

Increased non-linear locomotion alters diaphyseal bone shape

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Summary

Comparative studies of vertebrate morphology that link habitual locomotor activities to bone structural properties are often limited by confounding factors such as genetic variability between groups. Experimental assessment of bone's adaptive response to altered activity patterns typically involves superimposing exercise onto a normal locomotor repertoire, making a distinction between qualitative changes to locomotor repertoires and quantitative increases in activity level difficult. Here, we directly tested the hypothesis that an increase in turning activity, without the application of exercise *per se*, will alter femoral cross-sectional shape. Thirty day-old female BALB/cByJ mice ($n=10$ per group) were single-housed for 8 weeks in custom-designed cages that either accentuated linear or turning locomotion or allowed subjects to freely roam standard cages. Consistent with a lack of difference in physical activity levels between groups, there were no significant differences in body mass, femoral length,

midshaft cortical area, and individual measures of mediolateral (ML) and anteroposterior (AP) bending rigidity. However, the ratio of ML to AP diaphyseal rigidity, an indicator of cross-sectional shape, was significantly greater ($P<0.05$) in turning subjects than in linear or control subjects. Considering that across all groups mice were genetically identical and had equivalent levels of bone quantity and physical activity, differences in femoral shape were attributed to qualitative differences in locomotor patterns (i.e. specific locomotor modes). These data indicate that increased turning can alter distribution of bone mass in the femoral diaphysis, and that turning should be considered in efforts to understand form–function relationships in vertebrates.

Key words: cortical bone, turning, femur, mouse, bone adaptation, force.

Introduction

Embracing behavioral variability within locomotor repertoires of free-ranging animals is an emerging topic among those who model the mechanics of animal performance (Dickinson et al., 2000; Alexander, 2002; Demes et al., 2006; Jindrich et al., 2007). One fundamental aspect of this variability is the ability of an organism to change its direction of travel, or maneuver through an obstacle-filled environment. Proficiency in turning would confer tremendous selective advantage to free-ranging animals through predator avoidance, resource acquisition or competitive exclusion strategies. Quadrupedal animals that inhabit spatially complex arboreal habitats or mountainous areas, particularly those characterized by rugged vertical relief, may benefit from adaptations facilitating the capacity to change travel direction. Despite the seemingly integral importance of turning to locomotor performance, and despite the development of a descriptive protocol for categorizing turning behavior (Eilam, 1994; Walter, 2003; Demes et al., 2006; Jindrich et al., 2006; Jindrich et al., 2007), turning as a locomotor mode is rarely integrated into consensus frameworks for recording locomotor behavior in free-ranging animals (e.g. Probst, 1965; Hunt et al., 1996; Thorpe and Crompton, 2006). The lack of attention devoted toward documenting turning frequency in free-ranging quadrupedal

vertebrates may be related to the dearth of attention received by this locomotor mode in experimental studies that model animal locomotion.

Since turning has received comparatively less attention than linear locomotion, the potential effects of elevated turning frequency on bending loads and external forces, as well as their potential effects on the appendicular skeleton, are not well understood. Higher bone strain magnitudes have been reported in the human tibia during zig-zag locomotion compared to linear locomotion (Burr et al., 1996). Strain orientations, however, were not documented in this study for methodological reasons, nor were site-specific strain magnitudes compared (e.g. anterior *versus* medial cortex). In a study of the radial midshaft of goats, higher strain magnitudes and less predictable loading overall were found during non-steady behaviors compared to steady state locomotion on a treadmill (Moreno et al., 2007).

Further inferences about mechanical loading of the appendicular skeleton during turning can be made using measurements of substrate reaction forces (SRFs) (Rubin and Lanyon, 1982; Biewener et al., 1983; Rubin and Lanyon, 1984; Biewener et al., 1988; Demes et al., 1998; Demes et al., 2001). High mediolateral (ML) forces have been observed in animals that adopt waddling gaits (e.g. penguins) or habitually abducted or sprawling limb postures (e.g. alligators) during linear

locomotion (Blob and Biewener, 1999; Griffin and Kram, 2000; Blob and Biewener, 2001). Lemurs that were enticed to perform turns of approximately 30° experienced elevated ML components of SRFs (Demes et al., 2006) compared to ML components experienced during linear locomotion (Carlson et al., 2005). Limb orientation relative to the SRF resultant was not quantified in these lemur studies so bending moments could not be ascertained. The lemurs, however, did appear to position their limbs more laterally during side-step turns to the opposite direction, probably through relatively greater abduction at proximal limb joints. In an experiment designed to demonstrate the effect of limb abduction on external forces, ML forces increased as abduction at proximal limb joints (i.e. shoulder and hip) progressively increased in lemurs that were enticed to walk along simulated arboreal substrates (Carlson, 2005b). Although not all quadrupeds may exhibit relatively high ML forces during turning, the available data on quadruped turning (Demes et al., 2006) indicate that elevated ML external forces are associated with redirection of travel paths (e.g. side-step turns). Presumably this magnifies ML bending loads experienced by long bone diaphyses relative to those experienced by long bone diaphyses when limbs are moving in parasagittal planes.

Mice share several gait characteristics of linear locomotion with other quadrupedal mammals (e.g. forelimb vertical SRF components exceed hindlimb vertical SRF components; hindlimb duty factors exceed forelimb duty factors) (Heglund et al., 1974; Biewener, 1983; Clarke and Still, 1999; Clarke and Still, 2001; Clarke et al., 2001; Zumwalt et al., 2006). This similarity extends to horizontal components of SRFs as well, including ML forces (Carlson et al., 2005; Zumwalt et al., 2006). Mice have been observed to abduct their limbs when initiating side-step turns (Walter, 2003), which presumably increases ML forces experienced by their limb bones during laterally directed applied forces that are responsible for redirecting travel paths. While the relative degree of ML bending, AP bending, or torsion that the mouse femur experiences during either turning or linear locomotion is undocumented to our knowledge, Wallace and colleagues [(Wallace et al., 2007), p. 1125] qualitatively described mice as tending to run on treadmills with their hindlimbs flared laterally. This led them to speculate that bending loads in the mouse femur may predominate over torsional loads (Wallace et al., 2007).

Not all vertebrates emphasize ML external forces during turning. Humans, by adopting a different gait (i.e. bipedalism) relative to most other vertebrates, utilize a different mechanism for turns. While high ML forces during lateral movements described as shuffling have been documented in human athletes (McClay et al., 1994), humans emphasize the anteroposterior (AP)-directed force during turning. Humans achieve side-step turns first by using AP-directed forces to unilaterally decelerate (i.e. counteract over- or under-rotation of the body), which consequentially rotates (yaw) the body about its longitudinal axis into a new direction of travel, and second by using AP-directed forces in order to accelerate into the new travel direction (Jindrlich et al., 2006). Not all bipeds, however, utilize a similar mechanism to change direction. Ostriches emphasize ML horizontal forces (Jindrlich et al., 2007), despite being a biped, similar to quadrupedal mammals (Demes et al., 2006).

It is commonly assumed that applied mechanical loads have to exceed a specific formation threshold to become anabolic (Rubin and Lanyon, 1987; Turner et al., 1994; Cullen et al., 2001), but an unusual loading condition by itself, without an increase in the level of loading, also may have an anabolic effect (Rubin and Lanyon, 1987). Thus, the appendicular skeleton of an animal that experiences changes in its locomotor patterns, such as increased turning activity, may adjust its diaphyseal morphology through bone functional adaptations (cf. Ruff et al., 2006).

The goal of this study was to test for the presence of specific, quantifiable bone functional adaptations to increased turning behavior (e.g. redistribution of bone mass resulting in femoral diaphyseal shape changes) by exposing a model species to different habitual locomotor modes. By choosing an inbred mouse strain as the animal model (e.g. BALB/cByJ), the potential influence of genetic variability on musculoskeletal adaptations can be eliminated. Furthermore, by emphasizing different habitual locomotor modes rather than differences in the overall activity levels, as induced by exercise, our experimental design differs from the approach of many animal models that investigate the response to exercise in terms of net bone mass gains or losses (e.g. Lee et al., 2002; Mori et al., 2003; Wu et al., 2004; Kelly et al., 2006; Wallace et al., 2007). In the absence of differences in the quantitative activity levels between groups, functional adaptations of the femur (e.g. shape changes) likely reflect osseous responses to behavioral differences (i.e. turning frequency) over the experimental period.

The overall hypothesis that an increase in habitual turning activity affects the cross-sectional shape of long bones is tested by two sub-hypotheses: (1) subjects with predominantly linear locomotion as well as subjects in which turning is emphasized in the locomotor repertoire will exhibit more elliptical femoral diaphyses than controls; (2) compared to the other two groups, linear subjects will have greater AP rigidity in their femoral diaphyses and turning subjects will have greater ML rigidity.

Materials and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Stony Brook University. Thirty female BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were assigned randomly to one of three groups ($n=10$ per group). All mice were single-housed in one of two experimental enclosures (Fig. 1) or in a control enclosure for the duration of the experimental period (57 days). One experimental enclosure was designed to emphasize linear locomotion while the other was designed to accentuate turning during locomotion. Enclosures for the control group consisted of standard laboratory mouse cages with wire tops and a suspended water source. For both experimental groups, the water source was attached to the outer surface of the cage top with a metal sipper tube protruding vertically downward into the cage. Use of a short vertically positioned metal sipper tube effectively limited climbing opportunities for the experimental groups. Custom-designed cages for the experimental groups also included flat acrylic crystalite tops that were fastened securely to the sides of the cages. Using acrylic crystalite tops in place of standard wire tops also limited climbing opportunities in the experimental groups. Mice in the control

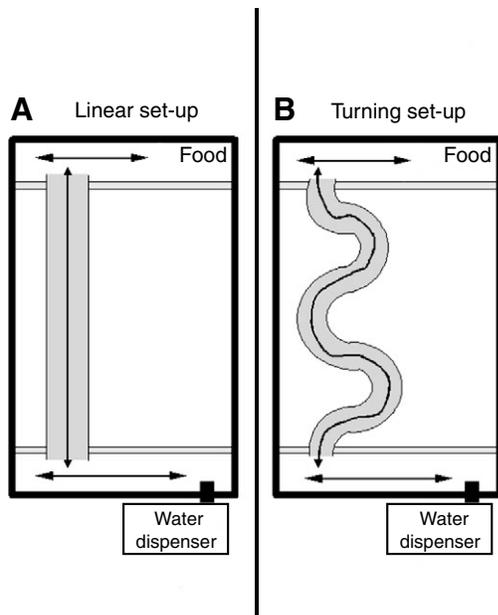


Fig. 1. The first experimental group (A; linear mice) was housed in a standard rat container with a modified tunnel apparatus designed to emphasize linear locomotion. The second experimental group (B; turning mice) was housed in the same type of container, but with a modified tunnel apparatus designed to emphasize changes in travel direction during locomotion (i.e. turning). Food and water sources were available at either end of the cages.

group were not discouraged from climbing locomotor activity. Climbing by control mice usually involved suspension by forelimbs followed by some 'brachiation' using predominantly forelimbs, or on occasion involved all limbs during 'inverted quadrupedalism' (Hunt et al., 1996). In the two experimental groups, food pellets (standard mouse chow) were placed on the floor at the end of the cage opposite the water source in order to stimulate use of the intervening tunnel.

All mice were introduced to enclosures approximately 30

days after birth. Mice were subject to 12 h:12 h (light:dark) cycles during the protocol period. Body mass and food intake were monitored each week for each individual to ensure acceptable health and activity levels over the duration of the protocol period. Monitoring individual body mass gains and food consumption also provided a mechanism for comparing mouse stress levels in response to enclosure type.

Daily behavioral assessments were performed over the course of the protocol period. Positional behavior (Prost, 1965) was documented approximately twice per day for each subject ($n=92$ observations per individual). Behavioral observations for a given day were usually performed at least 8 h apart, but always at least 5 h apart (mean difference in daily observation times= 10.8 ± 2.3 h). Observation times between days were varied in order to prevent oversampling particular periods of active or sleep cycles of subjects. Behavioral observations were recorded using an instantaneous focal sampling strategy (Altmann, 1974). Briefly, this consisted of carefully recording the behavior of a focal animal at a pre-arranged time. During a behavioral assessment, mice served sequentially as focal animals until the behavior of each subject was recorded quickly. Behavioral categories were constructed from standard positional behavior modes used previously for quantifying primate behavioral repertoires (Hunt et al., 1996; Carlson, 2005a; Carlson et al., 2006). Modes were devised to be self-descriptive and sufficient to represent an overwhelming majority of all observed behaviors. Behavioral categories were divided into locomotor behaviors (i.e. walk, run, jump, climb) and postural behaviors (i.e. lie, sit, stand using three or four limbs, stand using both forelimbs only, stand using both hind limbs only). Percentage locomotor behavior was used as a proxy measure for activity level (Table 1).

Additional behavioral observations using an instantaneous focal sampling strategy with 1 min intervals over 1 h were applied to randomly selected individuals from each experimental group. The purpose of this set of behavioral observations ($n=9$ h per experimental group) was twofold: to verify that rare behaviors in daily observations were not

Table 1. Comparison of activity profiles from behavioral assessments

	% Lie	% Sit	% Stand4L	% Stand2HL	% Postural
Linear	32.4 \pm 7.6	27.7 \pm 6.8	9.8 \pm 5.0	15.9 \pm 5.6	85.8 \pm 3.7
Turning	31.9 \pm 8.2	27.5 \pm 3.8	12.2 \pm 5.7	13.8 \pm 5.5	85.4 \pm 9.1
Control	39.9 \pm 6.0	27.8 \pm 3.8	11.0 \pm 4.9	8.4 \pm 2.9	87.1 \pm 4.2
	% Walk	% Run	% Climb [†]		% Locomotor
Linear	10.7 \pm 3.2	1.0 \pm 0.8	2.6 \pm 1.6		14.2 \pm 3.7
Turning	12.0 \pm 6.1	0.7 \pm 0.9	1.9 \pm 3.9		14.6 \pm 9.1
Control	9.1 \pm 2.7	0.1 \pm 0.3	3.7 \pm 2.3		12.9 \pm 4.2

Values are mean \pm 1 s.d. ($n=92$ per individual).

% Lie, % sit, % stand4L=stand four limbs, and % stand2HL=stand hindlimbs only. These four behaviors sum to % postural=static behavior. % Walk, % run and % climb sum to % locomotor behavior=dynamic behavior. % Locomotor=activity level. % Postural and % locomotor sum to 100% behavioral activity.

[†]Climbing activity in experimental groups was restricted to individuals jumping up to air holes in the cage top and suspending themselves using their forelimbs only. Other than the initial jump, which was similar to the jump exhibited by control subjects during climbing, climbing locomotor activity had minimal impact on the hindlimbs of experimental subjects. Hindlimb involvement in climbing by control subjects was also minimal compared to forelimb involvement. No significant group differences are observed between % postural or % locomotor activity.

Table 2. Descriptive statistics for group structural and shape properties

	Body mass (g)	Bone length† (mm)	Femoral 35% diaphysis					Femoral 50% diaphysis					
			Ps.Ar (mm ²)	Ct.Ar (mm ²)	I _y (mm ⁴)	I _x (mm ⁴)	I _{max} /I _{min}	I _y /I _x	Ps.Ar (mm ²)	Ct.Ar (mm ²)	I _y (mm ⁴)	I _x (mm ⁴)	I _{max} /I _{min}
Linear	22.0±1.3	12.7±0.3	1.572±0.087	0.899±0.061	0.240±0.028	0.107±0.012	2.249±0.150	2.239±0.136	0.857±0.052	0.214±0.025	0.109±0.012	1.995±0.107	1.955±0.109
Turning	22.8±1.3	12.6±0.1	1.571±0.071	0.896±0.066	0.244±0.032	0.104±0.009	2.344±0.211	2.337±0.214	0.857±0.054	0.218±0.027	0.105±0.010	2.091±0.121	2.065±0.123
Control	22.9±1.6	12.5±0.4	1.586±0.118	0.908±0.089	0.243±0.039	0.110±0.015	2.212±0.108	2.207±0.107	0.879±0.052	0.217±0.029	0.111±0.016	1.981±0.073	1.956±0.079
ANOVA													
P-value	0.330	0.299	0.921	0.930	0.954	0.611	0.198	0.195	0.556	0.923	0.607	0.053	0.049*

Values are means ± 1 s.d.
 *Statistically significant at the P<0.05 level.
 †Defined as femoral mechanical length (Carlson, 2005a).

underestimated in frequency (e.g. jump), and to assess whether experimental groups used tunnels with equivalent frequencies. Mice usually traversed tunnels 20 or more times within an hour regardless of the experimental group, though linear mice tended to traverse the tunnel at a higher frequency (linear group mean tunnel passes ±1 s.d.=63.3±29.8; turning group mean tunnel passes ±1 s.d.=40.7±22.8). While the linear tunnel was approximately 34.5 cm in length, the winding tunnel was approximately 48.0 cm in length. Thus, the higher frequency of tunnel use by the linear group was somewhat equalized by the longer distance the turning group traveled through the tunnel in one pass (mean linear travel distance per hour=2183.85 cm; mean turning travel distance per hour=1953.6 cm). Furthermore, three individuals per experimental group were observed twice in order to assess intraindividual variation in tunnel use. Ultimately, intraindividual differences in tunnel use (22.7±14.3) were similar in scale to intergroup differences in tunnel use (22.6). Thus, we believe that it is reasonable to assume similar tunnel use by subjects from the two experimental groups (i.e. no exercise effect was present).

At the termination of the experimental protocol (i.e. day 57), mice were sacrificed and limbs were disarticulated. The left femur was stored in 70% ethanol and scanned in a microCT system (Scanco µCT 40: Scanco Medical AG, Bassersdorf, Switzerland). To create 3D volumes, digital image stacks representing whole femora were exported as DICOM files into Amira™ imaging software (Berlin, Germany). Once 3D volumes representing individual femora were segmented, they were imported into custom-designed software (FoRM-IT) (Zollikofer et al., 1995). While we did not use commercially available software, such software is available (i.e. Amira™) and is preferable for subsequent studies. Digitally rendered volumes representing femora were positioned in a standardized fashion according to anatomically relevant orientations that have been used for similar structural comparisons among other taxa (Ruff, 2002; Carlson, 2005a). Femoral mechanical lengths (see Carlson, 2005a) were measured from 3D volumes after they were positioned since voxels were of known dimensions. Camera options of the software program were used subsequently to digitally re-slice elements in order to obtain cross sections at the desired regions of interest (ROIs): 35%, 50%, and 65% lengths. The most distal point of the femur while in the standardized position corresponded to 0% length.

Once a digital cross section was produced, the image was imported into Scion Image (release Beta 4.0.2) for analysis using custom-written macros modeled after the SLICE program (Nagurka and Hayes, 1980). Scion Image was ported from NIH Image for the Macintosh by Scion Corporation and is available on the Internet at <http://www.scioncorp.com>. Standard cross-sectional properties calculated for all femora included: subperiosteal area (Ps.Ar), cortical area (Ct.Ar), second moments of area about AP (I_y) and ML (I_x) anatomical axes, principal moments of area (I_{max}, I_{min}), and the principal angle (θ) (Parfitt et al., 1987). Since body mass (P=0.330) and bone length (P=0.299) did not differ significantly between groups (Table 2), comparisons of structural properties across groups were consistent with the definition of narrow allometry (Smith, 1980; Smith, 1984; Jungers, 1987). Unstandardized cross-

sectional properties, therefore, were used in comparisons of group structural properties.

Shape ratios (i.e. I_y/I_x and I_{\max}/I_{\min}) were calculated from several structural properties. The I_{\max}/I_{\min} ratio reflects deviation of cross-sectional shape from circularity, while the I_y/I_x ratio reflects differences in AP/ML rigidity (Carlson, 2005a). Theta (Θ), the principal angle, is defined as the angle between the ML anatomical axis and the maximum principal axis. Maximum divergence of principal and anatomical axes occurs when Θ approaches 45° , but when Θ approaches 0° these ratios will provide convergent measures of overall cross-sectional shape. Theta for femoral ROIs in this study ranged between 0° and 10° . Only second moments of area in anatomical planes (I_y and I_x), therefore, are reported in Table 2 since these offer intuitive anatomical interpretations.

Standard descriptive statistics were computed for all variables of interest. Normal distribution of data was verified with one-sample Kolmogorov–Smirnov tests. Since the distributions of behavioral variables (i.e. % postural behavior, % locomotor behavior), terminal body mass and cross-sectional properties did not differ significantly from normal distributions, parametric statistical analyses were selected for evaluating group mean differences in these properties. Equality of group variances for a variable of interest was verified using a Levene's test for homogeneity of variances. Since data fit the assumptions of a one-way analysis of variance (ANOVA), this statistical procedure was chosen to assess null hypotheses that group means were equal for given variables. If an ANOVA indicated statistically significant differences between groups for any variable, Fisher's least significant difference (LSD) *post-hoc* analyses were used to determine which groups differed significantly from one another. Statistical significance levels were set at $P < 0.05$ for all statistical analyses. All statistical analyses were performed within SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

One turning subject was consistently outside 2 s.d. from group mean shape ratios at each femoral region of interest (ROI). This individual was otherwise unremarkable in measures of body mass, activity level, or other structural properties. Since femoral shape ratios were of particular interest in this study, this individual was considered an outlier and excluded from subsequent analyses and comparisons.

Groups did not differ significantly in overall activity level as measured by percent locomotor behavior over the duration of the experimental period ($P = 0.82$) (Table 1), nor in average body mass at the end of the experimental period ($P = 0.33$) (Table 2). Measures of cortical bone quantity in the femoral diaphysis – total subperiosteal area (Ps.Ar) and cortical area (Ct.Ar) – also were not significantly different between groups ($P > 0.5$) (Table 2). Groups did not differ significantly in their second moments of area about the anatomical AP and ML axes ($P > 0.6$) (Table 2). These measures approximate ML (I_y) and AP (I_x) bending rigidity, respectively.

In contrast to measures of bone quantity or structural rigidity, diaphyseal shapes effectively distinguished groups (Figs 2, 3; Table 2). Although individual structural properties were not significantly different between groups, the femoral midshaft

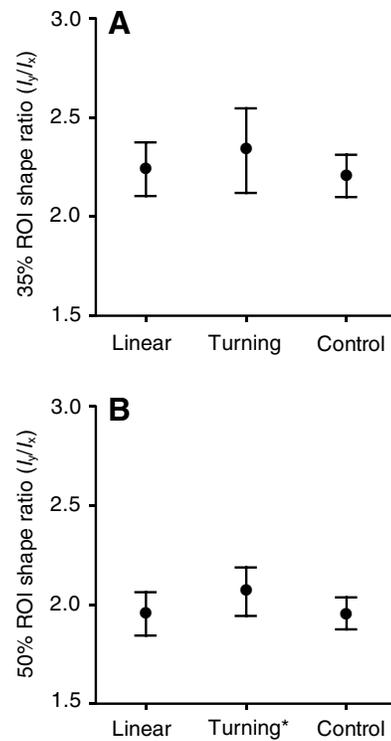


Fig. 2. Plots for group shape ratios (I_y/I_x) at femoral 35% diaphysis (A) and 50% diaphysis (B). Values are means \pm 1 s.d. Note that the highest mean values are observed in the turning group at both diaphyseal locations. *Group with statistically significant difference in the plotted shape ratio.

exhibited a significant shape difference according to I_y/I_x ratios ($P = 0.049$), and was borderline non-significant according to I_{\max}/I_{\min} ratios ($P = 0.053$). *Post-hoc* testing confirmed the hypothesis that the femoral midshaft shape of turning mice is different from the other two groups (i.e. turning mice had more elliptical cross sections than control mice and were most ML-elliptical of any group) (Fig. 3). The I_y/I_x ratio was 5.6% greater ($P = 0.030$) in turning mice than in linear mice and 5.6% greater ($P = 0.032$) in turning mice than in control mice (Fig. 3). The comparisons of the I_{\max}/I_{\min} ratio revealed essentially the same pattern as for the I_y/I_x ratio. In *post-hoc* testing, the I_{\max}/I_{\min} shape ratio was 4.8% greater ($P = 0.049$) in turning mice than in linear mice and 5.6% greater ($P = 0.025$) in turning mice than in control mice.

Although linear subjects on average had relatively less ML-elliptical femoral midshafts (i.e. higher I_x and lower I_y) than turning subjects, this was not the case when comparing linear and control subjects. Linear and control mice were differentiated neither by their structural properties (Fig. 2) nor by their diaphyseal shapes (Fig. 3). The I_y/I_x ratio of control mice was within 0.1% ($P = 0.98$) of linear mice, which did not support the hypothesis that control subjects would have the most circular femora (i.e. lowest shape ratios) and that linear subjects would have more AP-elliptical femora. Similarly, the I_{\max}/I_{\min} shape ratios of linear and control mice were not significantly different ($P = 0.750$) and within 1% of each other.

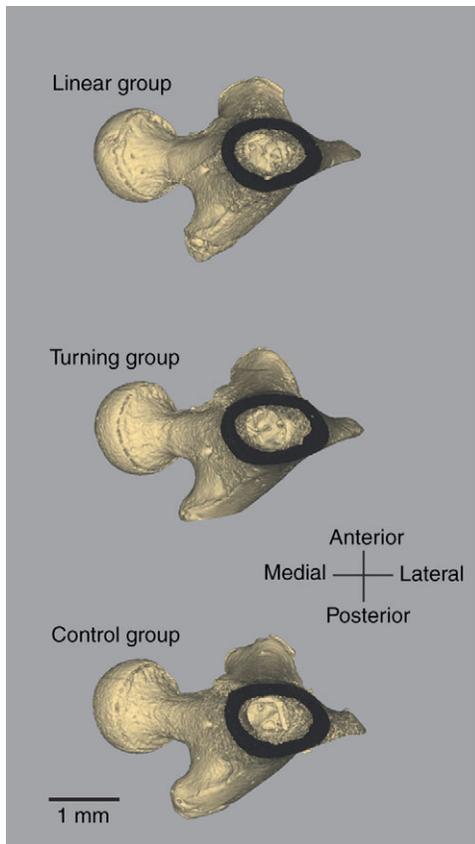


Fig. 3. Representative femoral midshaft cross sections from linear group (top), turning group (middle) and control group (bottom). Note the slightly greater mediolateral distribution of bone in the turning subject relative to subjects from the other two groups, which both have slightly more circular cross sections (black areas).

Discussion

Effects of locomotor modes on femoral morphology

Direct structural consequences of turning behavior on the skeletal system have not been documented previously. Using a carefully controlled experimental model that minimized confounding variables (e.g. genetics, sex, activity level) to accentuate the qualitative behavioral signal (e.g. linear locomotion *versus* turning), we demonstrated that growing mice performing an increased frequency of turns in their locomotor repertoire developed a femoral midshaft that was different in shape from growing mice in which the linear component of their locomotor repertoire was emphasized. Importantly, the observed shape divergence between groups was not due to differences in overall activity level, but rather it was more likely attributable to differing consequences of the two emphasized locomotor behaviours: linear locomotion and turning. While velocity during tunnel passage was not quantified in this study, both experimental groups exhibited similar frequencies of walking and running during quadrupedal locomotion (Table 1).

Since ML and AP bending rigidities of the mouse femoral diaphyses consistently corresponded to maximum and minimum rigidities, respectively, it is appropriate to discuss general diaphyseal shape differences between the groups in

terms of the more intuitive anatomical properties (Carlson, 2005a). Relative to mice in the linear group or mice in the 'free-ranging' control group, mice in the turning group exhibited a significantly more elliptical femoral midshaft. This shape change was attributable to a combination of small (non-significant) but cumulative differences in both higher ML rigidity among turning subjects relative to other groups, and lower AP rigidity among turning subjects relative to the other groups. This result is consistent with studies reporting higher strain magnitudes (presumably in ML directions) and higher ML external forces during turning relative to linear locomotion (Burr et al., 1996; Demes et al., 2006). The drop in AP rigidity among turning subjects may have been a consequence of reduced opportunities for linear locomotion relative to other groups.

Relative to subjects in the turning group, subjects in the linear group exhibited shape differences in the predicted direction – higher AP rigidity and lower ML rigidity. When comparing control subjects and the lower AP and ML rigidities of linear subjects, however, group differences were not significant. Furthermore, linear and control groups did not differ significantly in overall shape differences of the femoral diaphysis. This contradicted the hypothesis that linear subjects would exhibit more elliptical diaphyses, and specifically more AP-elliptical diaphyses than control subjects. An explanation for similarity in the structural properties and shape ratios of these groups is unclear. That the frequency of turning behaviors in 'free-ranging' conditions (i.e. control subjects) is unknown, however, could be relevant. For example, the possibility that turning frequency was performed by control subjects to the same extent as the restricted turning frequency of linear subjects cannot be excluded. Data on turning frequency in 'free-ranging' animals would be extremely helpful in evaluating this possibility.

Turning mice were the most distinctive group in terms of shape ratios. While linear locomotion could not be eliminated entirely from the locomotor repertoire of turning mice in either end of the modified enclosures, they experienced substantially reduced opportunities for linear locomotion relative to linear subjects (Fig. 1). Likewise, while turning behavior could not be eliminated entirely from the locomotor repertoire of linear mice upon entering or exiting tunnels, they had substantially reduced opportunities for altering travel direction relative to turning mice (Fig. 1). Inclusion of what are probably low frequencies of these restricted behaviors in their locomotor repertoires ultimately could be responsible for some of the overlap in measures of structural rigidity (e.g. I_y or I_x) and shape ratios of the experimental groups (Fig. 2A,B), and thus may be partially responsible for the relatively low absolute magnitude of femoral shape difference between groups (i.e. 5–6%).

Factors other than behavioral differences have the potential to confound comparisons of femoral shape differences between groups. Bone mass and structural properties are regulated in part by heredity (e.g. Judex et al., 2004a; Judex et al., 2004b; Wergedal et al., 2005). All mice used in this study were from the same genetic inbred strain, and thus genetic variability, a potentially confounding variable in some functional morphology studies, did not play a role here. Levels of physical activity also are known to affect bone mass and morphology

(e.g. Forwood and Burr, 1993). On average, however, groups did not differ in body mass or activity level (i.e. no significant group differences in % locomotor behavior) and thus exercise *per se* could not account for the observed shape differences. Since the sample was composed of only female individuals from a single inbred strain, variation in hormonal levels (e.g. Devlin and Lieberman, 2007) likely had at most only a minor contribution to shape differences between groups that expose femora to different mechanical loading environments. Similarities in average Ps.Ar and Ct.Ar of groups is consistent with the view that our experimental design eliminated most confounding variables. Therefore, in our view, group differences in the shape of the femoral midshaft, particularly between turning subjects and other subjects, are most parsimoniously attributable to differential distributions of bone resulting from locomotor behavior differences between groups (i.e. mode differences).

Simultaneous documentation of bone functional adaptations and changes in the mechanical loading environments of the femur was technically not feasible within the experimental design of this study. Considering the mechanical similarities in locomotion (including turning) between mice and other mammalian quadrupeds (Rubin and Lanyon, 1984; Biewener and Taylor, 1986; Clarke and Still, 1999; Clarke and Still, 2001; Clarke et al., 2001; Walter, 2003; Carlson et al., 2005; Demes et al., 2006; Zumwalt et al., 2006), we hypothesize that, compared to the other two groups, turning mice experienced elevated ML external forces during turning that in turn may have generated their distinct femoral cross-sectional shape. Verification of such differences in the mechanical loading environment (i.e. differences in ML bending), however, is required to verify this hypothesis.

Implications for comparative studies of bone morphology

The observed group differences in average shape ratios have two potential implications. First, we are unaware of published data reporting turning frequencies for any 'free-ranging' quadrupedal animal. It was anticipated that control subjects (i.e. 'free-ranging' individuals) would display intermediate frequencies of both turning and linear locomotor behaviors in their locomotor repertoire compared to the two experimental groups. Given greater structural similarity between control and linear subjects than was observed between control and turning subjects, 'free-ranging' control subjects presumably performed turning behaviors at frequencies more similar to linear subjects. In other words, turning behavior may have been relatively less frequent within the overall behavioral repertoire of 'free-ranging' subjects than was expected. A necessary caveat here is that the experimental 'free-ranging' conditions did not incorporate natural incentives for changing direction such as predator avoidance and intraspecific competition. Clearly it would be helpful to quantify turning frequency and its context as part of behavioral data collection in observational studies of free-ranging animals.

A second implication is that femoral cross-sectional shape may be more sensitive to certain locomotor modes compared to others, although expected shape outcomes remain difficult to predict for most modes. Arboreal locomotor modes such as scrambling, which is characterized specifically by multiple

rapid changes in the direction of travel in both vertical and horizontal planes (Hunt et al., 1996), could be particularly relevant to diaphyseal shape adaptations in the musculoskeletal system of arboreal quadrupeds, particularly among primates (Carlson, 2005a; Carlson et al., 2006). If load magnitudes are high enough, even a small number of loads (e.g. five or more) have been shown to evoke an anabolic response in the musculoskeletal system (Umehara et al., 1997). Local strain magnitudes may be greater during turning in comparison to linear locomotion (Burr et al., 1996). Furthermore, fewer load cycles are sufficient to initiate an osseous response when the load regime is novel in orientation (Turner, 1998; Burr et al., 2002), as may be the case during turning if load orientations vary widely relative to linear locomotion. Our study did not attempt to address whether the distinctive shape of the femoral midshaft in turning subjects is driven by higher local strain magnitudes associated with turning or whether potential novel orientations of the load regime associated with this behavior are more relevant to cross-sectional shape change. Either or both impetuses are plausible in the current study design.

Since the absolute effect of turning on diaphyseal shape difference was relatively small in this study (i.e. around 5–6% difference), it is not surprising that significant shape differences were observed only at the femoral midshaft. Experimental studies have shown that bone responses to load stimuli vary between diaphyseal locations (Gross et al., 1997; Judex et al., 1997). The midshaft is theoretically the location experiencing the greatest bending strains in long bone diaphyses (Martin et al., 1998) and hence, changes in cellular activity driven by altered mechanical loading can be expected to be most visible at this location. Accordingly, shape differences, particularly those of small absolute magnitudes (i.e. 5–6%), may be difficult to discern at other diaphyseal locations (i.e. 35% or 65% diaphyseal locations). In a more genetically diverse population than an inbred strain of laboratory mouse, or in a sample generated from museum specimens, shape signals of turning frequency even in the femoral midshaft could be more subtle, and afflicted with greater data variability, than those that were observed in the present study.

In order to fully appreciate the potential importance of turning to interpretations of skeletal morphology in free-ranging taxa, its context within the locomotor repertoire of a taxon should be contemplated. Turns performed by extant 'free-ranging' animals likely occur across a broad range of angles. Winding turns, such as those elicited in the present study, may involve different mechanical consequences than turns of 90° (Walter, 2003) or approximately 30° (Demes et al., 2006). Habitat variability may be linked to variability in turning frequency (e.g. higher frequency in closed forest *versus* lower frequency in open savanna). Furthermore, habitually abducted limb postures have been used as explanations for increased ML rigidity in extinct taxa [e.g. *Megaladapis edwardsi* (Jungers and Minns, 1979), multituberculate mammals (Kielan-Jawarowska and Gambaryan, 1994)]. One also must be mindful of limb postural differences, therefore, when attempting to infer the frequency of turning behaviors in locomotor repertoires, particularly when behavioral observations are no longer possible (i.e. extinct taxa).

Though the absolute effective shape change we observed may be relatively small, this proof-of-principle study demonstrates

that increasing the ratio of turning to linear locomotion alters diaphyseal cross-sectional shape in a predictable fashion. In order to further explore the usefulness of this relationship for comparative biologists, several points would be worthwhile for investigation. The effect of turning could be more dramatic in larger quadrupeds if they encounter higher strain magnitudes relative to their body size during changes in direction, though studies of linear locomotion find clear evidence for dynamic strain similarity across adult animals of varying body sizes (Rubin and Lanyon, 1984; Biewener and Taylor, 1986). Additionally, subjects encountering a longer experimental period (e.g. 12 weeks rather than 8 weeks) could produce a more exaggerated shape change as bone modeling and remodeling continues into adulthood. Ultimately, extending the experimental period may provide a more appropriate context for comparisons of extant or extinct vertebrate adults, whose femoral diaphyseal shapes reflect a behavioral contribution from summed locomotor input over a lifetime. Shape changes also could be more dramatic in mouse strains that are known to exhibit greater sensitivity in osteogenic responses to given functional loads than those exhibited by BALB/cByJ mice (Judex et al., 2004b; Wergedal et al., 2005), or in mouse strains that exhibit higher levels of physical activity (Kaye and Kusy, 1995). Even in the absence of any potential improvement to the strength of the form–function signal demonstrated here, these data provide direct evidence that increased turning can alter the distribution of bone mass in the femoral diaphysis and that, in addition to quantitative exercise-based differences, qualitative differences between locomotor repertoires (e.g. amount of turning *versus* linear locomotion) should be considered among the factors that determine bone morphology of an individual.

List of abbreviations and symbols

ANOVA	analysis of variance
AP	anteroposterior
Ct.Ar	cortical area
I_{\max}, I_{\min}	principal moments of area
I_y, I_x	second moments of area about AP and ML anatomical axes, respectively
LSD	least significant difference
ML	mediolateral
Ps.Ar	subperiosteal area
ROI	region of interest
SRF	substrate reaction force
Θ	principal angle

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