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Variation in estradiol level affects cortical bone growth in response to mechanical loading in sheep

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Summary

Although mechanical loading can stimulate cortical bone growth, little is known about how individual physiology affects this response. This study demonstrates that *in vivo* variation in estradiol (E_2) level alters osteoblast sensitivity to exercise-induced strains, affecting cortical bone responses to mechanical loading. Subadult sheep were divided into treatment groups that varied in terms of circulating E_2 levels and loading (exercised and sedentary). After 45 days, periosteal cortical bone growth rates and cross-sectional properties were measured at the midshafts of hindlimb bones and compared with strain data. The results indicate significant interactions between E_2 and strain. Cortical bone growth in exercised animals with elevated E_2 levels was 27% greater in the femur, 6% greater in the tibia, and 14% greater in the metatarsal than in exercised animals with lower E_2 levels, or sedentary animals regardless of E_2 dose ($P<0.05$). There was also a trend toward greater resistance to deformation in the tibia, but not the metatarsal, in the exercised, high-E_2 group compared to the other treatment groups. These results demonstrate that E_2 plays a role in mediating skeletal responses to strain, such that physiological variation in E_2 levels among individuals may lead to differential growth responses to similar mechanical loading regimes. Efforts to model the relationship between environmental strain and bone morphology should include the effects of physiological variation in hormone levels.

Key words: bone, estradiol, estrogen receptor-alpha, periosteal modeling, sheep, strain.

Introduction

Understanding how the growing skeleton adapts to its mechanical environment is a fundamental problem in vertebrate bone biology. While cyclic applied loads often stimulate cortical growth, leading to changes in cross-sectional geometry that can improve a bone's resistance to loading, the mechanisms involved remain the subject of much ongoing research (for reviews, see Pauwels, 1980; Lanyon and Rubin, 1984; Lanyon and Rubin, 1985; Martin et al., 1998; Carter and Beaupré, 2001; Currey, 2002; Pearson and Lieberman, 2004). Strain often stimulates formation of new cortical bone (modeling), but may also provoke no response (stasis), Haversian remodeling, or bone resorption by osteoclasts, depending on the magnitude, frequency and duration of the strain signal and the age of the individual (for a review, see Currey, 2002). Bones are particularly sensitive to high-magnitude cyclic loads and to intermittent loading bouts (Hsieh and Turner, 2001; Robling et al., 2001), and exhibit the greatest growth response prior to sexual maturity (Jones et al., 1977; Lieberman et al., 2003; Pearson and Lieberman, 2004).

In addition, there is evidence for a trade-off between growth and repair in tapered limbs, with more modeling in response to strain in proximal limb elements and more Haversian remodeling in distal limb elements (Lieberman and Crompton, 1998; Lieberman and Pearson, 2001; Lieberman et al., 2003). This trade-off may reduce the kinetic energy cost of accelerating additional bone mass in distal segments (e.g. Hildebrand, 1985; Myers and Steudel, 1985; Bertram and Biewener, 1988; Marsh et al., 2004), but at a cost of higher strains and higher potential for fatigue-induced microcracks, which may be repaired *via* Haversian remodeling. For example, in juvenile sheep, average total strain magnitudes in the metatarsal are over 50% higher than in the tibia (1850 ± 132 $\mu\epsilon$ *vs* 1162 ± 122 $\mu\epsilon$), and rates of Haversian remodeling are about 250% higher in the metatarsal than in the tibia (16.31 ± 4.71 *vs* 4.67 ± 2.79 secondary osteons mm^{-2}) (Lieberman et al., 2004).

At the cellular level, there are multiple pathways by which strain influences osteoblasts and osteocytes. Potential sensory mechanisms include fluid flow and communication at gap junctions between osteocyte canaliculi (Cowin et al., 1995; Saunders et al., 2001; Cherian et al., 2003), Ca^{2+} flux through stretch-activated ion channels in osteoblast cell membranes (Guggino et al., 1989; Davidson et al., 1996), small electrical charges known as strain-generated potentials (SGPs) (Cowin and Moss, 2001), and the primary cilium, a cell process involved in mechanosensation in other tissues (Whitfield,
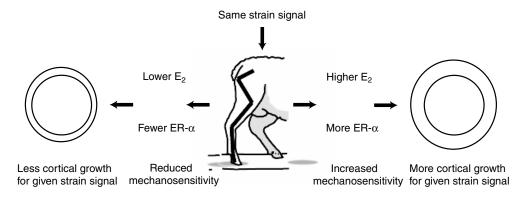
Fig. 1. Hypothesized mechanism of interaction of E_2, ER-α and strain in osteoblasts. Left, E_2 levels are lower and osteoblasts express fewer ER-α receptors, decreasing strain sensitivity. Right, higher E_2 upregulates osteoblast expression of ER-α, increasing strain sensitivity and causing greater osteogenic response to identical mechanical loading.

2003). Such extracellular signals initiate a variety of osteogenic intracellular responses within bone cells, including production of nitrous oxide (NO) and prostaglandin E_2 (PGE$_2$) (Bakker et al., 2001; Bakker et al., 2003; Jessop et al., 2002), and upregulation of Runx2 (also known as Cbfa1), a transcription factor necessary for cortical bone matrix secretion and osteoblast differentiation from precursor cells (Karsenty, 1999; Olsen et al., 2000).

This study tests a mechanism for mechanotransduction that involves interactions between estrogen receptor-alpha (ER-α), estradiol (E_2) and strain. Recent *in vitro* experiments indicate that strain in osteoblasts causes phosphorylation of ER-α, allowing it to function as a mechanosensory structure (Damien et al., 1998; Damien et al., 2000; Zaman et al., 2000; Zaman et al., 2006; Jessop et al., 2001; Cheng et al., 2002). Osteoblast response to mechanical stimuli also varies with ER-α density. Transfecting osteoblasts with additional ER-α increases strain-induced proliferation by 40%, while ER-α knockout (ERKO) mice exhibit markedly reduced cortical growth in response to *in vivo* mechanical loading, compared to normal controls (Zaman et al., 2000; Lee et al., 2003). The relationship between ER-α number and strain sensitivity in osteoblasts is of particular interest because ER-α transcription depends in part on E_2 level. ER-α transcription is decreased by estrogen deficiency and increased by E_2 treatment in humans (Hoyland et al., 1999) and murine models (Lim et al., 1999; Zhou et al., 2001; Zaman et al., 2006).

Although more research is needed on the effects of E_2 on ER-α transcription, particularly in cortical bone, there is evidence that more estrogen leads to more estrogen receptors (Hoyland et al., 1999; Zhou et al., 2001; Zaman et al., 2006), with more receptors generally producing a greater osteogenic response to strain (Zaman et al., 2000; Lee et al., 2003). A reasonable prediction that follows from these results is that variation in E_2 and ER-α could alter cortical bone response to mechanical stimuli. If so, then the same strain stimulus could produce a range of osteogenic responses in different individuals, depending on their E_2 levels and ER-α density.

Here we test a model of the effects of variation in E_2 on *in vivo* cortical responses to strain in limb bone midshafts (Fig. 1). We focus on the midshaft because it is the site of maximum bending within a diaphysis (Biewener et al., 1986), and because previous studies have measured strain distributions at the midshafts of the tibia and metatarsal in sheep (Lieberman et al., 2003; Lieberman et al., 2004), allowing comparisons between local strain environment and bone growth (Fig. 2). The general hypothesis is that estradiol (E_2) affects osteoblast responses to loading by increasing their sensitivity to strain signals. Although this effect presumably occcurs *via* upregulation of ER-α density, it is important to note that this study does not include direct measurement of ER-α, but rather tests for correlations between E_2 level and cortical response to loading. Future studies will include direct quantification of ER-α.
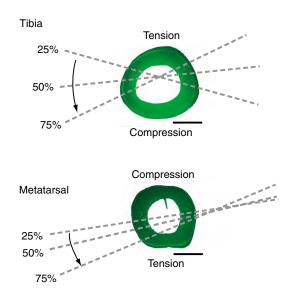

Fig. 2. Neutral axis (NA) location at 25%, 50% and 75% of stance phase in tibia and metatarsal (after Lieberman et al., 2004). Anterior is at top, lateral to the left. Scale bar, 10 mm. Despite NA rotation, strains remain higher on the anterior and posterior than the medial and lateral cortices, 300–800 $\mu\epsilon$ *vs* 0–300 $\mu\epsilon$, respectively.

Hypotheses to be tested

The general hypothesis that estradiol (E_2) affects the capacity of osteoblasts to respond to mechanical loading *in vivo* leads to two sets of hypotheses. The first is that there will be interactions between E_2 level and mechanical loading that have varying effects on cortical bone growth, depending on skeletal location.

Effects on periosteal appositional bone growth

Hypothesis 1. There will be an interaction between E_2 and mechanical loading. Exercised animals with elevated E_2 levels will have more bone growth than those with normal or suppressed E_2 levels. Sedentary animals will exhibit little difference in periosteal bone growth, regardless of E_2 level, because of the low levels of strain stimulus.

Hypothesis 2. Interactions between E_2 and mechanical loading will vary by skeletal location. In mammals such as sheep with tapered limbs, such interactions will follow a proximo-distal gradient, with the greatest growth response in the femur and the least in the metatarsal. Although one might generally expect the most periosteal growth in the bones subject to the greatest loads, previous studies on sheep limbs reveal the opposite pattern, with more modeling proximally and less modeling distally, regardless of applied loads (see discussion above). Accordingly, we predict that interactions between estrogen, strain, and bone growth will follow the same overall pattern.

Effects on midshaft cross-sectional geometry

A second set of hypotheses relates to how interactions between E_2 and strain affect bone strength. The general hypothesis is that by increasing mechanosensitivity at the level of the osteoblast, E_2 may allow localized effects of strain on cortical growth, which leads to the following specific predictions.

Hypothesis 3. There will be an interaction between E_2 and bone strength. Exercised animals with higher E_2 levels will have greater overall resistance to deformation, as measured by section moduli, than those with normal or low E_2 levels. Sedentary animals will exhibit little difference in resistance to bending deformation, regardless of E_2 level, because of the low levels of strain stimulus.

Hypothesis 4. Interactions between E_2 and mechanical loading will be larger on surfaces subjected to tension and compression during locomotion, and smaller near the neutral axis of bending, where strains are lower during locomotion. For this study, periosteal growth was measured on the outer surface of the cortex at the furthest distance from the neutral axis (NA) at peak strain, for two reasons. First, it is reasonable to expect the largest growth responses will occur where strains are highest, i.e. perpendicular to the NA at peak strain. Second, although the position of the NA rotates counterclockwise and migrates caudally during stance phase (Fig. 2), the cortex in the measured locations remains in tension or compression throughout stance phase (Lieberman et al., 2003; Lieberman et al., 2004).

Materials and methods

Subjects and exercise training

Subjects

Experiments to test the above hypotheses used 32 ewe lambs (*Ovis aries* Dorset), aged approximately 120 days old at the start of the 45-day treatment period (Table 1). Domestic sheep are used as the model animal for this study for several reasons: (1) normative growth and limb bone strain data for sheep are available from previous studies (e.g. Lieberman et al., 2003; Lieberman et al., 2004); (2) the animals are of sufficient size and body mass to conduct *in vivo* loading experiments; (3) sheep are easily trained to run on the treadmill; and (4) their rapid pubertal growth involves some of the same hormones that mediate human growth, including growth hormone (GH), insulin-like growth factor (IGF) and E_2 (Turner, 2002). The animals were housed in an outdoor paddock at the Concord Field Station, Harvard University. All animals received the same diet of hay and high-protein grain (Rumilab®, PMI Nutrition International, St Louis, MO, USA), and water *ad libitum*. The protocol was approved by the Harvard University IACUC, protocol #22-13.

Hormonal treatment

The 32 sheep were divided into two E_2 treatment groups in Experiment 1, low E_2 (*N*=8) and high E_2 (*N*=8), and three treatment groups in Experiment 2, low E_2 (*N*=4), normal E_2 (*N*=8) and high E_2 (*N*=4) (Table 1). The low-E_2 animals were vaccinated [4 ml intramuscularly (IM)] on day 1 and day 22 against GnRH (gonadotrophin releasing hormone) using Protherics immunoneutering vaccine (Protherics PLC, Cheshire, UK). In previous studies, vaccination against GnRH suppressed production of gonadal steroids, including E_2 (Brown et al., 1995). The high-E_2 animals were implanted on day 1 with subcutaneous capsules that release 61.5 μg E_2 day^{-1} (Encore©, VetLife, Inc, Norcross, GA, USA). No side effects were observed from the vaccine or the estradiol implant, and all treated animals exhibited normal appetite, activity levels and weight gain.

Exercise treatment

Half of the animals in each E_2 treatment group were sedentary and half were exercised, for a total of six treatment groups: low E_2-sedentary (LS), normal E_2-sedentary (NS), high E_2-sedentary (HS), low E_2-exercised (LE), normal E_2-exercised (NE), and high E_2-exercised (HE) (Table 1). Prior to the start of the experiment, animals assigned to exercise groups were habituated to running in an enclosed box on a treadmill at a moderate trot, a Froude number of approximately 0.5 (1.67 m s^{-1}) (Alexander, 1977). During the experiment, animals exercised for 40 min day^{-1}, generating approximately 4000 loading cycles per limb per day. Exercise was divided into two bouts of 20 min, separated by 4–6 h, as bone cells lose their sensitivity to mechanical stimuli after 20–30 min, and only regain this sensitivity after several hours' rest (Robling et al., 2002). The sedentary animals were not exercised.

Table 1. *Subjects and exercise training*

		Body mass (kg)			Treatment[†]		
	N	Initial	Final	Age (days)	Duration (days)	$\hat{u}$	Number of bouts
Experiment 1							
Sedentary							
Low E$_2$	4	19.41±1.23	24.68±2.54	134±28	45	None	
High E$_2$	4	20.68±1.30	23.62±1.66	147±30	45	None	
Exercised							
Low E$_2$	4	19.80±2.85	24.41±2.47	106±21	45	0.5	2
High E$_2$	4	18.03±2.60	24.03±1.00	128±26	45	0.5	2
Experiment 2							
Sedentary							
Low E$_2$	2	26.25±0.35*	37.25±0.21*	121±1	45	None	
Normal E$_2$	4	26.20±3.81	39.03±3.37	132±19	45	None	
High E$_2$	2	27.40±3.11*	38.10±3.39*	132±16	45	None	
Exercised							
Low E$_2$	2	28.25±0.92*	37.90±2.40*	124±10	45	0.5	2
Normal E$_2$	4	24.88±3.91	37.18±6.28	131±20	45	0.5	2
High E$_2$	2	25.50±1.41*	37.90±4.81*	127±12	45	0.5	2

Values are means ± 1 s.d.

[†]Froude number $\hat{u}=v^2(\boldsymbol{g}h)^{-0.5}$, where v=speed (m s^{-1}), $\boldsymbol{g}$=acceleration constant, h=hip height (m) (Alexander, 1977).

1 treatment bout = 20 min day^{-1}.

*Significantly different from same treatment group in Experiment 1 ($P<0.05$, ANOVA, *post-hoc* Fisher's LSD test).

Growth measurements

Cortical bone growth during the treatment period was labeled using calcein (20 mg kg^{-1} on day 1), a fluorochrome dye that incorporates into bone mineral. All animals were weighed biweekly on a digital scale. Blood samples were collected at the beginning, midpoint and conclusion of the experiment for measuring serum E$_2$ levels.

Analysis
Histology

At the end of the treatment period, the animals were euthanized and their limbs prepared for histological analysis. Lengths of the femur, tibia and metatarsal were measured *post-mortem* using digital calipers. Femoral length was measured from the most proximal point on the femoral head to the line connecting the two distal condyles; tibial length was measured from the center of the lateral condylar surface to the center of the distal articular surface; metatarsal length was measured from the center of the proximal articular surface to the most distal point of the distal articular surface. Midshaft cortical bone sections were prepared following the protocol in Lieberman et al. (Lieberman et al., 2003). Specifically, a 1-cm cylinder was cut from the midshafts of the femur, tibia and metatarsal, cleaned of soft tissue, fixed in ethanol and cleared in xylene, and embedded in Epotek 301 epoxy resin (Epoxy Technology, Billerica, MA, USA). Two sections were cut from each embedded midshaft using an Isomet 1000 low-speed saw (Buehler Ltd, Lake Bluff, IL, USA), mounted on slides, ground and polished to a thickness of 100 μm using a Buehler Petrothin grinder, and coverslips placed on top.

Images of each slide were captured at 3.5–11.25× under fluorescent light using a Retiga 1300 camera (QImaging, Burnaby, BC, Canada) attached to an Olympus SZH10 stereozoom microscope (Olympus, Melville, NY, USA) and imported into IPLab imaging software (Scanalytics, Rockville, MD, USA).

Bone growth

Periosteal appositional bone growth during the treatment period was measured as the total area added (mm^2), from the initial calcein line marking day 1 to the outer surface of the bone, in IPLab (Scanalytics, Rockville, MD, USA).

Cross-sectional properties

Midshaft cross-sectional properties were measured in NIH Image 1.63 (http://rsb.info.nih.gov/nih-image/) for the tibia and metatarsal using the experimentally determined neutral axis (Fig. 2) and a custom NIH Image macro (for details, see Lieberman et al., 2004). Second moments of area, I_N and I_{Ny}, and the polar moment of area, J_N, were calculated by the macro. The section moduli of tension and compression, Z_{Nc} and Z_{Nt}, were calculated as I_N/a_c and I_N/a_t, where a_c and a_t are the greatest perpendicular distances from the experimentally derived NA to the outer perimeter subject to compression and tension in the plane of bending. Linear cortical bone growth was measured from the calcein line to the outer cortex, at the points where the neutral axis (NA) and the perpendicular axis intersect the bone surface, in IPLab.

Hormonal assays

Serum estradiol measurements were obtained at the beginning, midpoint, and conclusion of the experiment *via*

Table 2. *Body mass, estradiol (E_2) and bone growth*

	N	Body mass (kg)		$[E_2]$ (pg ml^{-1})	Bone added (mm^2/$M_b^{0.66}$)		
		Initial	Final		Femur	Tibia	Metatarsal
Pooled by exercise							
Sedentary	16	24.37±2.86	32.14±5.85	21.14±28.10	2.14±0.45	1.36±0.82*	0.91±0.53
Exercised	16	21.92±1.90	31.33±3.44	16.92±23.22	2.73±0.74	1.99±0.45*	1.23±0.30
Pooled by hormone							
Low E_2	12	22.12±0.15	28.72±1.08	5.53±3.25	1.84±0.12*	1.19±0.42*	0.82±0.12*
Normal E_2	8	25.60±2.76	37.06±2.49	2.98±1.15	2.77±0.18*	2.32±0.01*	1.51±0.01*,†
High E_2	12	21.72±2.58	29.43±0.32	48.58±6.85	2.70±0.93	1.53±0.90	0.89±0.56†

		N	Body mass (kg)		$[E_2]$ (pg ml^{-1})	Bone added (mm^2/$M_b^{0.66}$)			Residual of bone added vs M_b		
			Initial	Final		Femur	Tibia	Metatarsal	Femur	Tibia	Metatarsal
Sedentary											
Low E_2	(LS)	6	22.01±1.27	27.95±2.75	7.83±2.31[a]	1.75±0.43[a]	0.89±0.41[a,b]	0.73±0.27[b]	–3.57±2.44[a]	–3.78±1.60[a,b]	–1.1±1.47
Normal E_2	(NS)	4	27.55±1.54[a]	38.82±2.29[a]	2.16±0.46[a]	2.64±0.32	2.31±0.13	1.51±0.24	–3.34±4.09[a]	0.15±1.62	0.64±3.22
High E_2	(HS)	6	23.54±1.54	29.65±3.52	53.42±11.27	2.04±0.36[a]	0.89±0.37[a,b]	0.49±0.17[a,b]	–2.03±1.58[a]	–5.12±2.73[a,b]	–4.10±0.78[a,b]
Exercised											
Low E_2	(LE)	6	22.22±2.36	29.48±3.91	3.23±0.40[a]	1.92±0.47[a]	1.48±0.43	0.90±0.31	–2.35±2.87[a]	0.85±2.35	0.04±1.73
Normal E_2	(NE)	4	23.65±0.74	35.30±2.06	3.79±0.40[a]	2.90±0.42	2.32±0.15[b]	1.50±0.21[b]	2.06±3.82	2.97±1.66[b]	2.33±2.09[b]
High E_2	(HE)	6	19.89±1.59[a]	29.20±2.42[a]	43.73±5.13[a]	3.36±0.38[a]	2.16±0.22[a]	1.28±0.13[a]	8.80±2.93[a]	5.97±2.01[a]	2.80±1.41[a]

Values are means ± 1 s.e.m.

Symbols indicate significant differences between pairs; letters indicate significant difference with [a]HE or [b]NE ($P<0.05$, ANOVA, Fisher's LSD).

radioimmunoassay (Prairie Diagnostic Services, University of Saskatchewan, SK, Canada).

Standardization and data pooling

Histological measurements were standardized by body mass (proportional to volume). Therefore, areas were standardized to body mass $(M_b)^{0.67}$, while linear measurements were standardized to $M_b^{0.33}$. Because the animals gained mass rapidly during the treatment period, we standardized periosteal bone area added by average body mass at the midpoint (mM_b) and conclusion (cM_b) of the experiment $[(mM_b+cM_b)/2]^{0.67}$, and linear bone growth by $[(mM_b+cM_b)/2]^{0.33}$. Cross-sectional properties were standardized by $\{[(mM_b+cM_b)/2]\times$limb length$\}$ (Lieberman et al., 2003).

The data reported here come from two separate experiments of identical duration, exercise protocol, and hormonal treatments, using subjects of the same age and breed (Table 1). Although there is a significant difference in initial and final body mass between the experiments (Table 1), all measurements of bone growth are standardized by body mass, allowing us to pool data from the two experiments in all analyses.

Hypothesis testing

Given the interactions examined here, ANOVA and pairwise comparisons with Fisher's LSD tests in Statistica (Statsoft, Tulsa, OK, USA) were primarily used for hypothesis testing, using E_2 treatment and exercise as nominal variables and bone growth (standardized by body mass) as a continuous variable. In addition, bone growth was regressed against average body

mass in Statview (SAS, Cary, NC, USA) to obtain the residual for each individual. ANOVA was then used to test for significant differences among treatment groups, using E_2 treatment and exercise as nominal variables and the residual of bone growth vs body mass as a continuous variable.

Results

Estradiol

As expected, mean serum E_2 levels in the estrogen-implanted animals were significantly higher (48.58 pg ml^{-1}, $P<0.05$, Table 2) than in the vaccinated (5.53 pg ml^{-1}) or normal (2.98 pg ml^{-1}) animals. However, vaccinated animals actually had higher circulating E_2 than normal controls (5.53 pg ml^{-1} vs 2.98 pg ml^{-1}), despite being immunized against GnRH. Both the vaccinated and normal sheep were well within the expected E_2 range of 2–15 pg ml^{-1} (Bartlewski et al., 1999a; Bartlewski et al., 1999b). This unexpected finding has several implications for our results, which are discussed below.

Periosteal growth

Periosteal appositional growth varied with activity level, with estrogen treatment, and with interactions between activity and estrogen. Overall, exercised animals grew more bone than did sedentary animals (Table 2). Exercise increased bone growth by 27% in the femur ($P=0.09$, ANOVA, Fisher's LSD), 46% in the tibia ($P<0.05$), and 35% in the metatarsal ($P=0.11$) relative to sedentary controls. The overall effects of estrogen treatment on cortical growth, with animals of differing activity levels pooled, were less clear (Table 2). As noted above,
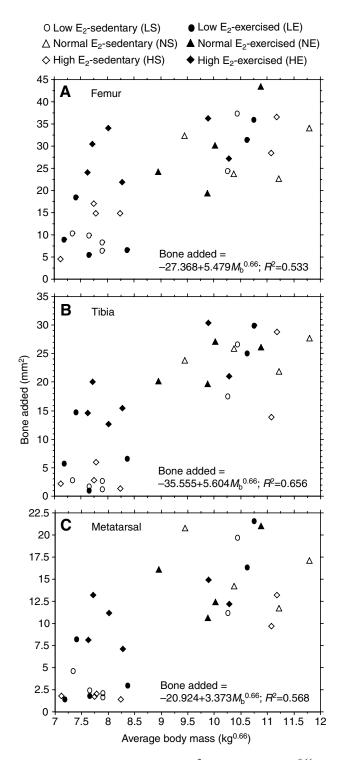
Fig. 3. Regression of bone added (mm^2) *vs* body mass ($kg^{0.66}$). (A) Femur, (B) tibia, (C) metatarsal.

Fig. 4. Residuals of bone added *vs* body mass. (A) Femur, (B) tibia, (C) metatarsal. Letters indicate significantly different from [a]HE, [b]NE group. ($P<0.05$, ANOVA, *post-hoc* Fisher's LSD test).

circulating E_2 levels were actually higher in vaccinated than in untreated animals (6 pg ml^{-1} *vs* 3 pg ml^{-1}). However, vaccinated animals had 34–49% less cortical growth in the femur, tibia and metatarsal than untreated controls ($P<0.05$, ANOVA, Fisher's LSD). High estrogen levels had similarly
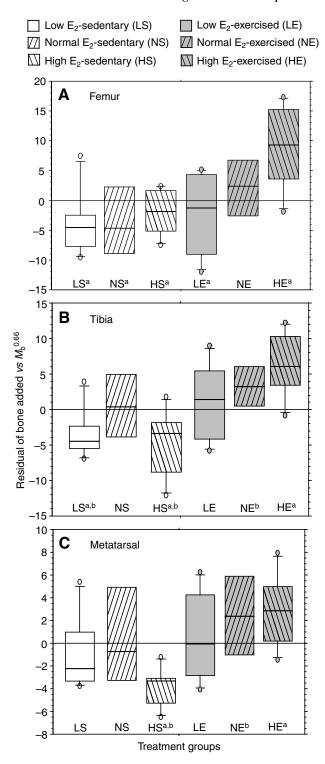
complex overall effects. Periosteal growth in high-E_2 animals was similar to that of normal controls in the femur, but 35–40% less than normal controls in the tibia ($P=0.06$) and in the metatarsal ($P<0.05$). To summarize, while exercise clearly

Table 3. *Cross-sectional properties and bone growth*

	N	Cross-sectional geometry					Linear bone growth (μm $M_b^{0.33}$)			
		$I_N M_b^{-1} l^{-1}$	$I_{NY} M_b^{-1} l^{-1}$	$J M_b^{-1} l^{-1}$	$Z_{Nt} M_b^{-1}$	$Z_{Nc} M_b^{-1}$	Anterior	Posterior	Medial	Lateral
Tibia										
Sedentary										
Low E_2 (LS)	6	3.40±0.29	3.60±0.21	6.99±0.47	11.52±0.78	7.87±0.37	5.20±1.97[a,b]	4.04±1.41[a,b]	8.75±3.64[a]	5.95±2.45[a,b]
Normal E_2 (NS)	4	3.37±0.26	3.62±0.29	6.99±0.54	11.32±1.08	7.99±0.52	13.36±1.38	9.68±1.00	17.90±1.52	12.38±1.88
High E_2 (HS)	6	3.62±0.35	3.91±0.46	7.53±0.81	12.08±1.37	8.83±1.10	3.85±1.23[a,b]	5.83±2.27[a,b]	6.69±3.09[a,b]	4.64±1.64[a,b]
Exercised										
Low E_2 (LE)	6	3.44±0.30	3.64±0.29	7.08±0.57	11.23±0.86	7.75±0.38	9.40±2.47	7.94[a]±2.07	15.52±3.50	7.00±2.55[a,b]
Normal E_2 (NE)	4	3.81±0.19	3.95±0.16	7.76±0.26	12.02±0.67	8.51±0.51	12.65±1.07	12.85±1.26[b]	17.30±1.93[b]	15.04±2.47[b]
High E_2 (HE)	6	3.79±0.31	3.99±0.48	7.78±0.78	12.76±1.01	8.82±1.10	13.33±1.45	15.20±2.50[a]	20.90±1.57[a]	15.23±1.64[a]
Metatarsal										
Sedentary										
Low E_2 (LS)	6	3.03±0.68	2.93±0.68	5.96±1.35	11.55±1.58	4.94±1.07	2.06±0.48	10.52±3.16	6.05±2.25	4.92±2.46[†]
Normal E_2 (NS)	4	6.13±0.78[a]	6.55±1.14[a]	12.67±1.91[a]	9.52±2.27[a]	10.72±1.77[a]	3.29±0.82	13.25±2.70	7.78±1.63	10.65±2.09
High E_2 (HS)	6	3.39±0.59	3.08±0.59	6.47±1.17	16.88±1.22	5.37±1.05	2.50±0.75	6.38±1.60[a,b]	3.57±1.09[a,b]	3.85±1.32[a,b]
Exercised										
Low E_2 (LE)	6	3.17±0.72	3.14±0.74	6.32±1.46	11.24±1.55	5.26±1.16	4.93±1.53	8.99±2.66[a]	6.67±2.24	5.38±1.80[b]
Normal E_2 (NE)	4	4.68±0.16	4.60±0.36	9.28±0.49	10.37±0.53	7.75±0.69	3.28±0.26	15.94±3.37[b]	11.03±2.04[b]	15.64±3.69[b]
High E_2 (HE)	6	3.09±0.58[a]	2.89±0.61[a]	5.98±1.18[a]	14.11±1.55[a]	4.99±1.03[a]	4.09±1.49	16.93±2.05[a]	9.59±1.27[a]	11.08±2.58[a]

Values are means ± 1 s.e.m.

Letters indicate significant difference with [a]HE or [b]NE (*P*<0.05, ANOVA, Fisher's LSD).

stimulates periosteal bone growth, it would appear that estrogen has no effect, or a suppressive effect, on bone growth.

In reality, estrogen does increase periosteal bone growth, but only in response to mechanical stimuli. When the effects of estrogen are tested with regard to activity level, significant interactions between E_2, strain and bone growth are revealed. As predicted, the most bone growth occurred in high-estrogen, exercised (HE) animals. The femur showed the clearest interaction between estrogen and mechanical loading, with the HE group adding an average of 27% (range +16% to +92%) more cortical bone than any other group (Table 2). In addition, in exercised animals, there was a trend toward more cortical deposition with increasing E_2 dose. Despite an identical loading regime, there was 75% more bone growth in the HE *vs* LE group (*P*<0.05, ANOVA, Fisher's LSD), but only 16% more *vs* the NE group (n.s.). In contrast, the NE group did not add significantly more bone than any other group.

In the tibia, the pattern was less consistent. The HE group added, on average, 6% (range –7% to +143%) more bone than the other groups, but the relationship between E_2 dose and bone growth was less clear. While there was 46% more bone growth in HE *vs* LE animals (*P*<0.05, ANOVA, Fisher's LSD), there was 6–7% less growth in HE *vs* NE or NS animals (Table 2). Also, both the HE and NE animals added significantly more bone than did LS and HS animals (*P*<0.05).

In the metatarsal, interactions between E_2 dose and bone growth were more modest. The HE group added about 14% (range –15 to +171%) more bone than the other groups, and in particular, 42% more bone than the LE group (*P*<0.05, ANOVA, Fisher's LSD). However, as in the tibia, the greatest growth response occurred in the NS and NE groups, both of which had about 15% more growth than the HE group. As a result, both HE and NE animals had significantly more growth than did LS and HS animals (*P*<0.05).

Given the significant difference in body mass between animals in experiments 1 and 2, we regressed cortical bone added *vs* body mass (Table 2, Fig. 3A–C) and compared the residuals for each experimental group (Table 2, Fig. 4A–C). Although body mass explained 53–65% of the variance in added bone (Fig. 3A–C), box plots of the residuals demonstrate that they were not randomly distributed among the treatment groups, but instead revealed a significant interaction between E_2 and exercise (Fig. 4A–C). In the femur (Fig. 4A), the residual of bone added *vs* body mass was highest in the HE group, which differed significantly from the sedentary groups and the LE group (*P*<0.01, ANOVA), but not the NE group (*P*=0.13). In the tibia, the pattern was more varied (Fig. 4B), with a significantly higher residual in the HE group *vs* the LS and HS groups (*P*<0.01), but not the NS (*P*=0.07) or LE (*P*=0.08) groups. Finally, in the metatarsal (Fig. 4C), the HE group did not differ significantly from the other groups except for the HS group (*P*<0.01).

Cross-sectional properties

Table 3 and Figs 5–8 present cross-sectional properties in the tibia and the metatarsal, for which midshaft strains in sheep of similar size and age have been experimentally determined (Fig. 2) (Lieberman et al., 2003; Lieberman et al., 2004). The analysis excluded the femur, for which *in vivo* strain data are unavailable.

In general, midshaft cross-sectional properties were somewhat elevated in exercised animals, particularly at higher E_2 doses. In
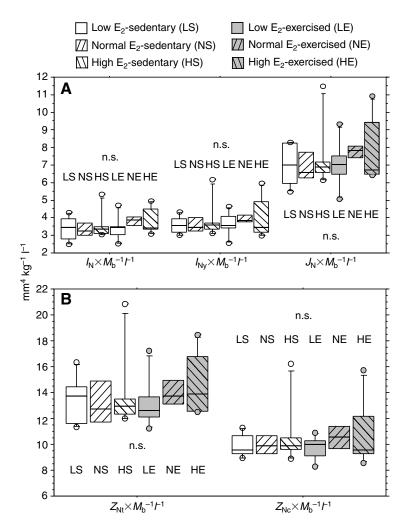
Fig. 5. Cross-sectional properties of tibia, relative to body mass and limb length. (A) I_N, I_{Ny}, J. (B) Z_{Nt}, Z_{Nc}. n.s., not significant.

the tibia, moments of area and section moduli in the HE group were, on average, 8% greater (range 0–14%) than in the other groups (Table 3, Fig. 5A,B), although these differences were not statistically significant. Within each treatment group, tibial second moments of area about the neutral axis (I_N) and about the axis perpendicular to the neutral axis (I_{Ny}) were similar, suggesting equal resistance to deformation in the anteroposterior plane, which is tensed and compressed at midstance, and in the mediolateral plane, in which strains are low at midstance (Fig. 5A). However, about the neutral axis, the section modulus of tension, Z_{Nt}, was higher than the section modulus of compression, Z_{Nc}, indicating increased resistance to tension *vs* compression during bending (Fig. 5B).

To compare bone growth to strain distribution, cortical apposition from the calcein label to the outer bone surface was measured at the neutral axis (I_N) and the perpendicular axis (I_{Ny}). On all tibial bone surfaces, there was generally more periosteal apposition with exercise and with increasing E_2 dose (Table 3), with an average of 95% more growth in the HE group than in other groups (range 0 to +275%, $P<0.05$ where indicated; Fig. 6). However, HE animals did not grow significantly more bone than NE or NS animals on any surface, and local rates of periosteal bone apposition within the cortex did not appear to be correlated with strain distribution at midstance. Within each treatment group, the extent of bone growth on the anterior (cranial) and posterior (caudal) surfaces, which are, respectively, tensed and compressed during stance phase, was similar to the extent of growth on the medial and lateral surfaces, where strains are about 50% lower (0–300 $\mu\epsilon$ *vs* 300–800 $\mu\epsilon$, Fig. 2).
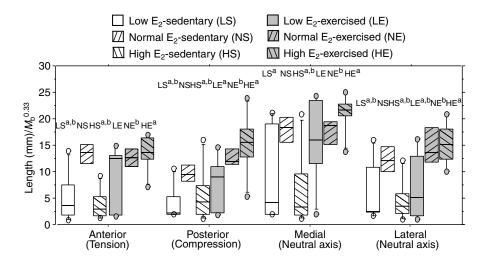

Fig. 6. Linear bone growth of tibia. Anterior and Posterior indicate growth at sites of maximal strain on anterior (cranial) and posterior (caudal) cortices; Medial and Lateral indicate growth at intersection of neutral axis (NA) with medial and lateral cortices. Letters indicate significantly different from [a]HE or [b]NE ($P<0.05$, ANOVA, *post-hoc* Fisher's LSD test).
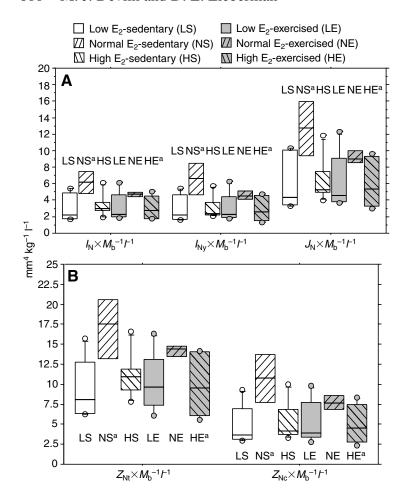
Fig. 7. Cross-sectional properties of metatarsal, relative to body mass and limb length. (A) I_N, I_{Ny}, J. (B) Z_{Nt}, Z_{Nc}. Letters indicate significantly different from [a]HE or [b]NE ($P<0.05$, ANOVA, *post-hoc* Fisher's LSD test).

In the metatarsal, in contrast to the tibia, there was no trend toward greater resistance to deformation with increasing E_2 or exercise (Table 3). In fact, moments of area and section moduli were 40–50% higher in the NS group than in the HE group ($P<0.05$, Table 3, Fig. 7A,B). As in the tibia, I_N and I_{Ny} were similar in the metatarsal, suggesting comparable resistance to anteroposterior and mediolateral deformation, and the section modulus of tension, Z_{Nt}, was higher than the section modulus of compression, Z_{Nc}, indicating greater resistance to tension than to compression during bending.

In terms of linear bone growth at the intersection of the cortex with the neutral axis (I_N) and the perpendicular axis (I_{Ny}), there was less apparent effect of E_2 or exercise in the metatarsal. Overall, the HE group added an average of 54% more bone (range –78% to +160%, $P<0.05$ where indicated, Fig. 8) than did the other groups. However, there were no significant differences between HE, NE, and NS animals. As in the tibia, within each group, similar growth occurred on the posterior (tensile) surface and on the medial and lateral surfaces near I_N (Table 3, Fig. 8), thereby maintaining a rounded cross-sectional shape.

Discussion

This study tests a model for interactions between estradiol (E_2) and *in vivo* responses to strain that likely involves upregulation of estrogen receptor alpha (ER-α). The hypothesis is that exercise-induced mechanical loading will have greater effects on bone growth and cross-sectional properties in individuals with higher E_2
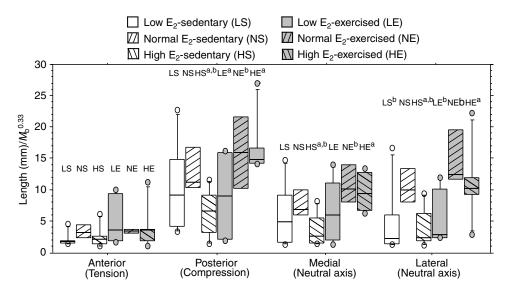

Fig. 8. Linear bone growth of metatarsal. Anterior and Posterior indicate growth at sites of maximal strain on (cranial) and posterior (caudal) cortices; Medial and Lateral indicate growth at intersection of neutral axis (NA) with medial and lateral cortices. Letters indicate significantly different from [a]HE or [b]NE ($P<0.05$, ANOVA, *post-hoc* Fisher's LSD test).

levels, due to increased osteoblast sensitivity to strain stimuli. The data indicate support for three of the four specific components of this hypothesis. Hypothesis 1, that exercised animals with higher E_2 levels (HE) will have more bone growth than those with normal E_2 levels, is strongly supported in the femur, in which HE animals had 16–92% more bone growth than any other treatment group. However, the effects of reduced E_2 level on bone growth remain ambiguous. Estrogen levels in the vaccinated and normal E_2 groups were similar, yet exercise-induced bone growth was reduced by up to 50% in vaccinated animals. It is not clear how the anti-GnRH vaccine caused diminished bone growth without affecting circulating E_2. Although the mechanism for diminished growth in the vaccinated animals requires further study, it is clear that increasing estrogen availability makes bone more sensitive to strain, as predicted.

Hypothesis 2, that the effects of E_2 will follow a proximo-distal gradient within a limb, is also supported. Residual plots demonstrate significant differences in growth response among treatment groups in the femur, with more moderate differences among groups in the tibia and the metatarsal (Fig. 4). In other words, the interaction between E_2 and mechanical loading is extensive in the femur, intermediate in the tibia, and minimal in the metatarsal, despite the fact that bone strains are likely lowest in the femur, intermediate in the tibia, and highest in the metatarsal. Thus estrogen-mediated bone growth follows the trade-off previously documented in tapered sheep limbs between increased resistance to deformation and increased cost of locomotion (Lieberman et al., 2003). This pattern suggests that in the periosteum, at least, estrogen (and perhaps ER-α) has a specific role in mediating skeletal responses to strain, as opposed to upregulating overall bone growth.

In terms of cross-sectional geometry, Hypothesis 3, that exercised animals with higher E_2 levels (HE) will have greater overall resistance to deformation, as measured by cross-sectional geometry, than normal controls (NE) or vaccinated (LE) animals, is modestly supported in the tibia, but not in the metatarsal. In the tibia, there is an 8% average increase in resistance to deformation in the HE group compared to the other groups, although this difference did not reach significance. In the metatarsal, the greatest resistance to deformation was actually in the normal, sedentary (NS) animals. Finally, the data relevant to Hypothesis 4, that E_2-induced periosteal bone growth will coincide with areas of higher strain during locomotion, are more difficult to interpret. While the HE group generally added more bone than did the other treatment groups in both the tibia and the metatarsal, this effect occurred on all bone surfaces, rather than corresponding to areas subjected to high tensile or compressive loads, as predicted by the hypothesis. As a result, in both bones the second moments of area in the anteroposterior plane, I_N, and the perpendicular plane, I_{Ny}, are similar, despite the fact that strains are much higher anteroposteriorly than mediolaterally during the stance phase of locomotion. There are several possible reasons for this similarity. Although strains on the medial and lateral surfaces are about 50% lower than on the anterior and posterior surfaces (0–300 $\mu\epsilon$ vs 300-800 $\mu\epsilon$), they may be sufficient to stimulate some bone growth. Additionally, the experimental animals were growing rapidly during the treatment period, and the apposition we observed around the entire tibia and metatarsal may simply reflect normal cortical drift.

Overall, our results support the hypothesized interaction between estrogen, strain and bone growth. Raising circulating E_2 levels increases the sensitivity of growing bone to mechanical signals, but has little effect on bone growth in sedentary animals, in the absence of strain signals (Fig. 4). To our knowledge, this is the first *in vivo* study to demonstrate that physiological variation in E_2 level among individuals can produce differential growth responses to an identical mechanical loading regime. The finding that interactions between E_2 and mechanical loading follow a proximo-distal gradient, with larger effects in the femur than in the metatarsal, warrants future study. If, as we hypothesize, E_2 increases the sensitivity of osteoblasts to mechanical stimuli *via* upregulation of ER-α, then ER-α transcription may vary within a limb, with more receptors in proximal elements and fewer in distal elements. Such a mechanism may underlie the observed higher modeling rates in proximal *vs* distal segments (Lieberman et al., 2003), a hypothesis that must be tested in future experiments.

Finally, the results presented here are interesting to consider in light of two well-documented trends in human skeletal evolution: that recent humans are less robust than earlier modern humans, and that humans from warm climates have less robust limbs than humans from cold climates (Ruff et al., 1993; Trinkaus, 1997; Pearson, 2000). If osteogenic responses to mechanical loading vary among individuals or populations, perhaps because of differences in hormone levels (e.g. Churchill, 1998), then there may not be a simple relationship between patterns of skeletal robusticity and individual loading history. This finding has significant implications for attempts to model the relationship between environmental strain and bone morphology.

List of symbols and abbreviations

A	anterior (cranial)
M_b	body mass
cM_b	body mass at conclusion of treatment
mM_b	body mass at midpoint of treatment
BMP	bone morphogenetic protein
E_2	estradiol
ERKO	estrogen receptor knockout
ER-α	estrogen receptor alpha
GH	growth hormone
GnRH	gonadotrophin releasing hormone
HE	high E_2-exercised
HS	high E_2-sedentary
IGF	insulin-like growth factor
IM	intramuscular
I_N	second moment of area about neutral axis
I_{Ny}	second moment of area about axis perpendicular to neutral axis

J_N	polar moment of area
l	limb (element) length
L	lateral
LE	low E_2-exercised
LS	low E_2-sedentary
M	medial
NA	neutral axis
NE	normal E_2-exercised
NS	normal E_2-sedentary
n.s.	not significant
NO	nitrous oxide
P	posterior (caudal)
PTH	parathyroid hormone
PGE_2	prostaglandin E_2
SGP	strain-generated potential
û	Froude number
Z_{Nc}	section modulus of compression
Z_{Nt}	section modulus of tension
$\mu\epsilon$	microstrain

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