Effects of Reproduction on Sexual Dimorphisms in Rat Bone Mechanics

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Abstract:

Osteoporosis most commonly affects postmenopausal women. Although men are also affected, women over 65 are 6 times more likely to develop osteoporosis than men of the same age. This is largely due to accelerated bone remodeling after menopause; however, the peak bone mass attained during young adulthood also plays an important role in osteoporosis risk. Multiple studies have demonstrated sexual dimorphisms in peak bone mass, and additionally, the female skeleton is significantly altered during pregnancy/lactation. Although clinical studies suggest that a reproductive history does not increase the risk of developing postmenopausal osteoporosis, reproduction has been shown to induce long-lasting alterations in maternal bone structure and mechanics, and the effects of pregnancy and lactation on maternal peak bone quality are not well understood. This study compared the structural and mechanical properties of male, virgin female, and post-reproductive female rat bone at multiple skeletal sites and at three different ages. We found that virgin females had a larger quantity of trabecular bone with greater trabecular number and more plate-like morphology, and, relative to their body weight, had a greater cortical bone size and greater bone strength than males. Post-reproductive females had altered trabecular microarchitecture relative to virgins, which was highly similar to that of male rats, and showed similar cortical bone size and bone mechanics to virgin females. This suggests that, to compensate for future reproductive bone losses, females may start off with more trabecular bone than is mechanically necessary, which may explain the paradox that reproduction induces long-lasting changes in maternal bone without increasing postmenopausal fracture risk.

Keywords: Sexual dimorphism, reproduction, lactation, bone microarchitecture, bone mechanical properties, puberty.

1.Introduction:

Osteoporosis, a disease of low bone mass and deteriorated microarchitecture, most commonly occurs in women after menopause. Although approximately 2.8 million men in the United States have osteoporosis, the prevalence of osteoporosis in women over age 65 is at least 6 times greater than that of men in the same age group (Department of Health and Human Services, 2004). This is largely due to the increased rate of bone remodeling resulting from the drop in estrogen levels that occurs during menopause, which leads to rapid bone loss.

However, another important determinant of osteoporosis risk is the peak bone mass that is attained during young adulthood. Studies have shown that the variance in bone structure developed early in life is ~10 times greater than the variance in the rate of bone loss occurring in old age (Hui et al.1990, Wang and Seeman 2008). Furthermore, when longitudinal measurements are made at multiple ages, the bone mass of an individual relative to an age- and sex-matched population remains highly consistent (Loro et al.2000, Emaus et al.2006), suggesting that individuals with a higher bone mass at young adulthood are less likely to develop osteoporosis later in life. In addition to sex-based differences directly related to menopause, men and women also attain different peak bone masses, which may play a role in the sexual dimorphism of osteoporosis risk. Men generally develop larger, more robust bones than women (Gilsanz et al.1994, Nieves et al.2005). However, when normalized by muscle size, females tend to have greater bone mass than males (Ferretti et al.1998, Schiessl et al.1998, Wang et al.2003, Ashby et al.2011).

In addition to postmenopausal bone loss, women undergo substantial skeletal changes during pregnancy and lactation. Reproduction causes significant maternal bone loss, which occurs through both elevated osteoblast/osteoclast-based bone remodeling, as well as direct

removal of mineral from the perilacunar spaces by osteocytes (Kent et al.1990, Sowers et al.1993, Zeni et al.1999, VanHouten and Wysolmerski 2003, Liu et al.2012, Qing et al.2012, Kaya et al.2017). Both types of reproductive bone loss substantially alter skeletal mechanical properties. After weaning, the maternal bone undergoes a period of recovery (Bowman et al.2002, Miller and Bowman 2004, Qing et al.2012, de Bakker et al.2017, Kaya et al.2017). However, the extent of post-weaning recovery remains debated: although reproductive history has no adverse effects on future osteoporosis/fracture risk (Kovacs 2016), multiple studies also demonstrate that, even after a lengthy post-weaning period, deficits in maternal bone structure and/or mechanics remain (Affinito et al.1996, Bowman and Miller 1999, More et al.2001, Bowman et al.2002, Ardeshirpour et al.2007, Liu et al.2012, Bornstein et al.2014, Bjornerem et al.2016, de Bakker et al.2017), indicating that pregnancy and lactation can permanently alter the maternal skeleton. However, despite the substantial impact of reproduction on maternal bone, the effect of reproductive bone loss and recovery on the peak bone mass that is attained, and its impact on skeletal sexual dimorphisms, remain incompletely understood. Furthermore, reproduction may alter the relationship between bone structure and mechanical properties. For instance, previous studies have demonstrated that, in individuals with different bone properties, structural and material properties can compensate for each other to maintain the skeleton's mechanical function (Tommasini et al.2009, Epelboym et al.2012). However, the effects of reproductive history and sex on the skeleton’s structure-function relationships are not known.

The rat is a commonly used preclinical model in the investigation of skeletal physiology, and its skeletal response to pregnancy/lactation has been well characterized (Bowman and Miller 1999, Zeni et al.1999, Vajda et al.2001, de Bakker et al.2017). However, the impacts of reproduction on sexual dimorphisms in the rat skeleton are unclear. Therefore, the objective of

this study was to investigate the impacts of sex and reproductive history on rat bone structure and mechanics at multiple skeletal sites and three different ages. By establishing the age- and site-specific effects of sex and reproduction on bone microarchitecture and mechanics, we aim to gain insight into the mechanisms that protect maternal bone against long-term risk of postmenopausal fracture/osteoporosis.

2.Methods:

2.1Animal Protocol:

All animal experiments were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Experiments were performed for three age groups of Sprague Dawley rats (Charles River, Wilmington, MA): pre-pubertal rats at age 1 month, and two adult groups at ages 6 and 15 months.

For the pre-pubertal groups, 12 1-month-old rats were used: males (n=6) and females (n=6). One month of age in a rat corresponds to the human pre-pubertal phase (Sengupta 2013).

For the adult groups at age 6 months, 21 rats were assigned to three groups: reproductive female (n=6), virgin female (n=6), and male (n=9). Reproductive females were mated at age 3.5 months, became pregnant, lactated for 3 weeks, recovered for 6 weeks post-weaning. All rats were euthanized at age 6 months.

For the adult groups at an average age of 15 months, 33 rats were assigned to 3 groups: reproductive female (n=12), virgin female (n=12), and male (n=9). Starting at age 4-5 months, reproductive female rats underwent 3 repeated reproductive cycles, each consisting of a 3-week pregnancy, 3-week lactation, and 3-6 weeks of post-weaning recovery. 2 reproductive rats failed to become pregnant, 3 rats died prior to the end of the experiment, and 2 rats developed mammary tumors, resulting in a final sample size of n=7 reproductive females (age 17±2

months), n=10 virgin females (age 17±2 months), and n=9 males(age 14±0 months). All rats were euthanized 6±3 months after the end of the last reproductive cycle for the reproductive females. The right tibiae, L2 and L4 vertebrae, and right femurs were dissected immediately after sacrifice. The tibiae and L4 were stored in 70% ethanol, while the femurs and L2 were wrapped in PBS-soaked gauze and frozen at -20ºC.

2.2 μCT Scans and Bone Microstructural Analyses:

The right proximal tibia, right femur midshaft, and 4th lumbar vertebra (L4) were scanned by μCT (Scanco vivaCT40, Scanco Medical AG, Brüttisellen, Switzerland) at 10.5 μm isotropic resolution, with 145 μA current, 55 kVp energy, and 200 ms integration time. At the proximal tibia, a 150-slice-thick volume of interest (VOI) was identified in the trabecular compartment, 2.5 mm distal to the growth plate. At the L4 vertebra, a trabecular VOI, which occupied the center 1/3 of the vertebral body, was identified, resulting in a 130-slice-thick VOI for pre-pubertal rats and a 200-slice-thick VOI for adults. Within each trabecular VOI, the μCT images were Gaussian filtered (sigma=1.2, support=2) and bone was identified by applying a global threshold (544 mg HA/cm^3 for adult and 350 mg HA/cm^3 for pre-pubertal rats), determined using an adaptive threshold function. Bone volume fraction (BV/TV), trabecular number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp), structure model index (SMI), and connectivity density (Conn.D) were quantified (Bouxsein et al.2010). A 50-slice-thick cortical VOI at the femur midshaft was thresholded (772 mg HA/cm^3 for adult and 540 mg HA/cm^3 for pre-pubertal rats). Cortical area (Ct.Area), cortical thickness (Ct.Th), polar moment of inertia (pMOI), tissue mineral density (TMD), periosteal perimeter (P.Perim), and endosteal perimeter (E.Perim) were quantified.

2.3Mechanical Testing of the Femur and L2 Vertebra:

A three-point-bending test was applied to the right femur (Instron 5542, Norwood, MA) at a displacement rate of 1.8 mm/minute. The resulting load-displacement curves were used to determine the peak load, whole-bone stiffness, and energy to failure (defined as area under the load-displacement curve up to the failure point). Estimated intrinsic mechanical properties, including ultimate stress, elastic modulus, and toughness, were determined by combining the mechanical testing data and μCT-derived structural parameters (Schriefer et al.2005).

The vertebral body L2 was imaged by μCT at 20 μm resolution to estimate the total cross-sectional area (CSA; including both bone tissue and marrow). The vertebral processes were removed and parallel cuts were made at the cranial and caudal ends of the vertebral body using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL), to isolate a section of the center 60% of the vertebral body. Samples were compressed to failure through uniaxial compression at a displacement rate of 1.8 mm/minute (Instron 5542), and the peak load, stiffness, and energy to failure were measured. The extrinsic properties were normalized by specimen height and CSA to derive apparent-level properties(Hogan et al.2000).

2.4 Statistics:

All results are presented as mean ± standard deviation (SD). For adult rats, comparisons among groups were made using 1-way ANOVA, with Bonferroni *post hoc* corrections. Comparisons between male and female pre-pubertal rats were made using Student's t-tests. In the presence of significant differences ($p<0.05$), the degree of variation between groups is reported as the percent difference, for all parameters except SMI. SMI ranges from -3 to 3; therefore, inter-group differences in SMI are reported as the absolute difference.

3.Results:

3.1 Trabecular bone microstructure

At age 1 month, males had 27% lower BV/TV, 18% greater Tb.Sp, and 40% lower Conn.D than females at the proximal tibia (Figure 1). The vertebra showed minimal sex-based differences in 1-month-old rats, with the exception of 6% lower Tb.N and 8% greater Tb.Sp in males than females (Figure 2).

By age 6 months, male and reproductive female rats had 56% and 40% lower BV/TV, respectively, than virgin females at the tibia. Additionally, males had dramatically 52% lower Tb.N, 126% greater Tb.Sp, 1.03 greater SMI, and 74% lower Conn.D than virgin females, and reproductive females had 32% lower Tb.N, 0.86 greater SMI, and 57% lower Conn.D than virgins. Tibial trabecular structure was highly similar between males and reproductive females, except that reproductive females had 43% greater Tb.N and 30% lower Tb.Sp than males (Figure 1). Similarly, at the L4 vertebra, males and reproductive females had 29% and 21% lower BV/TV, respectively, than virgin females. Furthermore, males had 19% lower Tb.N, 31% greater Tb.Sp, and 0.91 greater SMI than virgin females, while reproductive females had 0.73 greater SMI than virgins. There were no differences between 6-month-old male and reproductive female rats in any microstructural parameters at L4 (Figure 2).

At age 15 months, sex- and reproductive history-based differences in trabecular microstructure followed similar patterns to those found at age 6 months (Figures 1 and 2). At both sites, male and reproductive female rats had lower BV/TV, Tb.N, and Conn.D, and greater Tb.Sp, than virgin females. However, in contrast to younger rats, which showed no differences among groups in Tb.Th, 15-month-old males and reproductive females both had 17-18% greater Tb.Th at the tibia than virgin females. There were no differences in trabecular microstructure between 15-month-old male and reproductive female rats.

3.2 Vertebral body mechanics

No differences were seen between 1-month-old male and female rats in any vertebral mechanical properties (Figure 3). 6-month-old rats also showed no differences among groups in any whole-bone mechanical properties (Figure 3 A-C). However, apparent-level ultimate stress, elastic modulus, and toughness were 25%, 13%, and 29% lower, respectively, in male rats than virgin females (Figure 3 F-H). Moreover, ultimate stress and elastic modulus were 22% and 14% lower, respectively, in male rats than reproductive females. No differences were seen between the two female groups in apparent-level properties.

By age 15 months, males showed 47-68% greater energy to failure than both groups of females. In addition, the advantages in apparent-level mechanical properties of both virgin and reproductive female rats over male rats in the 6-month age group disappeared by age 15 months. However, 15-month-old virgin female rats had 3% greater TMD at the lumbar vertebra than males (Figure 3D).

3.3 Cortical bone structure and mechanics

At age 1 month, males had 11%, 5%, and 7% greater pMOI, P.Perim and E.Perim, respectively, than females at the femur midshaft (Figure 4). Surprisingly, 3-point bending indicated that males had 15% and 23% lower peak load and stiffness, respectively, in addition to 23% and 35% lower ultimate stress and elastic modulus, than females (Figure 4).

6-month-old male rats had 72-136% greater pMOI and 26-44% greater Ct.Area than virgin and reproductive females, in addition to 10-13%, 15-26%, and 17-35% greater Ct.Th, P.Perim, and E.Perim (Figure 4 A-F). Meanwhile, males had 2-3% lower TMD than virgin and reproductive females. Furthermore, males had 20% greater whole-bone stiffness, but 25% and 27% lower ultimate stress and elastic modulus, than virgin females (Figure 4G-L). Effects of reproductive history on cortical bone microstructure were mild compared to sex differences: 6-

month-old reproductive females had a 13% lower Ct.Area, and 8% lower P.Perim than virgin females, with no other reproductive history-based differences. However, reproductive females had 21% greater whole-bone stiffness and 61% elevated elastic modulus, than virgins.

At age 15 months, cortical bone structure at the femur midshaft was highly similar to that of 6-month-old rats. 15-month-old males showed more robust cortical bone structure, with a lower TMD, as compared to both virgin and reproductive females ($p<0.05$ for all parameters; Figure 4 A-F). Reproductive history continued to minimally affect cortical microstructure at age 15 months.

Sex-based differences in femur mechanics were more pronounced in 15-month-old rats than younger animals (Figure 4 G-L), as 15-month-old males had 35% greater peak load and 227% greater energy to failure than virgin females. Additionally, males had 35% and 62% lower ultimate stress and elastic modulus, respectively, but 102% greater toughness, than virgin females. The advantages in femur mechanical properties of reproductive rats over virgins observed at age 6 months disappeared in the 15-month age group, with reproductive females showing 19% lower ultimate stress than virgins.

3.4 Bone mechanics normalized by body weight

At all ages, male rats showed significantly greater body weight than females (Table 1). Differences were minimal (14%) at age 1 month, while 6-month-old male rats weighed 63-77% more than females and 15-month-old male rats weighed 83-87% more than females. No differences in weight were found based on reproductive history.

When normalized for body weight, male rats had 8-35% lower vertebral cross-sectional area than virgin females at all ages. At age 1 month, there were no significant differences between male and female rats in vertebral whole-bone mechanical properties normalized by body

weight. However, adult rats showed substantial sex-based differences in vertebral mechanics after normalizing for weight, with males having 37-44% lower normalized peak load and 43-47% lower normalized stiffness than virgin females at both 6 and 15 months of age.

At the femur, the sex-based differences in Ct.Area, peak load, and stiffness observed in adult rats were reversed when normalized for body weight, with males showing 14% and 22% lower normalized Ct.Area at ages 1 and 6 months, and 26-33% lower peak load and 26-45% lower normalized stiffness at all ages than virgin females. The only parameter that remained significantly greater for male rats after normalizing for body weight was energy to failure, as 15-month-old males had 71% greater normalized energy to failure than virgin females.

Reproductive females had similar normalized parameters of bone mechanics as virgin females at both the vertebra and femur, except that reproductive females showed 32% greater normalized femoral stiffness than virgins at age 6 months.

4.Discussion:

This study indicates substantial differences in rat trabecular and cortical bone structure and mechanics based on sex and reproductive history. Overall, male and reproductive female rats showed lower trabecular bone volume with reduced number and connectivity, and more rod-like morphology, relative to virgin females. At the femur mid-diaphysis, male rats had greater cortical bone size and strength than both groups of females. However, when normalized for body weight, female rats had greater bone strength than males at both the lumbar vertebra and femur midshaft.

At both trabecular sites that were investigated, male rats had a lower bone volume, with reduced connectivity, than virgin females. Sexual dimorphisms appeared earlier and were of greater magnitude at the tibia than the vertebra. Previous studies in rats found similar variations

between male and female trabecular bone (Hefferan et al.2003, David et al.2006). In contrast, the effects of sex on mouse trabecular bone appear to be highly strain-dependent, as BV/TV and Tb.N were reported to be higher in male C57BL/6 mice than females (Glatt et al.2007), while the opposite was found in BALBc mice (Willinghamm et al.2010). Clinical studies of the effect of sex on trabecular bone showed variable findings: some suggested site-specific effects, with young women (age 18-29) showing greater bone density at the spine while men of the same age had more robust trabecular microarchitecture in the peripheral skeleton (Riggs et al.2004, Nieves et al.2005, Sode et al.2010, Macdonald et al.2011), while others indicated no sex-based differences in vertebral bone density in young adults (Gilsanz et al.1994). However, the reproductive history of women included in these studies was not reported, complicating the interpretation of the results relative to the current evaluation.

In addition to sexual dimorphisms, we also saw substantial effects of reproductive history on trabecular microstructure. At both sites assessed, female reproductive rats had a lower BV/TV with an altered microarchitecture relative to virgin females. It is well established that lactation induces substantial skeletal deterioration, as the maternal skeleton forms an important source of calcium for infant growth (Kent et al.1990, Sowers et al.1993, Zeni et al.1999, VanHouten and Wysolmerski 2003, Liu et al.2012). However, multiple clinical studies have indicated that reproduction does not increase the risk of postmenopausal osteoporosis or fracture (Kovacs 2016), leading many to conclude that reproductive bone losses are fully recovered after weaning. Conversely, several rodent and clinical studies have indicated that, although the trabecular bone does undergo a period of recovery post-weaning, the total extent of the recovery is incomplete, resulting in long-term alterations (Affinito et al.1996, Bowman and Miller 1999, More et al.2001, Bowman et al.2002, Ardeshirpour et al.2007, Liu et al.2012, Bornstein et al.2014,

Bjornerem et al.2016, de Bakker et al.2017), consistent with the current study.

Interestingly, our results demonstrate minimal differences in trabecular bone structure between male rats and post-reproductive females. This suggests that, while reproductive bone losses in the trabecular compartment may not be fully recoverable post-weaning, reproduction does not appear to put female trabecular bone at a disadvantage as compared to that of males. This remained true both after a single reproductive cycle (in 6-month-old rats), and after 3 cycles (in 15-month-old rats). Furthermore, this finding also suggests that, prior to reproduction, the female rat skeleton may contain excess trabecular bone in order to ensure that a sufficient quantity of bone remains after reproduction to serve the skeleton's mechanical functions. This idea is consistent with studies by the Miller group, which suggested that virgin females may start off with more bone than is mechanically necessary, to compensate for reproductive bone losses (Bowman and Miller 1999, Bowman et al.2002). However, the relevance of these findings to the clinical setting remains to be determined, as no clinical studies have yet been performed to investigate the effects of reproductive history on sexual skeletal dimorphisms.

Reproductive history and sex both appeared to minimally affect whole-bone mechanical properties at the vertebra, despite substantial differences among groups in vertebral body size and trabecular microarchitecture. This is consistent with previous studies investigating the effects of reproduction on rat vertebral mechanics, which showed complete recovery of vertebral body strength and stiffness by 8 weeks post-weaning (Vajda et al., 2001), but contrasts with clinical findings that females had lower vertebral peak load than males (Ebbesen et al.1999). In the current study, the highly uniform whole-bone mechanical properties at the vertebra, combined with substantial differences among groups in trabecular microarchitecture, suggest the existence of compensatory mechanisms that allow the bone to maintain a constant load-bearing capacity

despite microstructural variations. It is likely that vertebral body size, microarchitecture, and material properties are coordinated to allow whole-bone strength to be maintained. Indeed, when evaluating the effects of sex on trabecular bone tissue-level properties, we found that female, 6-month-old rats had greater apparent-level vertebral body ultimate stress, elastic modulus, and toughness than males, while 15-month-old virgin females had greater TMD than males. These results are similar to previous findings that, among individuals with different bone properties, structural and material properties may covary, allowing different attributes to compensate for each other (Tommasini et al.2009, Epelboym et al.2012). Similarly, it is possible that reproduction may induce localized changes in bone tissue composition, which could compensate for reductions in bone mass. However, the current study found no reproductive history-based differences in the apparent-level mechanical properties or TMD of the vertebral trabecular bone. Thus, further studies to directly measure the effects of reproduction on trabecular bone material properties are required.

At the femur midshaft, adult male rats had larger, stronger bones than both virgin and reproductive females. Multiple studies have demonstrated that males, who tend to have larger body size and muscle mass, also have larger bones compared to females (Gilsanz et al.1994, Ebbesen et al.1999, Hefferan et al.2003, Nieves et al.2005, David et al.2006). Reproductive history also affected cortical bone mechanics, as 6-month-old reproductive females had greater whole-bone stiffness and derived elastic modulus than virgins. The mechanism behind this reproductive effect remains unclear. However, a recent microindentation-based evaluation of material properties of the mouse femur demonstrated complete recovery of lactation-induced reductions in elastic modulus after weaning, which was hypothesized to be associated with lactation-induced remodeling of the perilacunar and peri-canalicular spaces by osteocytes (Kaya

et al.2016). Thus, it is possible that the elevated elastic modulus found in post-reproductive, 6-month-old rat femurs in the current study resulted from osteocyte activities during reproduction. However, future investigations to directly evaluate material properties of reproductive bone through micro- or nano-indentation, and track osteocyte activities during reproduction, will be required to further explain this finding.

When normalized for body weight, comparisons between male and female rats indicate that females may build stronger bones relative to their size than males. In addition, adult female rats had greater cortical TMD than males. Similarly, clinical studies indicate that pre-menopausal women have greater bone mineral content relative to lean mass than men of the same age group (Ferretti et al.1998, Schiessl et al.1998, Ashby et al.2011). In the current study, the greater bone size, stiffness, and strength in female rats when normalized for body weight appear to provide a margin of safety to protect from possible reproduction-associated reductions in bone properties.

This study provides a thorough evaluation of the impact of sex and reproductive history on bone microarchitecture and mechanical properties in a rat model. In addition, the combination of mechanical and morphometric data allows a unique insight into the effects of reproduction on skeletal structure-function relationships. However, this study was not without limitations. Although the rat is a commonly used preclinical model, important differences exist between rat and human physiology, notably in patterns of longitudinal growth, and in the number of offspring. Thus, clinical studies to directly evaluate the effects of reproductive history on sexual dimorphisms in bone structure and mechanics in humans are required. In addition, the precise mechanisms behind the reproductive history- and sex-based differences in skeletal morphology and mechanics reported here remain to be elucidated. Future studies would include direct, material-level characterization of bone tissue composition and mechanics, as well as

measurement of bone cell activities and remodeling rates to evaluate the cellular mechanisms responsible for reproduction- and sex-based differences.

In summary, this study demonstrates that virgin female rats have greater trabecular bone mass and microarchitecture and, relative to their body weight, have a greater cortical size and greater bone strength than males. Reproduction induced long-lasting deterioration of the trabecular microarchitecture, with minimal effects on cortical bone size and minimal impact on bone mechanics. Trabecular bone structure in post-reproductive females was highly similar to that of male rats, and, when normalized for their body size, the mechanical properties of post-reproductive female bone remained greater than those of males. Thus, despite persistently altered trabecular microstructure relative to virgin females, reproductive females appear to have no skeletal deficits compared to their male counterparts, which suggests that virgin females may start off with more trabecular bone than is mechanically necessary to compensate for possible future reproductive bone losses. This may help to explain the paradox that reproduction induces long-lasting changes in maternal bone without increasing postmenopausal fracture risk.

5.Acknowledgements

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Figure Captions:

Figure 1. (A) Representative renderings of tibial trabecular bone of 6-month-old rats. (B-G) Comparisons among virgin female, reproductive female, and male rats in (B) BV/TV, (C) Tb.N, (D) Tb.Th, (E) Tb.Sp, (F) SMI, and (G) Conn.D at the proximal tibia. * indicate significant differences among groups at a given age ($p<0.05$).

Figure 2. (A) Representative renderings of trabecular bone at the 4th lumbar vertebra (L4) of 6-month-old rats. (B-G) Comparisons among virgin female, reproductive female, and male rats in (B) BV/TV, (C) Tb.N, (D) Tb.Th, (E) Tb.Sp, (F) SMI, and (G) Conn.D at L4. * indicate significant differences among groups at a given age ($p<0.05$).

Figure 3. Differences among virgin female, reproductive female, and male rats in vertebra mechanics as measured through uniaxial compression testing. (A-C) Extrinsic mechanical properties, including (A) peak load, (B) stiffness, and (C) energy to failure; (D) Tissue Mineral Density; (E-H) Vertebral body apparent-level properties, derived by normalizing extrinsic properties by (E) total cross-sectional area, including: apparent (F) ultimate stress, (G) elastic modulus, and (H) toughness. * indicate significant differences among groups at a given age ($p<0.05$).

Figure 4. Comparisons among virgin female, reproductive female, and male rats in (A-F) cortical bone structure at the femur midshaft, including: (A) pMOI, (B) Ct.Area, (C) Ct.Th, (D) TMD, (E) P.Perim, and (F) E.Perim; (G-I) whole bone mechanical properties, including: (G) peak load, (H) stiffness, and (I) energy to failure. (J-L) Intrinsic mechanical properties were derived based on 3-point bending results and μCT-based cortical structure: (J) ultimate stress, (K) elastic modulus, and (L) toughness. * indicate significant differences among groups at a given age ($p<0.05$).

Table 1. Vertebral and femoral mechanical properties normalized by body weight. All measurements shown as mean ± standard deviation [a]: significantly different from virgin female ($p<0.05$), [b]: significantly different from reproductive female ($p<0.05$), [c]: significantly different from male ($p<0.05$).

		1-Month-Old Rats		*6-Month-Old Rats*			*15-Month-Old Rats*		
		Virgin Female	**Male**	**Virgin Female**	**Reproductive Female**	**Male**	**Virgin Female**	**Reproductive Female**	**Male**
	Body Weight (kg)	0.11 ± 0.01 [c]	0.13 ± 0.01 [a]	0.36 ± 0.04 [c]	0.33 ± 0.02 [c]	0.59 ± 0.04 [a,b]	0.41 ± 0.07 [c]	0.42 ± 0.07 [c]	0.77 ± 0.06 [a,b]
Normalized Vertebral Mechanics	**CSA (mm^2/kg)**	43.0 ± 1.3 [c]	39.6 ± 1.6 [a]	17.6 ± 1.6 [c]	18.2 ± 1.2 [c]	13.0 ± 1.6 [a,b]	16.2 ± 4.0 [c]	15.0 ± 2.4 [c]	10.6 ± 0.8 [a,b]
	Peak Load (N/Kg)	555 ± 104	559 ± 121	945 ± 107 [c]	942 ± 121 [c]	532 ± 130 [a,b]	653 ± 172 [c]	595 ± 184	411 ± 106 [a]
	Stiffness (N/mm/kg)	4911 ± 1350	4541 ± 635	3297 ± 361 [c]	3436 ± 343 [c]	1877 ± 159 [a,b]	2654 ± 456 [c]	2523 ± 568 [c]	1417 ± 194 [a,b]
	Energy to Failure (mJ/kg)	58.8 ± 18.7	69.6 ± 26.5	167 ± 29 [c]	164 ± 27 [c]	100 ± 40 [a,b]	103 ± 39	89.2 ± 32.2	79.9 ± 31.4
Normalized Femur Mechanics	**Ct.Area (mm^2/kg)**	20.4 ± 2.0 [c]	17.6 ± 1.3 [a]	18.4 ± 1.6 [c]	17.5 ± 0.8 [c]	14.3 ± 1.7 [a,b]	16.9 ± 3.4	16.0 ± 2.7	14.1 ± 1.6
	Peak Load (N/kg)	239 ± 39 [c]	177 ± 10 [a]	582 ± 74 [c]	584 ± 50 [c]	390 ± 66 [a,b]	555 ± 119 [c]	455 ± 71	392 ± 50 [a]
	Stiffness (N/mm/kg)	535 ± 110 [c]	361 ± 34 [a]	805 ± 41 [b,c]	1063 ± 101 [a,c]	595 ± 45 [a,b]	902 ± 180 [c]	798 ± 107 [c]	492 ± 32 [a,b]
	Energy to Failure (mJ/kg)	321 ± 63	246 ± 59	367 ± 70	282 ± 35	269 ± 105	262 ± 66 [c]	276 ± 79 [c]	449 ± 120 [a,b]

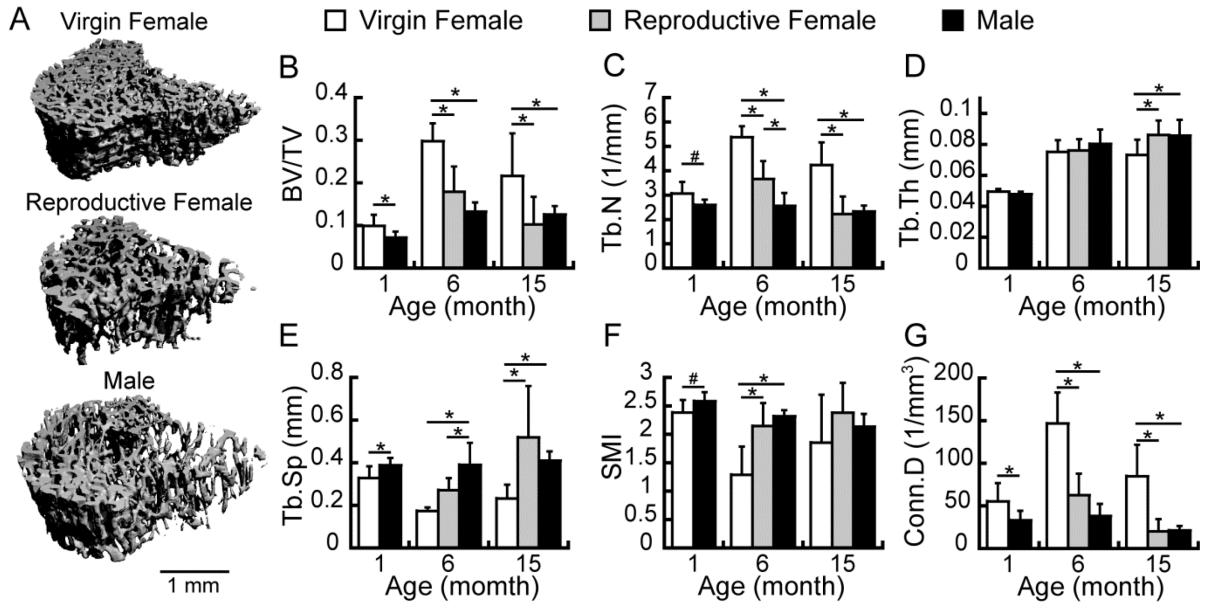

Figure 1. (A) Representative renderings of tibial trabecular bone of 6-month-old rats. (B-G) Comparisons among virgin female, reproductive female, and male rats in (B) BV/TV, (C) Tb.N, (D) Tb.Th, (E) Tb.Sp, (F) SMI, and (G) Conn.D at the proximal tibia. * indicate significant differences among groups at a given age ($p<0.05$).

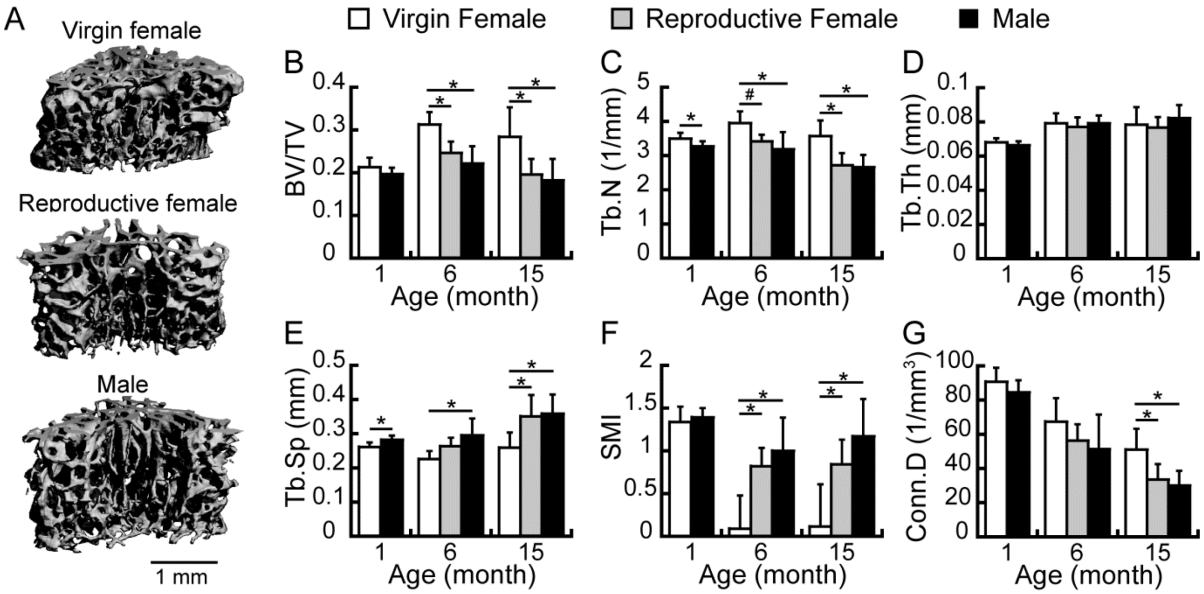

Figure 2. (A) Representative renderings of trabecular bone at the 4th lumbar vertebra (L4) of 6-month-old rats. (B-G) Comparisons among virgin female, reproductive female, and male rats in (B) BV/TV, (C) Tb.N, (D) Tb.Th, (E) Tb.Sp, (F) SMI, and (G) Conn.D at L4. * indicate significant differences among groups at a given age ($p<0.05$).

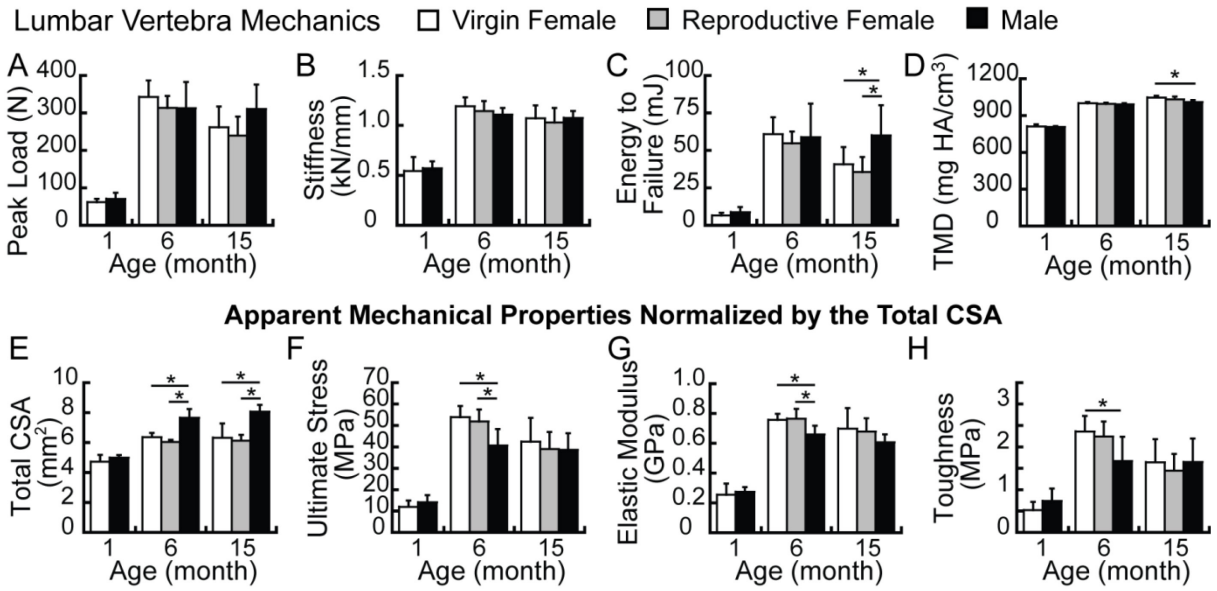

Figure 3. Differences among virgin female, reproductive female, and male rats in vertebra mechanics as measured through uniaxial compression testing. (A-C) Extrinsic mechanical properties, including (A) peak load, (B) stiffness, and (C) energy to failure; (D) Tissue Mineral Density; (E-H) Vertebral body apparent-level properties, derived by normalizing extrinsic properties by (E) total cross-sectional area, including: apparent (F) ultimate stress, (G) elastic modulus, and (H) toughness. * indicate significant differences among groups at a given age ($p<0.05$).

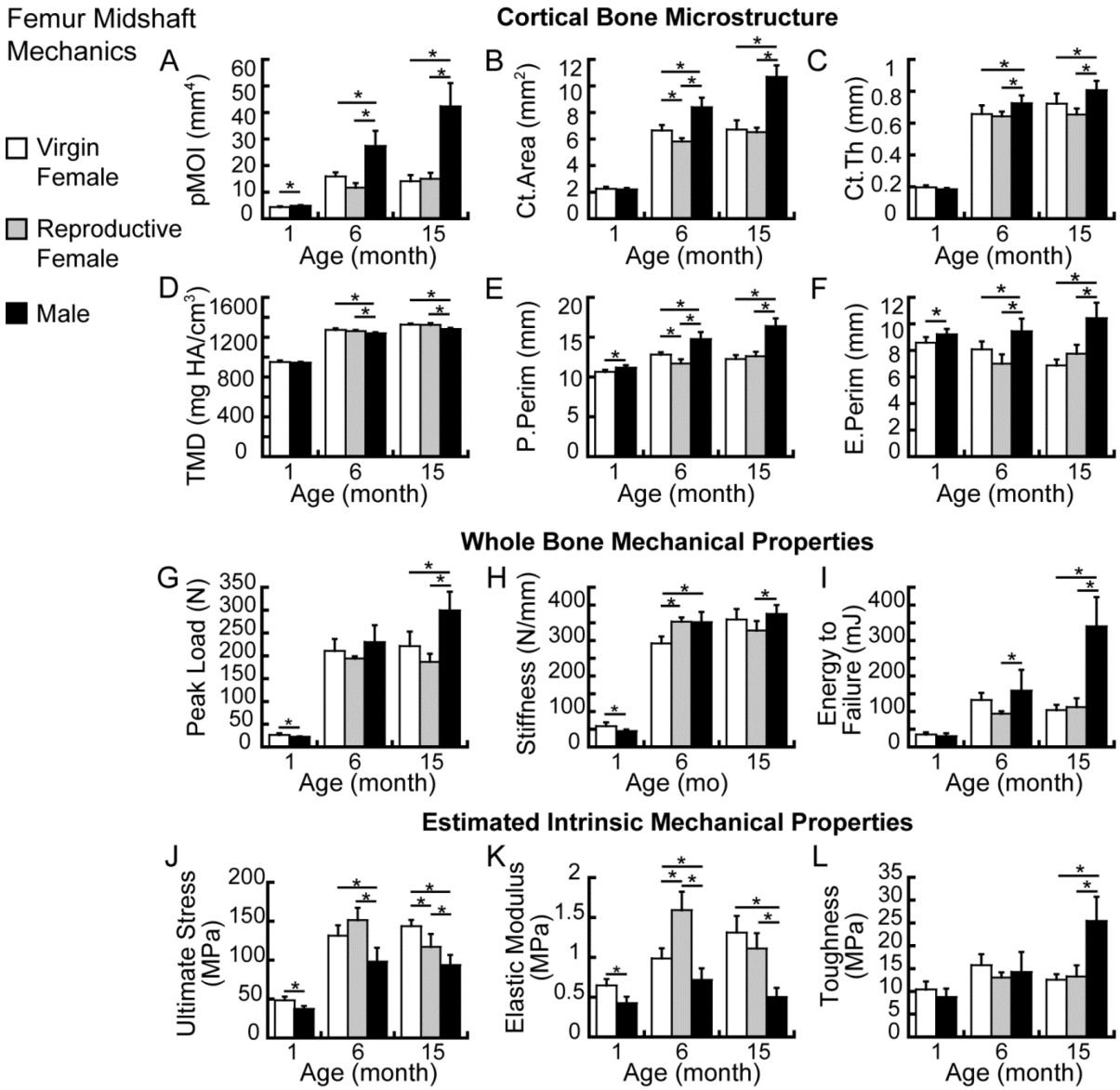

Figure 4. Comparisons among virgin female, reproductive female, and male rats in (A-F) cortical bone structure at the femur midshaft, including: (A) pMOI, (B) Ct.Area, (C) Ct.Th, (D) TMD, (E) P.Perim, and (F) E.Perim; (G-I) whole bone mechanical properties, including: (G) peak load, (H) stiffness, and (I) energy to failure. (J-L) Intrinsic mechanical properties were derived based on 3-point bending results and μCT-based cortical structure: (J) ultimate stress, (K) elastic modulus, and (L) toughness. * indicate significant differences among groups at a given age ($p<0.05$).

Conflict of Interest:
The authors declare that they have no conflict of interest.

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