposure to BPA, but not BPS, detrimentally impacts cortical geometry and trabecular microarchitecture in male adult offspring. We also found that maternal exercise increased cortical bone stiffness, and that BPA exposure and maternal exercise interact to negatively affect cortical bone toughness. These results warrant further study, given the current exposure rates of BPA in industrialized countries. As mice and humans have similar bone anatomy and physiology, the findings are likely to translate to humans. If so, these findings may have significant public health impacts on expecting women or those seeking to become pregnant.

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## CRediT authorship contribution statement

**Rebecca K. Dirkes:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Rebecca J. Welly:** Formal analysis, Investigation. **Jiude Mao:** Formal analysis, Investigation. **Jessica Kinkade:** Formal analysis, Investigation. **Victoria J. Vieira-Potter:** Conceptualization, Supervision, Project administration. **Cheryl S. Rosenfeld:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition. **Pamela S. Bruzina:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

All authors have nothing to disclose and no conflicts of interest.

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