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RESEARCH ARTICLE

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Maintenance of bone mass despite estrogen depletion in female common marmoset monkeys (*Callithrix jacchus*)

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Estrogen depletion leads to bone loss in almost all mammals with frequent regular ovarian cycles. However, subordinate adult female common marmosets (*Callithrix jacchus*) undergo socially induced anovulation and hypoestrogenism without clinically apparent adverse skeletal consequences. Thus, we speculated that this non human primate might have evolved a mechanism to avoid estrogen-depletion bone loss. To test this possibility, we performed three experiments in which lumbar-spine (L5-L6) bone mineral content (BMC) and density (BMD) were assessed using dual-energy X-ray absorptiometry: (i) cross-sectionally in 13 long-term ovariectomized animals and 12 age- and weight-matched controls undergoing ovulatory cycles; (ii) longitudinally in 12 animals prior to, 3–4 and 6–7 months following ovariectomy (ovx), and six controls; and (iii) cross-sectionally in nine anovulatory subordinate and nine dominant females. In Experiments 1 and 3, plasma estradiol and estrone concentrations were measured and uterine dimensions were obtained by ultrasound in a subset of animals as a marker of functional estrogen depletion. Estrogen levels, uterine trans-fundus width, and uterine dorso-ventral diameter were lower in ovariectomized and subordinate females than in those undergoing ovulatory cycles. However, no differences were found in L5-L6 BMC or BMD. These results indicate that estrogen depletion, whether surgically or socially induced, is not associated with lower bone mass in female common marmosets. Thus, this species may possess unique adaptations to avoid bone loss associated with estrogen depletion.

KEYWORDS
bone, estrogen, marmoset, osteoporosis, reproductive suppression

1 | INTRODUCTION

In almost all mammals with regular, frequent ovarian cycles, substantial, chronic reduction of circulating estrogen concentrations, whether occurring spontaneously (e.g., post-menopausally) or induced experimentally (e.g., by ovariectomy [ovx]), leads to reduction in bone mass (Bauss & Russell, 2004; Rodgers, Monier-Faugere, & Malluche, 1993). This occurs within 1 month following ovx in rats (Florio et al., 2016; Lelovas, Xanthos, Thoma, Lyritis, & Dontas, 2008; Li, Shen, & Wronski, 1997) and in as little as 3 months in rhesus monkeys (*Macaca mulatta*; Binkley et al., 1998; Fox et al., 2009). Among primates, estrogen-depletion bone loss has been documented in all species studied (Smith, Jolette, & Turner, 2009), including rhesus macaques (Binkley et al., 1998; Colman, Kemnitz, Lane, Abbott, & Binkley, 1999), cynomolgus macaques (*M. fascicularis*; Florio et al., 2016; Iwamoto et al., 2009; Jerome et al., 1994; Jerome, Lees, & Weaver, 1995; Jerome, Turner, & Lees, 1997; Mazess, Vetter, & Weaver, 1987; Miller, Weaver, McAlister, & Koritnik, 1986), baboons (*Papio sp.*; Havill, Levine, Newman, & Mahaney, 2008), and humans (Raisz, 2005; Riggs, 2000; Streicher et al., 2017).

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The link between estrogen depletion and bone loss in women was first observed nearly 80 years ago (Albright, 1940). Extensive subsequent research has established estrogen as a, if not the, major systemic regulator of bone metabolism in both women and men (Khosla, Amin, & Orwoll, 2008). Estrogen inhibits the activation of bone resorption. Thus, following estrogen withdrawal in women, whether natural or surgical, there is a marked increase in bone resorption and bone formation. However, resorption exceeds formation, leading to net bone loss (Khosla, Oursler, & Monroe, 2012). This bone loss has profound importance for postmenopausal women with up to 50% experiencing an osteoporosis-related fracture in their lifetime with associated loss of independence, increased risk for institutionalization, and death (Cosman et al., 2014; Morin et al., 2011, 2012).

Glucocorticoids, both natural and synthetic, are also known to impact bone metabolism. Although the mechanisms are not fully elucidated, glucocorticoids such as cortisol have a complex and dose-dependent role in bone health. At physiological levels, glucocorticoids promote bone formation (Zhou, Cooper, & Seibel, 2013), while elevated levels of cortisol blunt bone formation leading to lower bone density (Delany, Dong, & Canalis, 1994). Cortisol also affects bone though several mechanisms including inhibition of growth hormone and gonadal steroid production and reduction of intestinal calcium absorption, ultimately leading to reduced bone mass (Heshmati et al., 1998). Hypercortisolism, whether from endogenous or exogenous glucocorticoids, is clearly linked to a high prevalence of osteoporosis and increased fracture risk (Kaltsas & Makras, 2010).

In the common marmoset (*Callithrix jacchus*), a small New World monkey, female reproductive physiology is profoundly influenced by social status in both the field and captivity. Behaviorally subordinate females typically undergo prolonged periods of anovulation, associated with very low circulating levels of estrogen, progesterone, and chorionic gonadotropin (CG; the marmoset equivalent of luteinizing hormone) (Abbott & Hearn, 1978; Abbott, Barnett, Colman, Yamamoto, & Schultz-Darken, 2003; Abbott, Hodges, & George, 1988; Evans & Hodges, 1984; Saltzman, Digby, & Abbott, 2009; Saltzman, Schultz-Darken, Wegner, Wittwer, & Abbott, 1998). These endocrine consequences of subordination are both rapid and reversible: when a female undergoing ovarian cycles is introduced into a social group and becomes subordinate, plasma CG concentrations fall dramatically in 1–4 days and ovulatory cycles cease. Upon removal from the social group, anovulatory subordinate females show equally rapid reversal of these endocrine changes and usually ovulate within 2–3 weeks (Abbott et al., 1988). Subordinate females may remain reproductively suppressed for 2 years or more (Abbott & George, 1991). Thus, female marmosets undergo prolonged periods of hypoestrogenism, which may constitute 20% or more of their adulthood.

In view of this prolonged hypoestrogenism, it might be anticipated that anovulatory subordinate females would experience bone loss and high fracture rates. However, because reproductive suppression appears to be an adaptation for cooperative breeding in this species and may occur frequently in reproductive-age females, we hypothesized that marmosets might have evolved a mechanism to avoid bone loss associated with estrogen depletion. To test this hypothesis, we characterized bone mass in three groups of hypoestrogenic adult females: (i) long-term bilaterally ovariectomized (ovxed) animals, compared cross-sectionally to age- and weight-matched controls undergoing regular ovulatory cycles; (ii) females studied longitudinally before and after ovx, compared to age- and weight-matched controls studied at comparable timepoints; and (iii) anovulatory, subordinate females compared cross-sectionally to dominant females undergoing ovulatory cycles. In Experiments 1 and 3, we additionally characterized plasma estradiol and estrone concentrations and determined uterine dimensions by ultrasound to quantify functional estrogen depletion.

2 | METHODS

2.1 | Animals

All marmosets participating in this research were housed at the Wisconsin National Primate Research Center (WNPRC) at the University of Wisconsin-Madison, an AAALAC-accredited facility that meets USDA standards. The Graduate School Animal Care and Use Committee (ACUC) monitors housing and animal condition regularly to ensure all guidelines are met for the safety and health of the animals. This research was reviewed and approved by the University of Wisconsin Graduate School ACUC. Experiments were conducted in compliance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

A total of 51 animals were utilized in three experimental paradigms. All procedures were performed at the WNPRC. Each of the three experiments used adult (mean life expectancy approximately 12–14 years, mean age of puberty approximately 16 mo; Abbott & Hearn, 1978) female common marmosets that had been born in captivity and were housed at the WNPRC. All monkeys were socially housed, either in male-female pairs (12 ovxed, 5 control females in Experiment 1, all ovxed females in Experiment 2), in triads with two males (1 ovxed, 2 control females in Experiment 1), or in groups containing 2–3 unrelated adult females and 1–2 intact adult males (5 controls in Experiment 1, all controls in Experiment 2, all dominant and subordinate females in Experiment 3). Females paired with males and those that are socially dominant in groups with >1 female have similar physiological parameters (Abbott & George, 1991; Saltzman et al., 1998). Animals had free access to water, food (Zu/Preem Marmoset Diet #6920 [Premium Nutritional Products, Topeka, KS] or Mazuri Callitrichid High Fiber Diet #5MI6 [Purina Mills International, St. Louis, MO]) and supplemental nutrition. Overall the animals' daily diet contained 0.20–0.45% calcium and 700 IU vitamin D_3. Additional information on marmoset housing and husbandry has been published (Saltzman et al., 1998). Mean age and body mass for animals in each of the three experiments are presented in Table 1.

TABLE 1 Age and body mass (mean ± SEM) at the time of bone mineral determination (baseline in Experiment 2)

	N	Age (months)	Body mass (g)
Experiment 1: Cross-sectional ovx			
Control	12	49.6 ± 1.7	392 ± 18
Ovxed	13	52.6 ± 2.0	383 ± 12
**p*-value (*t*-statistic[df])		0.18 (1.37[24])	0.47 (0.74[24])
Experiment 2: Longitudinal ovx			
Control	6	28.45 ± 2.8	382 ± 8
Ovxed	12	35.11 ± 2.7	380 ± 16
**p*-value		0.15 (1.52[16])	0.92 (0.10[16])
Experiment 3: Social subordination			
Dominant	9	37.0 ± 1.9	398 ± 18
Subordinate	9	32.5 ± 2.1	403 ± 30
**p*-value		0.13 (1.60[16])	0.57 (0.58[16])

**p*-value from unpaired Student's *t*-test.

2.2 | Experimental design

2.2.1 | Experiment 1 (cross-sectional ovx)

The skeletal effects of estrogen depletion were evaluated cross-sectionally by dual-energy X-ray absorptiometry (DXA) in 13 long-term ovxed animals (mean ± SEM time since ovx: 16.3 ± 1.1 mo, range: 9.4–20.5 mo). These animals were sexually mature at the time of surgery (mean ± SEM age: 36.3 ± 1.7 mo, range: 28.5–47.7 mo). The control group consisted of 12 age- and weight-matched intact females undergoing ovulatory cycles. Each animal underwent skeletal assessment at one time-point. Additionally, we determined plasma estrone and estradiol levels in all animals and performed uterine ultrasonography to assess functional estrogen depletion in a randomly selected subset of animals (4 ovxed, 4 control) to secondarily confirm low circulating estrogen concentrations. Four ovxed animals that had been used in Experiment 2, 2 dominant females that had been used in Experiment 3, and 2 animals (1 ovxed, 1 control) that had been used in both Experiments 2 and 3, each underwent an additional DXA scan at least 12 months later for inclusion in this cross-sectional study. Data collection for this experiment was performed between August 1995 and May 1998.

2.2.2 | Experiment 2 (longitudinal ovx)

The skeletal impact of estrogen depletion was examined longitudinally by DXA in 12 adult female common marmosets compared to six age- and weight-matched control animals undergoing ovulatory cycles. The 12 experimental animals were assessed at baseline, then underwent bilateral ovx within 10 days and were again assessed at 3–4 and 6–7 months post-ovx. The six control animals followed a similar schedule. Three dominant females from Experiment 3 underwent subsequent DXA scans, 4–5 months later, for this experiment (2 as controls, 1 as ovxed). Data collection for this experiment was performed between March 1995 and August 1998.

2.2.3 | Experiment 3 (social subordination)

In this experiment, we cross-sectionally characterized bone mass in 18 nulliparous adult female common marmosets: nine anovulatory, socially subordinate females and nine age- and weight-matched dominant animals undergoing ovulatory cycles (Table 1). Additionally, within 1 month of DXA, plasma estradiol and estrone concentrations were determined in all animals, and uterine ultrasonography was performed on a smaller number of randomly selected dominant ($n = 6$) and subordinate ($n = 3$) females. Social groups had been formed as described previously (Saltzman et al., 1998), 10.4 ± 1.2 mo prior to skeletal assessment. Determination of dominant and subordinate status, based on behavior (Saltzman, Schultz-Darken, Scheffler, Wegner, & Abbott, 1994), was confirmed by the occurrence of ovulatory cycles in dominant females and anovulation in subordinate females, based on plasma progesterone concentrations (Saltzman et al., 1994; see below). At the time of bone mass measurement, subordinate females had been both anovulatory (plasma progesterone concentrations <10 ng/ml; see below) and socially subordinate for 6.1 ± 0.8 mo (range: 3.0–9.8 mo). Two subordinates had been anovulatory at least since we began monitoring their ovarian function when they were 1 year of age. The remaining seven subordinates were known to have ovulated at least once, before and/or after formation of social groups. Dominant females had been undergoing regular ovulatory cycles of approximately 28 days (Saltzman et al., 1994) for 10.5 ± 1.8 mo (range: 4.7–18.2 mo). Data collection for this experiment was performed between May 1996 and June 2002.

2.3 | Ovariectomy

Marmosets were anesthetized with Saffan (20 mg/kg, IM; 8.1 mg alphaxalone:2.7 mg alphadolone acetate, IM; Pitman-Moore, Harefield, Uxbridge, Middlesex, UK), and bilateral ovx was performed using a ventral midline approach and standard methodology.

2.4 | Blood sample collection, hormone assays, and monitoring of ovarian function

To monitor ovarian function in intact animals, femoral venipuncture was performed at 3- to 4-day intervals for measurement of plasma progesterone concentrations; estradiol and estrone were additionally assayed in some of these samples.

Plasma progesterone concentrations were measured in duplicate using a heterologous enzyme immunoassay (Saltzman et al., 1994). Assay sensitivity at 90% binding was 2.7 ng/ml, and intra- and inter-assay coefficients of variation (CVs) of a marmoset plasma pool were 5.9% and 21.0%, respectively. Ovulation was considered to have occurred on the day before a sustained (≥2 consecutive blood samples) progesterone elevation above 10 ng/ml (Harlow, Gems, Hodges, & Hearn, 1983), and animals were considered to be in the luteal phase of

the ovarian cycle or in early pregnancy whenever progesterone concentrations remained above this level (Saltzman et al., 1994). Representative progesterone profiles from two cycling (dominant) females and two anovulatory subordinate females are presented in Figure 1.

For each cycling female, we assayed estradiol and estrone in an “early follicular” pool (samples collected 6–8 days before ovulation), a “midcycle” pool (5 days before through 3 days after ovulation), and a “luteal/early pregnancy” pool (4–21 days after ovulation; Saltzman et al., 1998). Estrone and estradiol concentrations from six ovxed animals in Experiment 1 and from five subordinate animals and one dominant animal in Experiment 3 have been reported previously (Saltzman et al., 1998).

Plasma estradiol and estrone concentrations were measured by radioimmunoassay following extraction with ethyl ether and celite column chromatography (Saltzman et al., 1998). Assay sensitivity was 10.0 pg/ml for estradiol and 39.7 pg/ml for estrone, and the intra- and inter-assay CVs were 3.3% and 15.7%, respectively, for estradiol, and 3.5% and 14.3%, respectively, for estrone.

To prevent term pregnancies, each ovulatory female received an IM injection of 0.75–1.0 μg cloprostenol sodium (Estrumate, Mobay Corp., Shawnee, KS), a prostaglandin F2α analog (Harlow et al., 1983), 14–30 days after each ovulation. This treatment causes luteolysis and termination of the luteal phase or early pregnancy (Summers, Wennink, & Hodges, 1985). Ovxed and subordinate females received a comparable dose of cloprostenol at irregular intervals, approximately once every 6 months.

2.5 | Ultrasonography

Uterine size was determined in four ovxed and four cycling females (Experiment 1), and in three subordinate and six dominant females (Experiment 3). The ovxed females were 15.5 ± 1.6 months post-surgery, and the subordinates had been anovulatory (plasma progesterone concentrations <10 ng/ml) for 4.5 ± 2.3 mo at the time of uterine analysis. Intact animals were in the early to mid-follicular phase of the ovarian cycle during ultrasonography. Non-invasive evaluation of uterine dimensions was obtained as described previously (Saltzman, Severin, Schultz-Darken, & Abbott, 1997). Ultrasonography was performed using an Aloka SSD-650 real-time scanner equipped with an interoperative linear array probe (UST-5522L-7.5) operating at a frequency of 10 MHz. Using the scanner's calibrated, digitized calipers, uterine trans-fundus length (transverse uterine diameter), and dorso-ventral uterine diameter were measured from transverse sections, and fundus-cervix length was measured from sagittal sections.

2.6 | Dual energy X-ray absorptiometry (DXA)

We performed DXA using a DPX-L densitometer (GE/Lunar Corp., Madison, WI) and small animal software (version 1.0d). The lumbar spine (L5-L6) was selected as the region of interest for two main reasons: (i) in other species, this area contains a high proportion of cancellous bone which is very susceptible to estrogen depletion-induced bone loss and (ii) we were able to acquire reproducible scans of

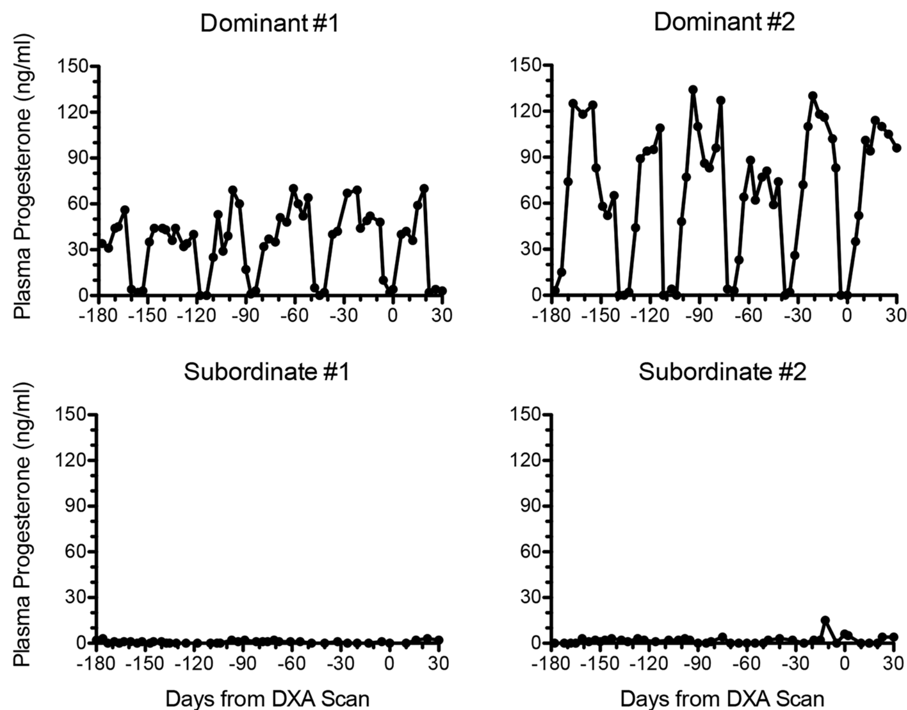

FIGURE 1 Experiment 3: plasma progesterone profiles of two representative dominant females and two representative subordinates, from 6 months before through 1 month after skeletal assessment (DXA scan). Animals were considered to be in the luteal phase of the ovarian cycle when progesterone concentrations exceeded 10 ng/ml in ≥2 consecutive samples

this region. Animals were anesthetized with Saffan (20 mg/kg, IM) and scans were performed using the appendicular mode with the animals placed supine upon the scanner bed. Scan analysis was performed using the "autoanalysis" feature after selecting a region of interest surrounding L5-L6. Precision (L5-L6) was determined as the average of the CVs of five scans performed on the same day on each of five different animals with repositioning between scans. L5-L6 BMC and BMD precision (CV) were 2.4% and 1.4%, respectively. Accuracy was determined by comparison of L5-L6 BMC to ash weight in 4 animals. Ash weight and BMC were highly correlated ($p < 0.0001$, $R^2 = 0.997$).

2.7 | Analysis

Plasma estradiol and estrone concentrations were log-transformed and analyzed by unpaired Student's *t*-test (cross-sectional comparisons) or ANOVA, with Tukey post-hoc testing (longitudinal comparisons). All other cross-sectional group comparisons were performed by unpaired Student's *t*-test. Repeated-measures ANOVA was performed on all longitudinal data. Because of small sample sizes, ultrasonographically determined uterine measurements were analyzed by Mann–Whitney tests. For all analyses, significance was assessed at the 0.05 level (two-tailed). Prospective power analysis indicated that with our sample sizes, we had >95% probability of detecting a 7% difference in BMD between groups. This difference was chosen based on rhesus macaque data showing a >7% decline in BMD 3 months after ovx (Binkley et al., 1998). Additional prospective power analysis indicated that with our sample sizes for uterine dimension analyses in Experiments 1 and 3, we had >97% probability of detecting differences between groups.

3 | RESULTS

3.1 | Experiment 1: cross-sectional ovariectomy

3.1.1 | Estrone and estradiol

We first evaluated changes in plasma estrone concentrations ($F[2, 34] = 42.23$, $p < 0.0001$) across the ovarian cycle in the 12 control animals. Estrone concentrations were similar between the early follicular and midcycle phases ($t[32] = -0.25$, $p > 0.05$) and rose in the luteal phase/early pregnancy (compared to early follicular $t[32] = 7.50$, $p < 0.0001$ and midcycle $t[32] = -8.15$, $p < 0.0001$; see Figure 2). Plasma estrone levels of cycling females at each phase of the cycle were higher than those of ovxed females (early follicular vs. ovx: $t[44] = -5.28$, $p < 0.0001$; luteal phase/early pregnancy vs. ovx: $t[44] = -14.07$, $p < 0.0001$; midcycle vs. ovx: $t[44] = -5.27$, $p < 0.0001$). Plasma estradiol levels, in contrast to estrone, did not change reliably across the ovarian cycle in control females ($F[2, 38] = 2.22$, $p > 0.10$), probably because the pre-ovulatory peak was subsumed within the lower values from most days within the "midcycle" pool. Mean estradiol levels of cycling females, however, were higher than those of ovxed animals ($t[13] = -14.34$, $p < 0.0001$).

3.1.2 | Uterine ultrasonography

Uterine trans-fundus length (control: median = 10.0 mm; ovx: median = 3.6 mm; $U = 0$, $p < 0.03$), dorso-ventral diameter (control: median = 6.0 mm; ovx: median = 2.5 mm; $U = 0$, $p < 0.03$), and fundus-cervix length (control: median = 10.0 mm; ovx: median = 5.0 mm; $U = 0$, $p < 0.03$) were significantly smaller in long-term ovxed marmosets than in intact controls, with no overlap between groups (Table 2), indicative of uterine atrophy. These findings demonstrate that ovx produced functional estrogen depletion.

3.1.3 | Bone mass

Overall, lumbar-spine (L5-6) bone mineral content (BMC) and bone mineral density (BMD) in female common marmosets averaged 0.298 g and 0.236 g/cm^2, respectively. No differences in L5-6 BMC ($t[24] = 0.43$, $p > 0.05$) or density ($t[24] = 0.84$, $p > 0.05$) were observed between long-term ovxed and intact females (Figure 3).

3.2 | Experiment 2: longitudinal ovariectomy

3.2.1 | Body mass

Body mass did not differ ($t[16] = 0.10$, $p > 0.50$) between the six control animals and the 12 ovxed animals at baseline (Table 1). Mean body

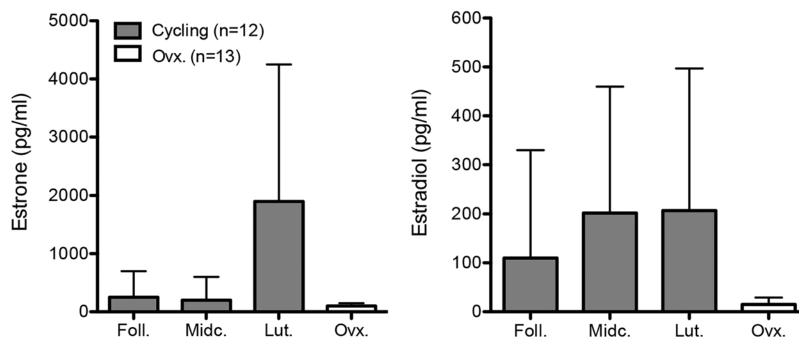

FIGURE 2 Experiment 1: plasma estradiol and estrone concentrations (anti-log of the mean + 95% confidence limits) of 12 cycling females in the early follicular phase of the ovarian cycle, midcycle, and luteal phase/early pregnancy, and in 13 long-term, bilaterally ovariectomized females

TABLE 2 Uterine dimensions (mean ± SEM [range]) as determined by uterine ultrasonography

	Fundus-cervix length (mm)	Trans-fundus width (mm)	Dorso-ventral diameter (mm)
Experiment 1: Cross-sectional ovx			
Control (*n* = 4)	14 ± 1 (12–7)	9 ± 1 (7–12)	6 ± 1 (4–8)
Ovxed (*n* = 4)	5 ± 0 (4–6)	4 ± 0 (3–5)	2 ± 0 (2–3)
**p*-value	<0.03	<0.03	<0.03
Experiment 3: Social subordination			
Dominant (*n* = 6)	15 ± 1 (12-17)	9 ± 1 (8–12)	6 ± 1 (5–8)
Subordinate (*n* = 3)	13 ± 1 (12–14)	6 ± 1 (4–7)	3 ± 1 (2–4)
**p*-value	>0.05	<0.03	<0.03

**p*-value from Mann–Whitney test.

mass did not change over the length of the study ($F[2, 48] = 1.42$, $p > 0.25$), nor did the control and ovxed groups differ ($F[1, 48] = 0.97$, $p > 0.32$, data not shown).

3.2.2 | Bone mass

L5-6 BMC and BMD (Figure 4) did not differ (BMC: $t[16] = 0.58$, $p > 0.56$; BMD: $t[16] = 0.19$, $p > 0.85$) between control and ovxed animals at baseline. Neither BMC nor BMD changed over time (BMC: $F[2, 48] = .09$, $p > 0.90$; BMD: $F[2, 48] = 0.34$, $p > 0.70$), nor did the control and ovxed groups differ over time (BMC: $F[2, 48] = .02$, $p > 0.90$; BMD: $F[2, 48] = 0.56$, $p > 0.50$). Although not statistically significant, ovxed animals experienced a mean 1.0% decrease in BMC (three animals increased, nine animals decreased) and mean 0.9% decrease in BMD (five animals increased, seven animals decreased) over the course of the study, compared to 1.0% decrease in BMC and 3.8% increase in BMD in the control animals. These data suggest that estrogen-depletion bone loss does not occur in ovxed marmosets, at least within the first 6–7 months following ovariectomy (ovx).

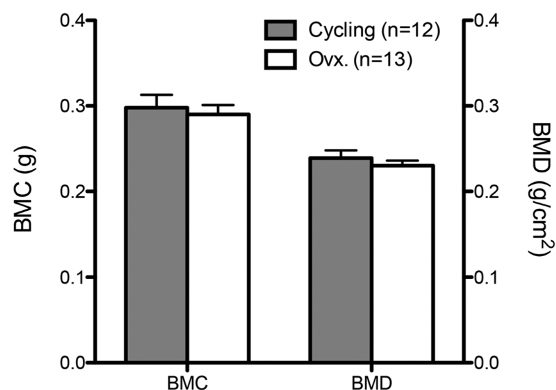

FIGURE 3 Experiment 1: mean + SEM L5-L6 bone mineral content and bone mineral density in 12 cycling and 13 bilaterally ovariectomized female marmosets, as determined by DXA. Ovariectomized animals were 9–20 months post-surgery

3.3 | Experiment 3: social subordination

3.3.1 | Estrone and estradiol

To compare circulating estrogen concentrations in dominant and subordinate females, we first evaluated changes in plasma estrone and estradiol levels across the ovarian cycle in dominant animals. Estrone levels changed significantly across the cycle ($F[2, 24] = 23.36$, $p < 0.0001$): estrone concentrations showed a nonsignificant tendency to decline from the early follicular to the midcycle phase ($t[8] = 2.20$, $p = 0.06$) and showed a significant rise in the luteal phase/early pregnancy (vs. follicular: $t[8] = 6.83$, $p < 0.001$, vs. midcycle: $t[8] = 11.61$, $p < 0.0001$; Figure 5). Plasma estrone levels of dominant females in both the follicular phase ($t[16] = 2.148$, $p < 0.05$) and luteal phase/early pregnancy ($t[16] = 16.00$, $p < 0.0001$), but not the mid-cycle period ($t[16] = 0.65$, $p > 0.1$), were significantly higher than those of subordinate females. Estradiol levels, in contrast to estrone, did not differ reliably across the phases of the ovarian cycle in dominant females ($F[2, 24] = 0.02$, $p > 0.9$); however, mean estradiol levels of dominant females were significantly higher than those of subordinate females ($t[16] = 2.22$, $p < 0.05$).

3.3.2 | Uterine ultrasonography

Among the nine animals for which ultrasonographically determined uterine measurements were available, dominant females had significantly larger trans-fundus lengths (dominant: median = 8.0 mm; subordinate: median = 7.0 mm; $U = 0$, $p < 0.03$) and dorso-ventral uterine diameters (dominant: median = 5.0 mm; subordinate: median = 4.0 mm; $U = 0$, $p < 0.03$) than subordinate females, and no overlap occurred between the two groups in these measures (Table 2). Fundus-cervix length did not differ reliably between dominant and subordinate animals (dominant: median = 15.0 mm; subordinate: median = 12.0 mm; $U = 4$, $p > 0.05$).

3.3.3 | Bone mass

In contrast with their differences in circulating estrogen levels and uterine sizes, dominant and subordinate female marmosets showed no

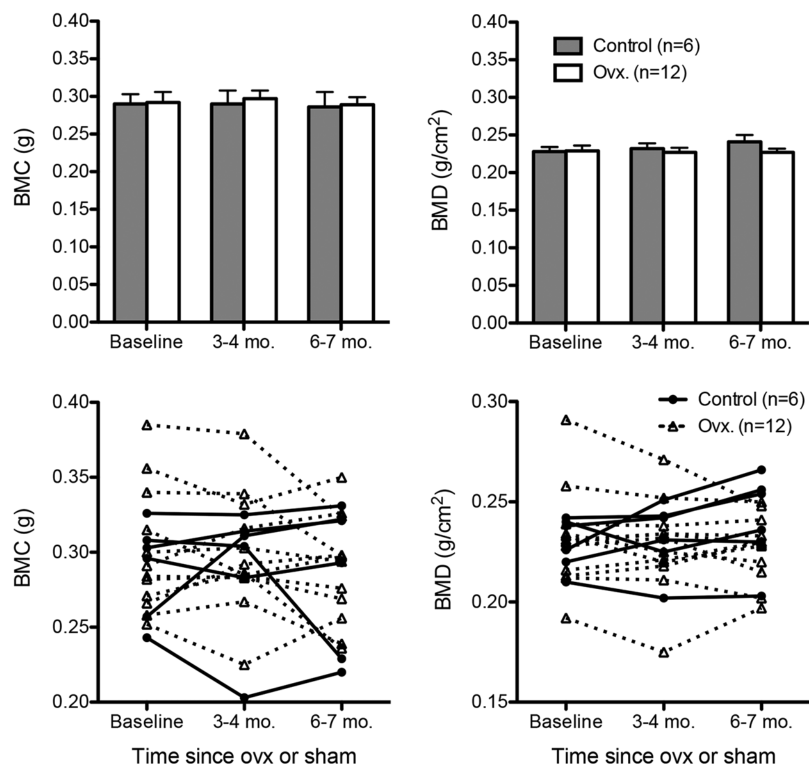

FIGURE 4 Experiment 2: longitudinal changes in L5-L6 bone mineral content and bone mineral density in 12 bilaterally ovariectomized marmosets at baseline and 3–4 and 6–7 months post-surgery, and six age- and weight-matched cycling controls at three comparable time points. Top panels represent mean + SEM L5-L6 bone mineral content and bone mineral density. Lower panels represent individual longitudinal change in control (closed circles and solid lines) and ovariectomized (open triangles and dashed lines) animals

significant differences in DXA-measured BMD ($t[16] = 0.77$, $p > 0.4$) or BMC ($t[16] = 0.68$, $p > 0.5$) of L5-6 (Figure 6).

4 | DISCUSSION

These studies confirm that both long-term ovxed and anovulatory, ovary intact socially subordinate female marmosets are estrogen deplete, and demonstrate that this estrogen depletion is not associated with lower L5-6 bone mass. This observation is unique among female primates and contrasts with findings in other mammals (Bauss & Russell, 2004; Binkley et al., 1998; Colman et al., 1999; Florio et al., 2016; Havill et al., 2008; Iwamoto et al., 2009; Komori, 2015; Smith et al., 2009), including humans (Khosla, 2013; Khosla et al., 2012), in which estrogen reduction leads to bone loss. Elucidation of the mechanism(s) that prevent estrogen-depletion bone loss in marmosets may provide important insight into approaches for preventing such loss in humans.

Our understanding of reproductive function and its suppression in free-living marmosets is limited, but it is clear that many socially subordinate adult females undergo periods of reproductive inactivity that may be associated with anovulation (Harris et al., 2014; Saltzman et al., 2009). If wild marmosets resemble their captive counterparts, in which subordinate females are reliably hypoestrogenemic

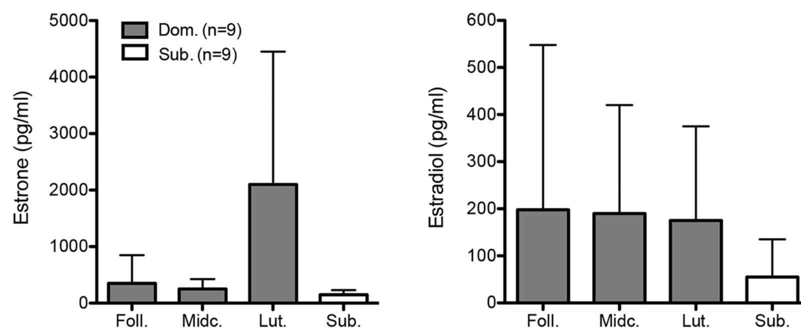

FIGURE 5 Experiment 3: plasma estrone and estradiol concentrations (anti-log of the mean + 95% confidence limits) of nine dominant females in the early follicular phase of the ovarian cycle, midcyle, and luteal phase/early pregnancy; and in nine anovulatory subordinate females

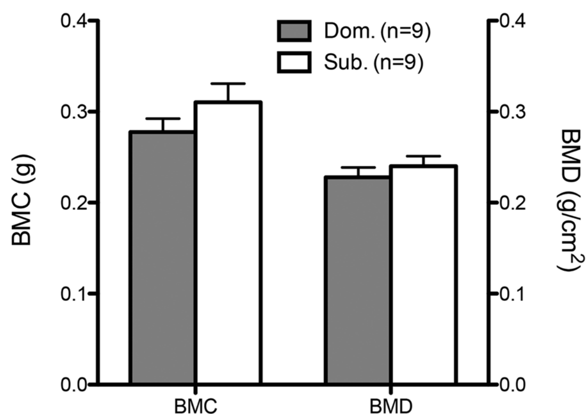

FIGURE 6 Experiment 3: mean + SEM L5-L6 bone mineral content and bone mineral density in nine dominant and nine subordinate females, as determined by DXA

(Abbott & Hearn, 1978; Abbott et al., 1988; Evans & Hodges, 1984; Saltzman et al., 1998), then most females are likely to spend a significant portion of their adult lives in an estrogen-deplete state. Thus, marmosets may have undergone strong natural selection for maintenance of skeletal integrity despite estrogen depletion.

The mechanism(s) preventing estrogen-depletion bone loss in marmosets are unknown. However, reduction of circulating glucocorticoid concentrations is one possibility, as both subordinate and ovxed females demonstrate suppression of basal cortisol concentrations. Specifically, plasma cortisol concentrations decline dramatically within 6–7 weeks following onset of subordinate status and anovulation and can remain suppressed for months to years (Johnson et al., 1996; Saltzman et al., 1994). In ovxed females, cortisol reductions may take several months to develop and are less drastic than those in subordinate females (Saltzman et al., 1998). Cortisol suppression in female marmosets appears to be at least partly mediated by reductions in ovarian hormones (Saltzman et al., 1998 but see Saltzman, Hogan, Allen, Horman, & Abbott, 2006), which may alter adrenocortical responsiveness to adrenocorticotropic hormone (Saltzman, Prudom, Schultz-Darken, & Abbott, 2000). It is intriguing to speculate that marmosets have evolved a mechanism to chronically reduce cortisol concentrations during sociophysiological conditions that are reliably associated with hypoestrogenism, enabling them to maintain bone mass during prolonged estrogen depletion. However, in several other species (e.g., cynomolgus macaques: Kaplan et al., 2010; Shively & Day, 2015 and rats: Kitay, 1963), ovx also reduces cortisol concentrations but does lead to bone loss. Thus, the significance of subordination- or ovx-induced cortisol reductions in marmosets remains unclear.

The absence of estrogen-depletion bone loss in female marmosets might be linked to their high circulating steroid hormone concentrations. Marmosets have circulating concentrations of several steroid hormones—including estrogens and 1,25-dihydroxyvitamin D_3—that are an order of magnitude higher than those in Old World primates (Adams, Gacad, Baker, Gonzales, & Rude, 1985; Coe, Savage, & Bromley, 1992). However, marmosets have been described as resistant to these steroids, apparently due to high expression of intracellular proteins that compete for binding with steroid receptors or response elements (Chen et al., 2004; Gacad, Chen, Arbelle, LeBon, & Adams, 1997). Thus, elevated levels of estrogens, 1,25-dihydroxyvitamin D_3, and other bone-active steroids seem unlikely to explain the absence of bone loss in estrogen-depleted female marmosets. Other mechanisms that might prevent estrogen-depletion bone loss in marmosets could involve cytokines or leptin, both of which are regulated by estrogen and contribute to estrogen-depletion bone loss in humans and rodents (Haberland, Schilling, Rueger, & Amling, 2001; Pacifici, 1996; Shimizu et al., 1997).

It is also possible that maintenance of bone mass in marmosets is regulated, at least in part, by potentially very low levels of estrogen that remain after ovx or by extra-ovarian estrogen (Kenealy et al., 2013; Terasawa, 2018) perhaps from bone (Miki et al., 2017), adipose tissue (DiSilvestro et al., 2014), or the hypothalamus (Guerriero, Keen, Millar, & Terasawa, 2012). Extra-ovarian estrogen can exert negative feedback regulation of gonadotropin release in female marmosets (Kraynak et al., 2017) suggesting that it might be involved in the bone preservation we observed. Studies examining the bone response to a reduction in both ovarian and brain estrogen may begin to address this possibility. Finally, it may be that development and maintenance of bone mass are not estrogen-dependent in marmosets. Such a phenomenon would, to our knowledge, be unique among mammals with regular ovarian cycles. Future studies examining the effects of estrogen on peak bone mass attainment are required to address this possibility. Given the contribution of estrogen-depletion bone loss to fracture risk in postmenopausal women, further research to define the mechanism(s) by which hypoestrogenic marmosets avoid bone loss is clearly warranted.

One might argue that subordinate female marmosets in this study did not exhibit lower bone mass because they were not fully estrogen-deplete. All subordinate females had detectable plasma estrone, and all but one had detectable estradiol. However, these estradiol and estrone concentrations were much lower (68% and 85%, respectively) than those in dominant animals. Furthermore, the subordinate females exhibited uterine atrophy, indicating that they were functionally estrogen-deplete. Finally, we found no evidence that female marmosets lose bone mass even after ovx, when estrogen levels are markedly lower than those of both dominant and subordinate females or undetectable (Saltzman et al., 1998).

The present studies had several limitations, including a lack of some measurements (uterine dimensions) in some animals and the use of cloprostenol sodium, a prostaglandin F2α ($PGF_{2\alpha}$) analog, to terminate the luteal phase or early pregnancy. Although little is known about the skeletal effects of $PGF_{2\alpha}$, a closely related prostaglandin, PGE_2, is known to have anabolic effects on bone (Zhang et al., 2015). In the only published report examining the skeletal effects of $PGF_{2\alpha}$ alone, in vivo (Ma et al., 1995), it was found to be 1/10th as potent as PGE_2 at stimulating bone formation in ovxed rats. Furthermore, these anabolic effects were demonstrated with daily injections of doses 1,000-fold higher than used in the present study. Thus, it seems unlikely that prostaglandin exposure explains our finding that estrogen-replete and -deplete marmosets did not differ in bone parameters.

In conclusion, this study is the first to document maintenance of female bone mass despite estrogen depletion in a primate species or, in fact, in any mammal. Marmosets may therefore provide a unique opportunity to investigate the endocrine control of skeletal physiology and mechanisms to circumvent estrogen-depletion bone loss.

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TWEETABLE SUMMARY

Female common marmosets maintain bone mass during estrogen depletion.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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