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The relationship between bone mechanical properties and ground reaction forces in normal and hypermuscular mice

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Abstract

Understanding the relationship between external load and bone morphology is critical for understanding adaptations to load in extant animals and inferring behavior in extinct forms. Yet the relationship bony anatomy and load is poorly understood, with empirical studies often producing conflicting results. It is widely assumed in many ecological and paleontological studies that bone size and strength reflects the forces experienced by the bone in-vivo. This study examines that assumption by providing preliminary data on gait mechanics in a hypermuscular myostatin-deficient mouse model with highly mineralized and hypertrophied long bones. A small sample of hypermuscular and wild-type mice were videorecorded while walking freely across a force platform. Temporal gait parameters, peak vertical and transverse (mediolateral) ground reaction forces, vertical impulse, and loading rates were measured. The only gait parameters that differed between the two groups were the speeds at which the animals traveled and the transverse forces on the hind limb. The myostatin-deficient mice move relatively slowly and experienced the same magnitude of vertical forces on all limbs and transverse forces on the forelimb as the wild-type mice, though the myostatin-deficient mice did experience lower mediolateral forces on their hindlimbs compared to the wild-type mice. These preliminary results call into question the hypothesis that skeletal hypertrophy observed in hypermuscular mice is a result of larger ground reaction forces experienced by the animals' limbs during locomotion and calls for further analysis and a cautious approach to inferences about locomotor behavior derived from bony morphology in extant and fossil species.

Keywords

myostatin; locomotor biomechanics; skeletal morphology; mouse model

Introduction

Understanding the relationship between the mechanical properties of animal bones and the forces generated during locomotion has long been a goal of morphologists, particularly those interested in understanding the selective pressures on bony anatomy in extant and extinct animals. Although there is extensive debate about the factors that influence bone anabolism (see Martin and Burr, '82; Bertram and Swartz, '91; Rubin et al. '94, 2001; Turner et al., '95; Jacobs et al., '98; Robling et al. 2000, 2002; Turner, 2002; Turner and Beamer, 2002;

Lieberman et al., 2003; Zumwalt, 2006; Main and Biewener, 2007), it is clear that ground reaction forces generated during locomotion influence the loading environment for any long bone. External ground reaction forces are not a direct surrogate for the strain experienced by bone tissue, but the magnitude and orientation of these forces must play a significant role in determining the strain and bending moments experienced by the bone. The actual strain experienced by the bone tissue is influenced by complex interactions between bone shape, bone mechanical properties, limb position, and ground reaction force magnitude and orientation (see Biewener, '89, '91).

Despite this complexity, many studies of fossil animals rely on an assumption that bone shape reflects in-vivo loading environment generated by many factors including ground reaction forces generated during locomotion, which are in turn assumed to reflect locomotor activity (see Thomason, '85; Trinkaus et al., '91, '94, '99; Heinrich et al., '93; Casinos, '96; Skerry, 2000; Churchill and Schmitt, 2003; Ruff, 2005 for a number of examples). This assumption explicitly underlies many paleontological reconstructions both in anthropology and broader areas of vertebrate paleontology. For example, Casinos ('96) specifically states that the shape of the long bones in *Megatherium* reflect stresses induced by loads associated with bipedalism. Similarly, Heinrich et al. ('93) use cross-sectional properties to infer the onset of bipedalism in *Dryosaurus* by assuming a relationship between forces and bone shape. Trinkhaus and colleagues ('91:272) assert that the shape of the Neanderthal femur reflects mediolateral bending stress whereas the shape of the femur of early modern humans reflect the loads "generated during a normal human striding gait." In a 1995 book dealing with fossil reconstruction, Weishampel ('95:38) argues explicitly that cross-sectional properties provide information on in-vivo mechanical loadings. In the same volume, Thomason ('95:260) discusses the complexities of the issue but still concludes that bone structure encodes information useful in "...reconstructing patterns of force that probably acted on fossil bones in-vivo."

As we discuss below there are good theoretical and empirical reasons to critically consider the assumption that bone responds to "changes in mechanical strain in a precise and systematic manner." (Heinrich et al. '93:179). Therefore, investigating the validity of the presumed correlation between bony morphology and peak ground reaction forces is critical to evaluating the strength of such inferences about the relationship between bone mechanical properties and load as well as reconstructions of locomotion in fossil animals.

It is difficult to collect the kind of data that is necessary to illuminate these complicated issues. Ideally, in order to infer locomotor behavior from long bone morphology, one should collect data on the local strain environment of the bone tissue, bone mechanical properties, limb movements, and ground reaction forces. Numerous studies of large and medium-sized animals such as horses, dogs, primates, and squirrels have provided important insights into the relationship between bone strength and loading (Biewener, '82, '83a, '83b; '89, '91; Budsberg et al. '87; Merckens et al. '93a, '93b; Riggs et al. '93; Demes et al. '98, 2001; Blob and Biewener, 2001). But until recently, it was exceedingly difficult to explore strain-based variation in load response in small animal models, despite the ready availability of rodent models of bone growth (Hamrick et al., 2000, 2002, 2003, 2007; Judex, et al. 2002, 2004a, 2004b; Amblard et al. 2003; Hamrick, 2003; Kelly et al. 2006; Carlson and Judex, 2007; Plochoki et al. 2008). A number of recent studies have explored gait patterns in mouse strains and mouse models of disease (Hampton et al. 2004; Herbin et al., 2004; 2006, 2007; Lepicard et al., 2005). The recent development of a small, durable, sensitive force plate (Fig. 1) and inexpensive high-speed cameras now allows collection of accurate ground reaction force values for small rodents (Zumwalt et al. 2006). The availability of a hypermuscular mouse model allowed this study to explore the loading environment of the rodent limb in order to examine the relationship between muscle mass, bone mineralization, and ground

reaction forces. The study presented here represents a preliminary dataset utilizing this unique mouse model.

The hypermuscular mice used in this study are deficient in myostatin, a growth factor that is a negative regulator of skeletal muscle growth (McPherron et al., '97). Because they lack myostatin, these animals have twice as much muscle in their limbs than do wild-type mice, and it has also been found that myostatin deficiency increases bone rigidity and biomineralization throughout the skeleton, including the limbs (Fig. 2), spine, and jaw (Hamrick et al., 2000, 2002, 2007; Hamrick, 2003; Ravosa et al., 2007). It has been proposed that the increased size and rigidity of the limb bones in these mice is a result of the mechanical influences of their large muscles (Hamrick et al., 2000; Hamrick et al., 2002), but the mechanism by which this relationship may occur has never been explored. It is presently unclear whether the large limb bones of myostatin-deficient mice develop as a response to large external loads experienced during locomotion, or to the action of the large muscles directly on the bones. It is also possible that the skeletal hypertrophy may be the result of a non-mechanical mechanism, such as the expression of osteogenic factors from the muscles or direct effects of myostatin deficiency on the differentiation or activity of osteoprogenitor cells.

In addition to the increased muscularity and overall bone rigidity, the diameters of some long bones in these mice are relatively expanded in the transverse plane. In the humerus, this mediolateral expansion is associated with increased periosteal circumference of the diaphysis (Figure 2a) (Hamrick et al., 2002). In the femur, Hamrick and his colleagues noted increased mediolateral diameter and reduced anteroposterior diameter of the diaphysis (Figure 2b) (Hamrick et al., 2003).

If a functional morphologist or paleontologist were to examine the bones of these mice, they might conclude that this animal experienced high peak ground reaction forces with a specific emphasis on vertical and transverse loads. Such a conclusion would lead to the development of postural and behavioral scenarios that would match the assumption of high ground reaction forces. This kind of thinking especially regarding mediolateral bending is evident in studies of fossil mammals (Casinos, '96) and early humans (Trinkhaus et al. '99), just as two examples. These reconstructions are ultimately based on the poorly tested assumption that limb biomechanical properties and shape reflect the external loading environment of the limb during locomotion. The mouse model presented here allows us to begin to test this assumption by examining the *in vivo* loading patterns of an animal that demonstrates clear skeletal hypertrophy.

Background

Vertical ground reaction forces reflect in large part an animal's body mass and running speed. Thus, researchers interested in understanding the mechanical principles and the underlying selective pressures that influence limb design have examined the relationship between bone mechanical properties, posture, and body size (see Alexander et al., '79; Biewener, '82, '83a, '83b; '89, '91; Bertram and Biewener, '88, '90; Bertram and Swartz, '91; Heinrich et al., '93; Christiansen, 2002). The fundamental finding of these studies is that bone cross-sectional properties (a measure of strength expressed as an area) generally scale isometrically or moderately positively with body mass (expressed as a volume). However, bone cross-section size does not increase proportionally (i.e. with enough positive allometry) with increases in mass, so larger animals have relatively weaker long bones than do smaller ones. Large animals compensate for this phenomenon by using standing and locomotor postures that reduce the bending moments on their limbs incurred by ground reaction forces (Biewener, '83a, '83b, '89, '91). As a result, many researchers interested in

inferring load in bones and joints in extant animals or in the reconstruction of posture and locomotor patterns in extinct animals (see Heinrich et al., '93; Trinkaus et al., '94, '99; Skerry, 2000; Churchill and Schmitt, 2003; Ruff, 2005) argue that increases in cortical area, bone cross-sectional area and bone mineral content reflect increased magnitudes of peak¹ loading that occurs during locomotion (see Bertram and Swartz, '91; Pearson and Lieberman, 2004; Ruff, 2005 for reviews of this complex issue).

However, detailed experimental studies have suggested that this relationship is not straightforward in all cases, and is often quite complex (see Rubin and Lanyon, '85; Demes et al., '98, 2001; McLeod et al., '98; Lieberman et al., 2003; Lieberman et al., 2004; Main and Biewener, 2007). Although it must be the case that forces generated by accelerating body and limb mass influence bone strain and bone mechanical properties, there remains no clear consensus concerning which aspect (peak magnitude, impulse, frequency, rate) of these ground reaction forces plays the most critical role, or the mechanism(s) by which this occurs (see Martin and Burr, '82; Bertram and Swartz, '91; Jacobs et al., '98; Robling et al., 2000, 2002a, 2002b; Skerry, 2000; Robling and Turner, 2002; Rubin et al., 2001; Lieberman et al., 2003, 2004; Zumwalt et al., 2006; Judex et al. 2007).

Although peak vertical and fore-aft forces² have figured prominently in previous analyses of limb design (e.g. Biewener, '83a, '83b), the transverse (mediolateral) component of the ground reaction force remains poorly studied primarily because this component is highly variable and relatively small, often less than 10% of an animal's body weight (Budsberg et al. '87; Ishida et al. '90; Merckens et al. '93a, '93b; Riggs et al., '93). However, although these forces are relatively low magnitude during average walking speeds chosen by the animal under relaxed conditions, they can still have a significant role in determining diaphyseal morphology. Studies of some non-mammalian bipeds such as penguins (Griffin and Kram, 2000), primate quadrupeds (Schmitt, 2003; Carlson et al. 2005) and quadrupeds with a sprawling limb posture, such as alligators (Blob and Biewener, 2001; Willey et al., 2004) have examined the transverse components of ground reaction forces with interesting results. For example, studies of primate quadrupeds have also suggested that transverse forces play an important part in maintaining stability during locomotion (Kimura et al., '79; Schmitt, '95, 2003; Demes et al. '98, 2001, 2007; Carlson et al. 2005) and may play an important role in determining limb morphology (Schmitt, '95, 2004; Demes et al. '98, 2001, 2007; Carlson et al., 2005). It is also likely that the limb is subjected to significant transverse forces during turning (Carlson et al. 2005, Demes et al. 2007). Several studies have documented the kinematics of turning in horses, primates, dogs, and mice (Walter, 2003; Carlson et al. 2005; Davies and Merritt, 2004) and some have argued that the anatomy of the lower limbs is influenced by transverse loads during turning (Carlson et al. 2005; Carlson and Judex, 2007).

There are few studies of the vertical forces applied by limbs of rodents, and no data on the transverse forces. Clarke and colleagues (Clarke and Still, '99, 2001; Clarke et al., 2001; Rochester and Clarke, '94) have collected peak vertical ground reaction force data on rats and mice, and Lee et al (2002) collected strain data from a single strain gauge on the mouse ulna. Experimental data on the loading environment of rodent limbs has recently been expanded to more complex questions such as the influence of turning on the rodent skeleton (Carlson and Judex, 2007; Walter, 2003), highlighting the importance of collecting transverse forces.

¹The ground reaction forces (GRF) that occur during locomotion may be broken down into three directional components (vertical, fore-aft and mediolateral). The vertical component of the GRF is the largest component applied by the limb (Winter, 1990; Biewener, 2003).

²We did not collect fore-aft forces for this study because the filtering methods used with this platform (Zumwalt et al. 2006) do not allow confidence in the output of braking and propulsive forces.

The study will document the vertical and transverse forces experienced by the limb in mice. We will compare these values in wild-type and myostatin-deficient mice, whose limbs have extra-large muscles and highly mineralized and hypertrophied limb bones in order to offer a preliminary test of the hypothesis that limbs of the myostatin-deficient mice are stronger because they experience higher peak vertical and transverse ground reaction forces, higher vertical loading rates, and greater vertical impulses than wild-type mice.

This project does not assume that increased muscularity increases forces. That is the hypothesis being explored in this study. There are a number of potential mechanisms by which the hypermuscular limbs might create higher vertical ground reaction forces during a normal stride. But before discussing this further, it is worth noting that we chose to test the hypothesis that there is a relationship between bone mechanical properties, muscle mass, and the external forces exerted during normal moderate-speed locomotion, rather than extreme forms of locomotion or broad activity patterns, for several reasons. First, this is the first step in a process and if this hypothesis is rejected we can explore additional areas including more extreme forms of locomotion. Secondly, McPherron and Lee (2002) have shown that myostatin knockouts do not differ from normal mice (relative to body weight) in metabolic rate, food consumption, or body temperature. Thus the myostatin-deficient mice do not appear to differ from wild-type mice in any significant physiological parameters and may differ only in muscle mass and bone mechanical properties. In addition, Hamrick (unpublished data) has shown, using an infrared in-cage monitoring system, that normal and myostatin-deficient mice do not differ significantly in activity level ($p=.75$). Third, as mentioned above, many of the arguments regarding the functional morphology of extinct animals rely on the assumption that bone mechanical properties reflect normal locomotor load levels.

In that light we recognize the fact that we are testing a specific hypothesis which is one of many possible ideas about what is reflected by different bone mechanical properties. Moreover, the mechanisms by which locomotor behavior might influence bone mechanical properties articulated below are based on mechanical models rather than specific data on limb forces in rodents.

As described above, ground reaction forces are affected by many factors, including the weight of the limbs, the force generated by the muscles during stance phase, and the posture adopted during locomotion. Since most of the muscle hypertrophy in these animals is localized to their limbs (Hamrick et al., 2002), the mechanism for increased load on heavier limbs is unclear and may have to do with higher muscle force generated by larger muscles during stance. It is also possible that the muscles themselves generate relatively high forces when firing to swing the limb forward, causing the limbs to strike the ground with higher initial loading rates and peaks of initial loads as the heavy limbs touch down and begin to bear weight. Additionally, in two animals with the same body mass, an animal with a relatively shorter stance phase for a given stride length (i.e., a shorter duty factor) experiences higher peak vertical forces during its stance phase (Schmitt, '99). It is possible that in the myostatin-deficient mice, swinging heavy limbs may increase the duration of their swing phases and concomitantly reduce stance phase, thereby leading to higher peak vertical forces.

The way in which differences in transverse (mediolateral) forces across strains of mice might be generated can also vary. It is possible that the hypertrophied muscles of these mice adduct the limb more forcefully than they do in wild-type mice and thus generate higher transverse ground reaction forces than those experienced by wild-type mice. It is also possible that increased muscle mass induces a sprawling gait that may require strong adduction forces and induce transverse forces that are at least somewhat higher than during

upright posture (Blob and Biewener, 2001). Therefore, there are too many potential mechanisms by which large GRFs might be created by hypertrophied limbs (or vice versa) for this project to clarify the mechanism at work in this model. Instead, this study can use force data to test the commonly held assumption that hypertrophied limb bones indicate high locomotor GRFs.

Materials and Methods

This is a preliminary study involving a small number of wild-type and myostatin-deficient mice walking on a newly developed force platform designed to collect data on small animals. The myostatin-deficient mice used in this study were bred on a CD1 background at the Medical College of Georgia (Augusta, Georgia) and the CD1 wild-type mice were obtained from Charles River Laboratories (Wilmington, MA). The animals used in this study were four months old and were housed at Duke University. The methods for kinetic and kinematic data collection are modified from Schmitt ('99) and Zumwalt et al.(2006). All data were collected at the Animal Locomotion Laboratory in the Department of Biological Anthropology and Anatomy at Duke University. All methods were consistent with the standards of the Institutional Animal Care and Use Committee of Duke University (IACUC protocol #A398-03-12).

The force platform is a small (15cm x 15cm), precise Hall-effects force platform capable of accurately recording small animals' ground reaction forces in all three directions, as well as impulse and loading rate in the vertical direction (Advanced Mechanical Technology, Inc; Watertown, MA). This plate was modified for collection of single-limb contacts and was tested for accuracy with mice as described previously (Zumwalt et al. 2006) (Figure 1). The mice (n=4 myostatin-null; n=3 wild-type) were allowed to walk freely along a 60cm x 15cm wooden runway. The force platform was positioned below the runway and isolated from contact except for a thin (15cm x 1cm x 1cm) strip of wood positioned to isolate single-limb contacts as the animals walked along the runway (Figure 1). Force data were collected into a personal computer using Bioanalysis with NetForce software (Advanced Mechanical Technology, Inc; Watertown, MA) and filtered with a 25Hz low pass filter using Autosignal wave analysis software (Systat Software Inc, Point Richmond, CA).

Subjects were filmed in lateral view at 125Hz with a Redlake Motionscope digital camera (Redlake, Tucson, AZ). Digital video data were processed in Motus 2000 and the x-y coordinate position of a marker on the body was collected for every frame and instantaneous and average horizontal velocity was calculated. Only walking (no aerial phase) strides showing no significant (less than 25%) acceleration or deceleration were used in the analysis. In addition footfall contacts were noted for each stride. Contact times (time of contact of the relevant limb) and stride times (time from one touch-down to the subsequent touch-down for the same limb) were from these data. Duty factors (contact time / stride time) were then calculated from these data. A complete stride was on average 31 frames long.

Force data were filtered with a 25Hz low pass filter using Autosignal wave analysis software (Systat Software Inc, Point Richmond, CA). Data were then processed in Kaleidagraph (Synergy Software; Reading, PA) and peak vertical (Fig. 3 points B and C) and transverse (Fig. 4) ground reaction force, vertical force loading rate (Fig. 3 point A), and vertical force impulse (area under the force curve) (Fig. 3 point D) were measured from these force traces. Loading rate was calculated by taking the first derivative of the force trace curve, which is equivalent to finding the slope of the curve at every point in time. The beginning of this period was defined as the point of foot or hand contact. The end of this period was defined when the slope became equal to or less than zero (Fig. 3 point B). The peak loading rate

(peak slope) within this initial loading phase of the limb contact was identified (Fig. 3, peak slope indicated by line A) and the average of all rates within 50% of this peak was calculated (modified from Gerlach et al., 2005). This value is an estimate of the average loading rate for the initial part of limb contact. The peak vertical force at the end of this initial loading period (Fig. 3, point B) and the peak vertical force for the entire limb contact period (Fig. 3, point C) were also recorded. Absolute peak forces are reported in Table 1. In addition, the vertical forces were standardized for body mass by dividing them by the animal's weight in Newtons.

All statistics were performed using the statistical package SPSS (SPSS Inc, Chicago, IL). All force data were divided by the animal's body mass to standardize for differences in body size. Student's t-tests were used to compare the group means for body mass. Analyses of covariance (ANCOVAs) were used to control for the influence of the potentially confounding factor of speed when comparing the group means of peak transverse forces experienced by the animals' limbs.

Results

There was no significant difference in body weights across the two groups (Table 1). The myostatin-deficient mice walked more slowly than the wild-type mice ($p=0.008$) (Table 1; Figs. 6 - 7). Despite the difference in speed, the two groups had similar duty factors (Table 1).

There was no difference in absolute or body-weight adjusted peak vertical forces between the groups of mice (Table 1). Once speed and body weight were accounted for, the two groups of mice produce statistically similar peak vertical ground reaction forces in both forelimbs and hind limbs (Table 1, Figs. 5 and 6) at a given speed during normal symmetrical walking chosen by the animal. Nor were there any statistical differences for loading rate, vertical force at end of initial load, vertical impulse (Table 1).

Peak transverse forces on the forelimb were the same across the two groups of mice (Table 1, Figure 7). This was not the case for the hind limb, where the transverse forces for the wild-type mice were significantly higher than those of the myostatin-deficient mice ($p=0.007$) (Table 1).

Discussion

The goal of this study was to collect preliminary data on the footfall patterns and ground reaction forces in wild-type and hypermuscular mice and to use these data to test the hypothesis that there is a relationship between external loading patterns and skeletal morphology. The walking velocity, footfall pattern, and duty factors found in the mice in this study are similar to those reported for other mice using symmetrical gaits (Herbin et al., 2004; 2006, 2007; Lepicard et al. 2005) and for most other animals (Hildebrand, '67, '76; Cartmill et al. 2002). Thus, although this is a small sample, it appears to reasonably represent the gait patterns of small rodents.

One of the significant difference between the wild-type and myostatin deficient mice documented in this study was the speed at which they walked (Table 1; Figs. 5 - 7). The myostatin-deficient mice in this study walked on average more slowly than their wild-type counterparts. In most animals, duty factor correlates strongly with walking velocity showing higher duty factors at lower walking speeds eventually shifting below 0.5 as they animals break into a run (Hildebrand, '67, '76; Biewener, '83b; Demes et al. '94; Cartmill et al. 2002). However, despite a significantly lower walking velocity, there was no significant difference in duty factor across groups. This suggests that the hyper-muscular mice may

actually have relatively shorter contact times at a given speed compared to the wild-type mice. This could be a result of restricted joint yield due to increased limb muscularity, but this cannot be verified without a detailed kinematic study.

The kinetic data reported here are also consistent with those for most other mammals. The peak vertical forces, which were consistent with those previously reported for mice and rats (Clarke et al., 2001; Clarke and Still, '99; Clarke and Still, 2001; Rochester and Clarke, '94) were roughly 60% of body weight with slightly higher values on the forelimb compared to the hind limb, as is the case in most nonprimate mammals (Kimura et al. '79; Demes et al. '94; Schmitt and Lemelin, 2002). The transverse forces were between 5% and 20% of body weight, as is the case with most other mammals recorded to date (Budsberg et al. '87; Ishida et al. '90; Merckens et al. '93a, '93b; Riggs et al., '93; Schmitt, '95, 2003; Demes et al., 2007; Carlson et al. 2005).

The mechanical properties of an animal's skeleton is often theorized to be strongly influenced by the external loading environment experienced by the animal's limbs during locomotion. Thus we predicted that the myostatin-deficient mice that have increased rigidity in their long bones would also experience higher external loads. But in this study, after accounting for velocity, there were no significant differences in loading of the limbs between the two groups. The myostatin-deficient mice do not appear to experience higher external loads on their limbs than do their wild-type counterparts. The limbs of the two groups were not loaded at different rates, nor did they experience higher vertical ground reaction forces at the end of initial loading periods (Table 1). Additionally, the peak forces (Table 1, Fig. 5 and 6) and vertical impulses (Table 1) experienced by the limbs of both groups were similar. The finding that peak vertical forces are the same in both groups is consistent with an earlier finding using a less sensitive force platform (Zumwalt et al. 2005). In addition the hypermuscular mice did not generate higher peak transverse ground reaction forces than the limbs of the wild-type mice did (Table 1, Fig. 7). In fact, the peak transverse forces in the hind limbs were actually lower in the knockout mice than in the normal mice (Table 1). Of course future studies with a larger sample and a wider range of gaits at higher speeds is needed to fully explore these interesting results.

At a given speed myostatin-deficient and wild-type mice experience the same magnitude vertical forces (Table 1, Fig. 5 and 6). However, because they adopt relatively slow speeds, the myostatin-deficient mice are able to habitually subject their limbs to lower peak vertical and transverse forces than they would experience were they to walk at the speeds wild-type mice use.

These results suggest that the increased bone formation in the limbs of these mice (Hamrick et al. 2007) is not the product of excessively large ground reaction forces created during normal locomotion. There are other aspects of gait-related loading patterns that may influence the bone and its response to load that were not explored in detail here. For instance, bones are subjected to bending strains during gait because joints are characteristically maintained in somewhat bent postures (Biewener, '83a, '83b, '89, '91). It is possible that the myostatin-null mice are adopting a more flexed-limb posture, and therefore creating greater moments around their joints and greater bending strains across their bones. Preliminary examinations of their shoulder and hip heights during gait indicate that these animals do not adopt more flexed joint positions during standing or locomotion (D. Schmitt, unpublished). However, it is impossible to precisely identify joint centers with the video data we have collected. A more thorough investigation, possibly using cineradiography, is needed in order to more accurately document the joint angles used by these animals during locomotion. Along the same lines, it is possible that the large agonist and antagonist muscles may stimulate increased bone robusticity by countering each other,

without affecting the GRF (see Blob et al. 2008). Unfortunately, directly measuring the force created by muscles on bone is extremely challenging, especially in small animals.

One possible explanation for the expanded transverse anatomy in the limb bones of hypermuscular mice in the absence of high transverse ground reaction forces is that the presence and/or action of muscles and muscle tissue may have a direct, local influence on bone formation and modification. Much of the mediolateral expansion of the myostatin-deficient mouse humeri and femora exists at or near muscle attachment sites (Figure 2) (Hamrick et al. 2002, 2003). Although a recent study suggests that a direct relationship between muscle mass and attachment site size does not necessarily exist in quadrupeds (Zumwalt, 2006), it is possible that in the case of these hypermuscular mice, the bones hypertrophy simply to provide surface area for the attachment of the large and numerous muscle fibers (Hamrick et al. 2000).

It is also possible that other physiological mechanisms also play an important role in regulating bone apposition. However, McPherron and Lee (2002) have shown that myostatin knockouts do not differ from normal mice in metabolism, food consumption, or body temperature. In addition, Hamrick (unpublished data) has shown no difference between the two groups in in-cage activity level. An interesting alternative explanation is that the large muscles of the myostatin-deficient mice function as paracrine organs that secrete critical growth factors along the periosteal surface and stimulate bone growth (Zacks and Sheff, '82; Montgomery et al., 2005). Muscle tissue is also likely to serve as a local source of muscle satellite cells capable of undergoing skeletal differentiation (Asakura et al., 2001), and myostatin deficiency itself may have direct effects on osteoprogenitor cells that increases their osteogenic potential (Hamrick et al., 2007). Recent work indicates that circulating myostatin may have direct effects on bone formation. The myostatin-receptor is expressed in bone-marrow stromal (stem) cells, and bone marrow stromal cells from mice lacking myostatin show increased osteogenic differentiation and enhanced mechanosensitivity (Hamrick et al., 2007). Mice injected with a decoy myostatin receptor, which binds circulating myostatin so that it cannot reach its target cells, show increased bone mass (Bialek et al., 2008). Thus, factors such as myostatin that influence muscle mass may also directly effect bone formation as well, suggesting that the integrated growth and development of these two tissues is regulated by a common molecular signals. Clearly further studies are still needed to elucidate the primary mechanism through which the skeletal hypertrophy observed in these animals is generated.

There are also myriad other mechanical influences on bone remodeling. These include the magnitude, frequency, and rate of strains experienced locally at and within the bone tissue, and the influence of local factors such as bone curvature (Lanyon et al., '82; Rubin and Lanyon, '85; Biewener and Bertram, '93; Mosley et al., '97; Judex and Zernicke, 2000; Fritton et al., 2000; Hsieh et al., 2001; Burr et al., 2002; Robling and Turner, 2002; Robling et al., 2002b; Warden and Turner, 2004).

It is also clear that muscle-derived stimuli are critical for the proper growth and development of limb bones, and we do not mean to suggest that this is not the case. For example, joints will not form in the absence of movement in utero, and mechanical loading stimulates chondrocyte proliferation in articular regions as well as in the growth plate. The key issue here is mechanism, specifically the role of peak locomotor forces in driving morphology as opposed to other stimuli, such as high frequency, low magnitude muscle-derived signals (see Rubin and Lanyon, '85; Fritton et al. 2000; Judex and Zernicke, 2000; Rubin et al. 2001; Judex et al. 2007 or changing intramedullary fluid pressure in bone emanating from muscle contraction working as a "pump" (Qin et al. 2003). Our data simply suggest that the unique skeletal phenotype of hypermuscular, myostatin-deficient mice is

unlikely related to large forces encountered during locomotion, but rather reflects other aspects of the muscle-bone relationship.

The role that all of these factors (external loads, strain, bone-muscle interactions etc.) play in bone formation in these mice will only be answered with more detailed study using bone strain gauges and other techniques on these mice. This was beyond the scope of this study, which focused on ground reaction forces and gait because many functional morphologists use bone morphology to infer locomotor behavior, often with a focus on peak ground reaction forces experienced during locomotion (e.g., Churchill and Schmitt, 2003; Heinrich et al., '93; Ruff, 2005; Trinkaus et al., '94; Trinkaus et al., '99). This study suggests that the assumptions used in such studies should be carefully considered when interpreting their results. The many alternative explanations for the skeletal morphology we observe in these mice reinforce the concept that the relationship between limb bone anatomy and *in vivo* loading is extremely complex. Although there is no doubt that external loads can influence skeletal morphology (Martin and Burr, '82; Frost, '87; Bertram and Swartz, '91; ; Turner et al., '95; Jacobs et al., '98; Robling et al., 2000, 2002b; Rubin et al., 2001; Turner, 2002; Turner and Beamer, 2002; Lieberman et al., 2003; Lieberman et al., 2004), this study reinforces the growing realization that this relationship is complex and difficult to predict.

The availability of novel animal models and improved and more accessible force plate technology has made it possible to investigate these questions in new ways. The preliminary data reported in this study call into question one commonly assumed mechanism for the moderation of bone morphology: a straightforward influence of locomotor ground reaction forces on skeletal morphology. The results of this study emphasize the need for further exploration of ground reaction forces in these unusual animals models as well as broader investigations into the myriad *in vivo* mechanisms regulating muscle-bone interactions.

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Figure 1.

A) Schematic diagram of the set-up for the mouse ground reaction force data collection. The runway covers but does not touch the force platform (black). Steps only register on the force platform when mouse paws touch the strip of wood covering the center of the plate (*). (B) Image of a mouse making a single forelimb contact with the wood strip (*).

Figure 2.

(a) Faxitron radiographs of the humerus in wild type (top row) and myostatin-deficient mice (bottom row) showing the expanded deltoid crest (asterisk) and transverse dimension of the diaphysis (arrows) in the mice lacking myostatin. (b) Radiographs of the femur in normal (top row) and myostatin-deficient mice (bottom row) showing the expanded third trochanter (asterisk) and transverse dimension of the diaphysis (arrows) in mice lacking myostatin.

Figure 3.

Demonstration of the various parameters measured on the vertical ground reaction force curve in this study. Shown is a force trace of the vertical ground reaction force of a single forelimb contact of a walking mouse, measured in Newtons. Parameters measured are (A) loading rate: the average slope of the initial loading period as defined in the text, (B) the peak vertical force at the end of the initial loading period, (C) the peak vertical force for the entire contact time, and (D) impulse: the area under the curve (shaded gray).

Figure 4.

A representative mediolateral (ML) force (N) trace for the forelimb (FL) and hind limb (HL) from a single individual walking at 0.18 m/s.

Figure 5.

Comparisons of some of the locomotor parameters for the wild-type (WT) and myostatin-knockout (KO) mice. The mean and one standard deviation for the walking speeds and peak vertical forces for forelimbs and hind limbs of the two groups of mice are compared. Significance values are indicated in the upper left corner. The designation “n.s.” indicates that the values are not significantly different from each other. Note that the myostatin-deficient (KO) mice walk significantly more slowly but the peak vertical forces are not significantly different from that of the wild-type (WT) mice.

Figure 6. Bivariate plot of peak vertical ground reaction force (divided by body weight) on the forelimb versus velocity (m/s) for the wild-type mice (dark gray triangles) and mysotatin-deficient knock-out mice (light gray circles).

Figure 7. Bivariate plot of peak transverse ground reaction force (in Newtons) on the forelimb versus velocity (m/s) for the wild-type mice (dark gray triangles) and mysotatin-deficient knock-out mice (light gray circles).

Table 1

Summary of the kinetic and kinematic data collected during this study. Means $\pm$ standard deviations are reported, followed by the range of observed data in parentheses. The reported significance results are from analyses of covariance, which controlled for the confounding effect of speed ($\alpha=0.05$; n.s. = not significant). N = newtons, BW = body weight (N), s = seconds

	<i>Mstn</i> ^{-/-}	<i>Wild Type</i>	<i>Signif at $\alpha=0.05$</i>
Individuals	4	3	
Body weight (N)	0.48 $\pm$ 0.03 (0.45 – 0.53)	0.46 $\pm$ 0.06 (0.39 – 0.49)	n.s.
Speed (m/s)	0.24 $\pm$ 0.06 (0.16 – 0.38)	0.33 $\pm$ 0.06 (0.18 – 0.66)	p<0.003
<i>Forelimb</i>			
Number of Steps	22	24	
Duty Factor (contact time [s] / stride time [s])	0.63 $\pm$ 0.05 (0.51 – 0.74)	0.61 $\pm$ 0.07 (0.47 – 0.74)	n.s.
Loading Rate (milliseconds)	16.6 $\pm$ 6.3 (6.1 – 30.6)	16.2 $\pm$ 5.5 (7.2 – 30.4)	n.s.
Peak vertical force / BW at end of initial load (N)	0.46 $\pm$ 0.12 (0.2 – 0.71)	0.48 $\pm$ 0.14 (0.23 – 0.72)	n.s.
Peak vertical force	0.30 $\pm$ 0.05 (0.20 – 0.40)	0.27 $\pm$ 0.05 (0.18 – 0.42)	n.s.
Peak vertical force (N) / BW	0.60 $\pm$ 0.1 (0.42 – 0.77)	0.64 $\pm$ 0.08 (0.47 – 0.82)	n.s.
Impulse (area under curve)	0.07 $\pm$ 0.02 (0.05 – 0.14)	0.06 $\pm$ 0.02 (0.04 – 0.09)	n.s.
Peak transverse force (N)	0.11 $\pm$ 0.03 (0.07 – 0.17)	0.10 $\pm$ 0.04 (0.05 – 0.2)	n.s.
Peak transverse force (N) / BW	0.22 $\pm$ 0.04 (0.14 – 0.35)	0.22 $\pm$ 0.055 (0.1 – 0.43)	n.s.
<i>Hind Limb</i>			
Number of Steps	17	14	
Duty Factor (contact time [s] / stride time [s])	0.67 $\pm$ 0.07 (0.51 – 0.84)	0.64 $\pm$ 0.1 (0.47 – 0.87)	n.s.
Loading Rate (milliseconds)	17.9 $\pm$ 7.8 (4.8 – 31.7)	19.1 $\pm$ 6.3 (11.7 – 31.2)	n.s.
Peak vertical force / BW at end of initial load (N)	0.51 $\pm$ 0.14 (0.23 – 0.86)	0.5 $\pm$ 0.13 (0.35 – 0.74)	n.s.
Peak vertical force (N)	0.28 $\pm$ 0.06 (0.20 – 0.44)	0.24 $\pm$ 0.06 (0.17 – 0.34)	n.s.
Peak vertical force / BW (N)	0.56 $\pm$ 0.12 (0.39 – 0.86)	0.55 $\pm$ 0.11 (0.36 – 0.74)	n.s.
Impulse (area under curve)	0.06 $\pm$ 0.02 (0.04 – 0.11)	0.08 $\pm$ 0.09 (0.02 – 0.39)	n.s.
Peak transverse force (N)	0.07 $\pm$ 0.02 (0.05 – 0.13)	0.11 $\pm$ 0.04 (0.06 – 0.19)	p=0.007
Peak transverse force (N) / BW	0.14 $\pm$ 0.05 (0.1 – 0.27)	0.23 $\pm$ 0.05 (0.13 – 0.41)	P=0.007