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Bone is functionally impaired in dystrophic mice but less so than skeletal muscle

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Abstract

The primary purpose of this study was to determine if tibial bone strength is compromised in dystrophic mice and if so, what geometric and material properties contribute. Results of three-point ending tests showed that tibia of *mdx* and *dco* (dystrophin- and utrophin-deficient) mice had up to 50% lower strength and stiffness compared to wild-type mice. Micro-computed tomography indicated that dystrophic tibia had reductions of 6–57% in cortical cross-sectional moment of inertia and cross-sectional area. Metaphyseal trabecular bone morphometry was also altered up to 78% in dystrophic mice. Bone-to-muscle functional ratios (i.e., three-point bending measures:muscle strength) indicated that bone strength was relatively high in 7-week-old dystrophic mice compared to muscle strength, but ratios were similar to wild-type mice by 24-months of age. Thus, as in boys with Duchenne muscular dystrophy, young dystrophic mice have compromised bone strength; these models may be useful for designing therapeutic regimens aimed at improving the skeleton.

Keywords

Duchenne Muscular Dystrophy; bone geometry; μCT ; bone strength; *mdx* mouse

Introduction

Bone fractures are on the rise in the nearly 8,000 boys in the United States affected by Duchenne muscular dystrophy (DMD), with little progress being made toward remediation [1–4]. DMD is characterized by progressive muscular weakness attributed to contraction-induced muscle injury followed by inefficient repair [5]. Thus as the disease progresses,

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boys become less physically active and unable to perform activities of daily living [6–8], both of which reduce the frequency and magnitude of mechanical loads placed on bone. As a result, bones should enter a stage of disuse, resulting in bone loss and structurally weakened bones that are more prone to fracture. Indeed, fracture incidence in boys with DMD is reported to be between 18–44%, where higher incidence rates parallel increases in age, physical limitation and disease state [1–3]. Fractures typically occur in the lower extremities after falling from standing or sitting height [3,9], indicating that bone strength is compromised. This loss of bone strength may be due to altered bone geometry and/or other extrinsic or intrinsic material properties (e.g., density or composition of the bone). Dual energy X-ray absorptiometry (DXA) has been utilized to monitor the loss of areal bone density in various skeletal regions with age in DMD [1,10], but the relative contribution of bone loss and that of compromised bone geometry and material properties to bone strength have not been evaluated in DMD. Delineating the roles of these factors will provide a better indication of whole bone function and fracture risk in the context of muscular dystrophy.

Mouse models of DMD are available and these could aid in the assessment of the disease's effect on bone strength and its underlying mechanical determinants including geometric and intrinsic material properties. There have only been two studies on bone in a DMD mouse model and both utilized the *mdx* mouse [11,12], the most commonly used animal model of DMD. Similar to boys with DMD, *mdx* mice have dysfunctional dystrophin protein, and as a result develop characteristic muscle weakness early in life, i.e., between 15–20 days of age [13]. Anderson et al. reported that the tibia is weaker in 4- and 18-week-old *mdx* mice, compared to that of age-matched, wild-type mice [11]. The bone weakness was indicated by lower ultimate load, smaller cross-sectional area, and thinner cortices. In contrast, Montgomery et al. found that 16-week old *mdx* mice had stronger, wider and denser femurs compared to those from wild-type mice [12]. In this latter study, after adjusting for the greater body weight of *mdx* mice, no differences in bone characteristics were detected between *mdx* and wild-type mice. Thus, it remains to be determined whether or not *mdx* bones are functionally compromised as a secondary consequence of the muscle disease.

DXA, X-ray, and histomorphometry are traditional methods used to analyze bone and were used in the two aforementioned studies. A newer and more sensitive technique is micro-computed tomography (μ CT), which simultaneously quantifies bone volumetric density and geometry at a higher resolution providing more sensitive assessment of bone. Furthermore, μ CT is a non-destructive imaging technique that allows for subsequent evaluations of the bone's composition and functional properties to be performed on the same specimen. Collectively, these approaches can be utilized to investigate bone strength and elucidate the mechanisms underlying a loss in bone strength that occur as a result of muscular dystrophy. This information may provide insight for designing strategies that could be used to offset the deleterious effects of the disease on bone.

Therefore, the primary objective of our study was to determine if *mdx* mice indeed have compromised bone compared to wild-type mice, and if so, what geometric and material properties contribute. Specifically, three-point bending tests, μ CT imaging and measurements of bone composition were utilized to assess bone strength, geometry and intrinsic material properties of the tibia, respectively. We also used these strategies to assess bone in a second model of DMD, *dco* mice. *Dco* mice lack functional dystrophin and utrophin proteins resulting in a more severe phenotype of the disease relative to *mdx* mice [14]; this phenotype is more similar to that seen in boys with DMD.

Another objective of the study was to directly evaluate the functional relationships between muscle and bone in this mouse model of muscle disease. Previous studies have suggested that skeletal muscle not only provides an anabolic stimulus to bone but is also required to

maintain the structural competence of bone [15]. Thus, according to the mechanostat theory [16], the loss of muscle function with DMD disease progression should translate to proportional declines in tibial bone size and strength. To address this theory, we calculated bone-to-muscle ratios of functional capacity using our measurements of ultimate load and stiffness for the tibial bone and force generating capacities for the extensor digitorum longus (EDL) muscle. We hypothesized that dystrophic mice would exhibit similar bone-to-muscle ratios as those of wild-type mice, indicating that their tibial bone function is diminished to a similar extent as the contractile function of the adjacent EDL muscle.

Methods

Animals and Experimental Design

Four wk-old wild-type (C57BL/10) and *mdx* mice (n=8 and 7, respectively) in addition to 8-mo-old wild-type and *mdx* retired breeders (n=9 each) were obtained from Jackson Laboratories (Bar Harbor, ME). Four wk-old *dko* mice (n=7) were obtained from our colony [17]. All wild-type and *mdx* mice were male; *dko* mice were male and female (n=4 and 3, respectively). Female *dko* mice were included due to the limited availability of male *dko* mice. Mice were housed in groups of four on a 12:12-h light-dark cycle at 20–23°C under pathogen-free conditions. All mice were provided food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

At the age of 7 wk or 24 mo, mice were weighed, anesthetized with sodium pentobarbital (100 mg/kg i.p.), and EDL muscles were dissected for contractile analyses. Mice were then sacrificed with an overdose of sodium pentobarbital (200 mg/kg) and tibial bones were excised. These bones were stored in phosphate-buffered saline at –20 °C until the time of analysis. The 7 wk age was chosen because it was thought to best reflect a time point with functional declines in both muscle and bone. Additional rationale for selecting the 7 wk time point was that it approximates the average lifespan of *dko* mice in our colony. Despite a report that the average lifespan of male *mdx* mice is 21.5 mo [5], all of our *mdx* retired breeders lived to 24 mo and remained apparently healthy. Therefore the 24-mo-old *mdx* mice in this study were mice that had lived with a chronic muscle disease.

Contractility of EDL Muscle

EDL muscle contractile function was measured as previously described [18,19]. Briefly, the muscle was placed in a 0.38-ml bath assembly filled with Krebs–Ringer bicarbonate buffer that was maintained at 25 °C; the proximal tendon was connected with 6-0 suture to the arm of a dual-mode muscle lever system (300B-LR; Aurora Scientific Inc., Aurora, ON, Canada). Optimal muscle length (L_0) was set and then maximal isometric tetanic force was determined by stimulating muscles with 0.5-ms pulses at 180 Hz and 150 V for 400 ms (Grass S48 stimulator connected to a SIU5D stimulus isolation unit; Grass Telefactor, Warwick, RI). The maximal isometric tetanic force was then normalized to the EDL muscle's physiological cross-sectional area (i.e. muscle mass divided by the product of muscle density (1.06 g/ml) and fiber length ($0.44 \cdot L_0$)) [20]. Peak eccentric force was determined by passively shortening the muscle from L_0 to $0.95 L_0$, and then simultaneously stimulating the muscle for 133 ms as the muscle lengthened to $1.05 L_0$ at $0.75 L_0/s$.

μCT of Tibial Mid-Diaphysis and Metaphysis

A μCT system (Scanco Medical μCT 40, Brüttisellen, Switzerland) was used to quantify cortical bone geometry and volumetric bone mineral density (vBMD) of the tibia at the mid-diaphysis; trabecular morphometry in the tibial metaphysis was also assessed. The scanner was set to a voltage of 55 kVp and a current of 145 μA, and bones were scanned using an

isotropic 12 μm voxel size with a 250 ms integration time. At the mid-diaphysis, 66 sequential two-dimensional grayscale slices (12 μm thickness) were obtained, as previously described [21]. After image segmentation for bone using a global threshold, an algorithm was used to compute the following morphometric outcome measures: cortical cross-sectional area, cortical thickness, periosteal diameter, cross-sectional moment of inertia (CSMI) and vBMD. These measures were assessed for each of the 66 slices, and then the average of the 66 slices was reported. The minimum principal CSMI ($\text{CSMI}_{\min}$) was utilized in the present study as it best corresponds to the CSMI about the bone bending axis during the three-point bending test (described below).

Trabecular bone in the proximal tibia metaphyseal region was imaged in ~50 sequential slices, beginning 5 slices distal to the last image containing the growth plate. The mean bone volume fraction (BV/TV), trabecular number, trabecular thickness, and trabecular spacing were quantified to characterize bone morphometry. Upon completion of imaging, bones were refrozen in PBS and stored at -80°C until the time they underwent mechanical testing.

Three-Point Bending Tests of Tibial Mid-Diaphysis

The procedures used to assess the functional capacities of the mouse tibia have been described in detail previously [21,22]. Briefly, tibial length was measured with digital calipers and the mid-diaphysis of the tibia was identified. Tibial bone functional properties were then determined by performing three-point bending at mid-diaphysis on a Mecmesin MultiTest 1-D test machine using a Mecmesin AFG-25 load cell (Mecmesin, West Sussex, UK). The tibia was placed onto a set of supports separated by 1 cm such that the lateral side of the bone was placed downward. Bones were placed in this position to provide the most stable loading position possible during the testing procedure. Quasi-static loading was applied to the central aspect of the bone on the medial surface using a displacement rate of 2 mm/min. The load applied to the bone was measured by a load cell with 5 mN resolution. The load and displacement outputs were sampled at 14 Hz by a computer and software (TestPoint version 7; Measurement Computing Corp., Norton, MA).

The load-displacement curve for each bone (e.g., Figure 1A) was analyzed using a custom-written TestPoint program [21]. The functional measures of tibial bone were quantified by ultimate load, stiffness, and deflection and energy absorbed to ultimate load. Ultimate load was determined by the program as the highest load obtained prior to failure (Figure 1A). Once the point corresponding to ultimate load was determined on the load-displacement curve, deflection and energy to ultimate load were determined. For stiffness, the program searched for the highest slope along the linear portion of the curve prior to ultimate load; the program required that the data segment contain at least 50 data points and that the correlation between load and displacement exceed 0.99.

In addition to bone geometric properties, other extrinsic material properties for each tibia were determined by quantifying dry and wet tibial masses. Intrinsic material properties for each tibia were also determined using vBMD (via μCT at the tibial midshaft and metaphysis), hydroxyproline concentration, ultimate stress and modulus of elasticity. Wet and dry tibial masses were measured immediately prior to measurement of the amino acid hydroxyproline, an amino acid unique to collagen. Hydroxyproline content was assayed following the procedures of Woessner [23]. As previously described [21], ultimate stress and modulus of elasticity were calculated for each tibial bone using classical beam theory. Ultimate stress was calculated using the following equation: $\text{ultimate stress} = (\text{UL} \cdot d) / (8 \cdot \text{CSMI})$, where UL, d and L are ultimate load, medial-lateral periosteal diameter, and bottom support span length, respectively. Modulus of elasticity was then calculated using: $\text{modulus of elasticity} = (k \cdot L^3) / (48 \cdot \text{CSMI})$, where k equals stiffness.

Bone and Muscle Relationships

To determine whether the tibia was more or less affected by the dystrophic condition relative to the EDL muscle's contractile strength, bone-muscle functional relationships were examined. Tibial bone's measures of functional capacity (ultimate load and stiffness) were divided by the EDL muscle's contractile capacity (maximum isometric and eccentric contraction forces) as previously described [21]. These bone-to-muscle ratios were done on a mouse-by-mouse basis, meaning that if a mouse did not have measurements for both its tibial bone and EDL muscle, the ratio was not calculated. Ratios that are higher than those for the corresponding age-matched control mice indicate that functional properties of dystrophic bone are relatively greater than those of muscle, while ratios that are lower than those for the control mice indicate the reverse.

Statistical Analyses

To analyze the effects of genotype (wild-type vs. *mdx*) and age (7 wk vs. 24 mo), two-way ANOVAs were utilized. When interactions were significant ($p < 0.05$), Holm-Sidak post hoc tests were performed to determine which combinations of conditions were different from each other. To assess possible effects of body mass on tibial geometric parameters at the midshaft (i.e., cortical cross sectional area, cortical wall thickness, and periosteal diameter) as well as tibial bone wet and dry mass, two-way ANCOVAs were run using body mass as the covariate.

To examine the effect of disease severity (i.e., the more severe dystrophin and utrophin deficient *dko* mouse model relative to both dystrophin deficient *mdx* mice and wild-type mice), 7 week-old *dko* mice were compared to wild-type and *mdx* mice of the same age. Before this analysis was done, independent *t*-tests were performed and percent differences were calculated to confirm that gender differences were not present between male and female *dko* mice. A one-way ANOVA was then done with genotype as the fixed factor. If an effect of genotype was present, Holm-Sidak post-hoc measures were used to determine differences among the genotypes. When assumptions of normality or equal variance were violated, Kruskal-Wallis One Way Analysis of Variance on Ranks was performed along with Dunn's post-hoc tests. All statistical analyses were carried out using SigmaStat version 3.5 (Systat Software Inc; Point Richmond, CA) with the exception of the two-way ANCOVAs which were conducted using SPSS version 12 (SPSS Inc., Chicago, IL).

Results

Effect of genotype on EDL muscle function

To demonstrate the severity of muscle functional impairments in the mouse models of DMD, *in vitro* measures of EDL muscle contractile forces of dystrophic mice were compared to those of wild-type mice. *Mdx* mice generated less maximal isometric tetanic forces at both 7 weeks and 24 months of age (41 % and 21 %, respectively) compared to their wild-type counterparts (Table 1). Peak eccentric force was also reduced in *mdx* mice (Table 1). In an effort to determine the source of these decrements in EDL muscle function in *mdx* mice, both the size of the EDL muscle (i.e., mass) as well as a surrogate for muscle quality (i.e., peak isometric force normalized to muscle cross-sectional area) were analyzed. *Mdx* and wild-type EDL muscle masses were equivalent at 7 weeks of age while *mdx* muscles were >50% larger at 24 months (Table 1). Peak isometric tetanic force normalized to muscle cross-sectional area was 20–40% less in *mdx* than wild-type mice (Table 1). Thus, despite *mdx* mice having equal or greater muscle masses, contractile function was compromised.

Data were not statistically different between male and female *dko* mice for any of the muscle or bone parameters measured (i.e., independent t-tests $P \geq 0.221$) with the greatest difference between genders being 21%. Therefore all muscle and bone results for the two genders were collapsed into a single *dko* group. Comparisons of EDL muscle from 7-week-old *dko* mice to those of age-matched, wild-type and *mdx* mice show that *dko* mice have functional deficits that are even greater than those of *mdx*. *Dko* mice had 42–67% lower peak isometric and eccentric forces relative to *mdx* and wild-type mice (Table 1). Unlike *mdx* mice, *dko* mice had decrements in both muscle size and muscle quality (Table 1), confirming that *dko* mice have a more severe phenotype than *mdx* mice in terms of muscle function.

Effect of genotype on tibial bone functional capacity

In general, the functional capacity of tibial bone was affected by both genotype and age. Figure 1B shows exemplar load-displacement curves from wild-type, *mdx* and *dko* tibia during the three-point bending test. Ultimate loads for tibia of *mdx* mice were 19 and 36% lower than those for wild-type mice at 7 weeks and 24 months, respectively (Figure 2A). The larger difference in ultimate load between *mdx* and wild-type mice at 24 months was attributed to an improvement with age in wild-type mice that did not occur in *mdx* mice. For bone stiffness, there were both significant genotype and age effects (Figure 2B). Tibial stiffness in *mdx* mice was 15–20% lower than wild-type mice, indicating that *mdx* bone was less resistant to bending. Additional functional properties, including energy absorbed to ultimate load and deflection to ultimate load, also reveal that *mdx* tibia are compromised. Both of these properties were 36–60% lower in 24 month-old *mdx* mice compared to those in 24 month-old wild-type mice (Table 2). Collectively, these results indicate that functional deficits are apparent in the tibial bones of *mdx* mice, confirming that bone strength is compromised.

The tibia of *dko* mice had even lower functional capacity. For example, bone from these mice had 34–50% lower values for ultimate load and stiffness compared to those of *mdx* and wild-type mice (Figure 2A and B). Energy absorbed to ultimate load and deflection to ultimate load were also smaller in *dko* mice than wild-type mice (61 and 17% respectively, Table 2), indicating that *dko* are less able to withstand loading.

Effect of genotype on tibial bone geometry

μCT was utilized to determine if properties reflecting bone geometry at the mid-shaft of the tibia were affected by genotype and thus potentially could account for the differences in bone mechanical properties observed among genotypes. For CSMI at the tibial mid-shaft, which reflects the moment of inertia during three-point bending, independent age and genotype effects were detected. *Mdx* bones had CSMI values that were approximately 25% lower than wild-type bones (Figure 3). Because cortical cross-sectional area usually parallels CSMI, it was not surprising to find that cortical cross-sectional area was correspondingly 6–17% smaller in *mdx* mice (Table 2). Specifically for the 24 month-old *mdx* mice, the smaller cross-sectional area can be largely attributed to reduced cortical wall thickness (Table 2). Body mass can influence bone geometry, therefore two-way ANCOVAs with body mass as the covariate were conducted. Results from those analyses were similar to those presented in Table 2, with the exception of periosteal diameter in which significant interactions between genotype and age were found ($p=0.002$). Specifically, with body mass as a covariate, periosteal diameters were smaller in 7-week old *mdx* mice (i.e., estimated marginal means for a body mass of 30.97 g was 1.25 mm vs 1.38 mm) and larger in 24 month-old *mdx* mice (1.26 mm vs 1.19 mm) compared to wild-type mice.

Tibial bones from 7 week old *dko* mice were smaller than wild-type and *mdx* bones for all geometric properties at the tibial midshaft (Table 2 and Figure 2). For example *dko* mice had 38–57% smaller CSMI and cross-sectional area values compared to *mdx* mice, further highlighting the impact of disease severity on tibial bone. These collective results indicate that alterations in bone geometry contribute to the compromised tibial bone strength in dystrophic mice.

μ CT was also used to determine if trabecular bone morphometry in the tibial metaphysis was affected by genotype. For trabecular bone volume fraction (i.e., trabecular bone volume per total volume of interest), independent genotype and age effects were present (Table 2). *Mdx* mice had at least 27% less trabecular bone than wild-type mice. In 7 week-old *mdx* mice, the reduction in bone volume fraction was largely attributed to having 17% fewer trabeculae of equivalent thickness, whereas in 24 month-old *mdx* mice, the number of trabeculae was not different from wild-type mice, yet the trabeculae were 19% thinner (Table 2). Consequent to these alterations in trabecular bone, the trabecular spacing was consistently 6–23% higher in *mdx* mice (Table 2).

Trabecular bone morphometry in the proximal tibial bone of 7 week-old *dko* mice was different from those of wild-type and *mdx* mice (Table 2). *Dko* mice had 78% lower bone volume fractions compared to wild-type mice, which was primarily due to having 34% fewer and 22% thinner trabeculae. These data for trabecular bone morphometry combined with the alterations seen in cortical bone geometry confirm that bone geometry is compromised in dystrophic mice.

Effect of genotype on tibial bone extrinsic and intrinsic material properties

Extrinsic material properties of the tibia, beyond geometry, were quantified by wet and dry tibial masses, and intrinsic material properties were quantified by vBMD, ultimate stress, modulus of elasticity and bone hydroxyproline concentration. Tibial bone wet and dry masses were lower in *mdx* mice relative to wild-type mice (Table 3); however after accounting for body mass as a covariate, tibial dry mass was similar between groups ($p=0.053$). Similarly, vBMD at both the tibial mid-shaft and tibial metaphysis was not different between the two genotypes (Table 3). Bone hydroxyproline concentration (i.e., hydroxyproline content relative to dry tibial mass) revealed that the concentration of collagen was higher in 24 month-old *mdx* mice compared to 24 month-old wild-type mice (Table 3). While ultimate stress values were similar between *mdx* and wild-type mice at 7 weeks of age, aged *mdx* mice had ultimate stress values that were 22% lower than wild-type mice at 24 months of age (Table 3). The modulus of elasticity within the tibial bone tended to be higher in *mdx* mice compared to wild-type mice (i.e., main effect of genotype was $p=0.051$) (Table 3). Combined, these data confirm that compared to wild-type mice, *mdx* mice have smaller sized bones of similar density. With age however, *mdx* mice developed higher hydroxyproline concentrations with a trend towards higher modulus of elasticity values while ultimate stress values were diminished.

Extrinsic tibial bone material properties (i.e., wet and dry tibial mass) of *dko* mice were much lower compared to both *mdx* and wild-type mice (Table 3). Despite the small bone size in *dko* mice, they had similar values for two of the intrinsic material properties, i.e., trabecular vBMD, hydroxyproline concentration and ultimate stress, compared to wild-type and *mdx* mice. Unlike *mdx* mice, *dko* mice had lower cortical vBMD values and higher modulus of elasticity compared to wild-type mice. In summary *dko* mice, the more severe model of DMD, have significant deficits in bone size and cortical vBMD but their modulus of elasticity was higher than that of wild-type mice.

Bone and muscle relationships

Ratios that were calculated between the functional properties of the tibia and the EDL muscle of 7-week old *mdx* mice were up to 1.44 fold of those for 7-week old wild-type mice (Figure 4 and Table 4). These data indicate that at this younger age in *mdx* mice, the functional capacity of the tibial bone is relatively high compared to that of the adjacent, diseased EDL muscle. As the mice aged to 24 months the functional relationships between bone and muscle in *mdx* mice became undistinguishable from those of wild-type mice. This observation can be attributed to two factors. First, there were greater EDL strength gains by *mdx* than wild-type mice with age (i.e., EDL muscles from 24-month *mdx* mice generated 47% more peak isometric force than those from 7-week *mdx* mice while wild-type muscle only improved by 9% with age; Table 1). Second, tibia ultimate load improved with age among wild-type (i.e., by 20%) but not *mdx* mice (Figure 2). The bone-to-muscle ratios in 7-week old *dko* mice were at least 1.33 fold of those for wild-type mice of the same age, again indicating that the functional capacity of *dko* tibial bone was relatively high compared to that of the neighboring muscle (Figure 4 and Table 4).

Discussion

We had three primary results from our study. First, mechanical testing of the tibia directly showed that tibial bones from dystrophic mice possess only 50–80% of the strength of that from wild-type mice, illustrating that the muscle disease has downstream functional effects on an associated tissue. Second, this low bone strength in dystrophic mice was mostly attributed to altered extrinsic material properties (i.e., bone geometry and bone mass) more so than alterations in bone mineral density or other intrinsic material properties. Third, the functional capacity of the tibial bone of young *mdx* and *dko* mice is greater than that of the adjacent EDL muscle as indicated by high bone-to-muscle functional capacity ratios relative to wild-type mice, but following improvements in muscle function in *mdx* mice with age the bone-to-muscle relationships in *mdx* mice become equivalent to those in wild-type mice. Combined, these data highlight that there are clear decrements in both bone and muscle tissues of dystrophic mice, as well as a distinct relationship between muscle disease and overall bone health.

In the past decade, the necessity of muscle force application for overall bone health has received increasing attention. Evidence from a hip prosthetic study suggested that 70% of the bending moments placed on bone originated from force produced via muscle contractions rather than from external reaction forces [24]. As a result, it has been hypothesized that muscular contractions produce the majority of mechanical load-induced stimuli sensed by bone's mechanosensory cells, which elicit adaptations in bone stiffness and strength if warranted. Several prospective studies have provided indirect evidence in favor of this hypothesis, by showing that bone loss followed muscle atrophy and conversely, that bone was regained after muscle mass recovered [25,26].

In muscular degenerative diseases that are progressive such as DMD, declines in bone strength and stiffness should theoretically follow decrements in muscle function (i.e., the magnitude and frequency of force production). This reduction in muscle load placed on bone in DMD is the combined effects of loss of contractile strength [27,28] and the progressive inability to perform activities of daily living [6–8]. In line with this, recent clinical evidence has shown that boys with DMD lose the greatest amount of bone following the loss of ambulation [1,10]. While this loss of bone mass is expected to be appropriate for the loss of muscle function, it likely increases the bone's propensity to fracture. This highlights the necessity of muscle-induced loading for proper maintenance of the skeleton in effort to prevent fractures. The present study, consistent with previous work in *mdx* and *dko* mice [14,27], confirmed that dystrophic muscle generated 20–60% lower levels of force

compared to that from wild-type mice. This reduction in force generating capacity in 24 month-old *mdx* mice may appear surprising due to their larger sized EDL muscles; however we are not the first to report this. Between the ages of 6 to 28 months, Lynch et al. [27] has shown that *mdx* mice have larger sized EDL and soleus muscles compared to wild-type mice, which is likely attributed to a lifelong accumulation of fibrosis within the muscle. The greatest deficits in muscle function we found were found in *dko* mice, which have been shown to have a more severe phenotype (i.e., lacking both dystrophin and its homolog utrophin) suggesting to better mimic muscle deterioration in boys with DMD. Likewise, we predicted and our data support that tibia from *mdx* mice were functionally compromised and those from *dko* mice were worse yet.

Our findings confirm and extend the work of Anderson et al. [11], showing that tibial bones from dystrophic mice are indeed functionally weakened. Specifically, we found that the tibial bone from *mdx* and *dko* mice were up to 50% lower for ultimate loads and 44% lower for stiffness than those of wild-type mice. These results are in parallel with the mechanostat theory, that is, one would have predicted that the progressive reductions in the magnitude and frequency of mechanical loading of dystrophic mice (i.e. weakened muscle contractions and reduced physical activity levels [17]) would translate to lower bone strength and stiffness compared to non-diseased mice. Contrary to the mechanostat theory, our bone-to-muscle ratios were not equivalent across genotypes in young mice, suggesting that the functional capacities of bone and muscle were not appropriately matched. Specifically, young dystrophic mice had up to 50% higher ratios compared to wild-type mice, implying that the tibial bone of these young mice was over-adapted relative to the function of the diseased muscle. Adaptations in bone are known to lag those of muscle, and because we selected the 7-week time point, *mdx* muscle had reached its peak rate of muscle deterioration (i.e., 3–4 weeks after weaning [29]); however, appropriate alterations to bone function had likely only been initiated. Alternatively, it is plausible that the lack of dystrophin caused a subtle prenatal bone effect that was apparent in the young, 7-week old *mdx* mice, but had resolved in the older *mdx* mice. This is speculation because how bone develops during the first three weeks of life in dystrophic mice has not been investigated. By 24 months of age, *mdx* mice muscle strength had increased by ~45% while their bone strength had remained unchanged, thereby resulting in bone-to-muscle ratios that were equivalent to those of wild-type mice. This improvement in muscle function likely reversed deleterious adaptations to bone which thereby preserved, but did not improve, its function between 7-weeks and 24-months of age. In summary, tibial bones from *mdx* and *dko* mice were found to be functionally impaired compared to wild-type mice, which was partially attributed to altered muscle contractile function; however, beyond the role of muscle-induced loading, it remains necessary to investigate other contributing factors of bone strength and function.

The skeletal factors that contribute to reductions in bone mechanical strength, namely bone geometry and material properties of the bone, have been minimally assessed in *mdx* mice previously [11,12]. Our data show that dystrophic tibial bones had smaller cortical cross-sectional area as well as reduced cortical wall thickness, both of which presumably contribute to their compromised bone strength. More importantly however, we found that dystrophic tibia have smaller CSMI compared to tibia from wild-type mice. Of the aforementioned parameters, CSMI is the most important determinant of a bone's mechanical properties when subjected to 3-point bending. We found CSMI to be reduced by 24–43% in dystrophic mice compared to wild-type mice. Montgomery et al. reported that *mdx* mice had higher femur bone strength presumably due to larger periosteal diameters, and equivalent cortical thickness and CSMI relative to wild-type mice *mdx* [12]. However after accounting for the larger body mass in *mdx* mice (i.e., performing ANCOVA with body mass as the covariate), differences between genotypes for periosteal diameter were eliminated in that study. Because body mass also varied among groups of mice in our study, we performed

similar two-way ANCOVAs with body mass held as the covariate. Our results on bone geometry indicate that muscular dystrophy independent of body mass was a detriment to bone geometry in *mdx* mice. The exception was periosteal diameter in the 24 month-old mice. The results of the two-way ANCOVA indicated that older *mdx* mice actually had higher periosteal diameters after adjusting for body mass compared to wild-type mice, which was dissimilar to the aforementioned findings of Montgomery et al.

These somewhat contrary findings for the *mdx* tibia and femur may be the result of either site-specific differences in bone geometry or due to the sensitivity of the techniques utilized to quantify geometry. μ CT was used in the present study, and this technique computationally assesses bone geometry by accounting for the contribution of each voxel and its position in bone, whereas Montgomery et al. utilized a caliper to quantify geometry. Thus, μ CT measurements provide greater sensitivity and precision when measuring bone geometry. Further studies utilizing μ CT are warranted to discern whether regional differences in geometry exist between *mdx* tibia and femur. Even less is known about geometric or material properties of bone from DMD patients. Consistent with our work on dystrophic mice, preliminary results on bone geometry in boys with DMD suggest that total cross-sectional areas are smaller than controls at various locations along the radial and tibial length [30,31].

In addition to altered bone geometry contributing to functionally weakened bone, it was important to determine if the muscle disease impacted other extrinsic as well as intrinsic material properties of bone. We found that dystrophic mice had reduced extrinsic material properties (i.e., tibial dry and wet mass). 24-month old *mdx* mice had alterations in two intrinsic material properties, i.e., hydroxyproline content and ultimate stress, which indicated that these mice have more collagen content (i.e., less mineralized bone tissue compared to wild-type mice) along with reduced whole bone strength. These data are in agreement with our results of tibial bone stiffness which, suggest that the stiffness of the tibia from 24-month old wild-type mice increases with their declining collagen content. Based on overwhelming evidence that boys with DMD undergo loss of areal bone mineral density [1,2,10,32,33], it may seem surprising that tibial cortical and trabecular vBMD was not different between *mdx* and wild-type mice, but this result is in agreement with findings from Montgomery et al [12]. They reported no differences in bone mineral density at the femur or spine between *mdx* and wild-type mice [12]. In addition, preliminary data from a DMD clinical trial showed that cortical vBMD, measured near the mid-diaphysis of the forearm and tibia, was actually higher in boys with DMD than age-matched controls, while significant deficits in vBMD were only apparent in distal regions of these bones [30,31]. In summary, the notion that overall vBMD is low as a result of dystrophin deficiency should be taken cautiously since four different bone sites of *mdx* mice and two bone sites of DMD patients do not show this. Despite the alterations in bone strength and geometry of *mdx* and *dko* tibia, a limitation in using these animal models is that dystrophic mice do not experience fractures like boys with DMD. A known shortcoming of the *mdx* mouse model is that they have a mild phenotype relative to boys with DMD both in terms of disease progression and muscle weakness, so it is not surprising that *mdx* mice do not experience fractures. The lack of clinical similarities between boys with DMD and *mdx* mice is attributed in part to elevated expression of utrophin by *mdx* mice [34]. *Dko* mice on the other hand, do not have this recovery due to the lack of utrophin, which translates to a progressive disease including truncated growth, limited physical abilities and muscle function, development of cardiomyopathies, and ultimately the mice suffer premature death better paralleling boys with DMD. *Dko* mice also have skeletal problems, the most notable being kyphosis of the spine [14]. Thus, utilization of the *dko* mouse for further bone investigations in relation to muscular dystrophy is reasonable and will likely prove very informative.

In summary, the present study has established that dystrophic mice, *mdx* and *dko*, do in fact have lower tibial bone strength along with deficits in muscle contractile functional capacity. Our findings indicate that bone weakness was predominately the result of adversely affected extrinsic material properties of bone (i.e., bone geometry and mass) rather than alterations to the bone's density or other intrinsic material properties. These mouse models of DMD provide a feasible approach for future research to test therapeutic regimens that ideally aim to simultaneously improve both skeletal and muscle tissues without causing further injury to either tissue.

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Figure 1.

(A) Representative load-displacement tracing obtained during a three-point bending mechanical test on a mouse tibial bone. The components of the curve that were analyzed for determining ultimate load, stiffness, energy to ultimate load, deflection to ultimate load and failure load are illustrated. (B) Representative load-displacement tracings obtained during three-point bending mechanical testing on tibial bones from wild-type (wt), *mdx*, and *dko* mice.

Figure 2.

Effects of genotype (wild-type, *mdx* and *dko*) and age (7wk vs. 24mo) on tibial bone (A) ultimate load and (B) stiffness. Values are mean $\pm$ SE. When significant main effects of two-way ANOVA ($p<0.05$) were present, the corresponding p-values are listed above the bars. When interactions between genotype and age were significant, results Holm-Sidak post hoc tests ($p<0.05$) are indicated using the lowercase letters above the bars; values with the same letter are not significantly different. Significant one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p<0.05$) are indicated y: * Significantly different from wt-7wk; †Significantly different from *mdx*-7wk.

Figure 3.

A) Effects of genotype (wild-type, *mdx* and *dko*) and age (7wk vs. 24mo) on tibial bone cross sectional moment of inertia (CSMI). Values are mean $\pm$ SE. Significant main effects of two-way ANOVA ($p < 0.05$) and the corresponding p-values are listed above the bars. Significant one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p < 0.05$) are indicated y: * Significantly different from wt-7wk; [†]Significantly different from *mdx*-7wk. (B) Exemplar μ CT images from 7-wk old wild-type, *mdx* and *dko* mice.

Figure 4.

Effects of genotype (wild-type, *mdx* and *dko*) and age (7wk vs. 24mo) on tibial bone to EDL muscle ratios. Values are mean $\pm$ SE. Statistically significant effects are indicated above the bars. Significant interactions between genotype and age detected by Holm-Sidak post hoc tests ($p < 0.05$) are indicated by the lowercase letters above the bars; values with the same letter are not significantly different. Significant one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p < 0.05$) are indicated by: * Significantly different from wt-7wk.

Table 1
Effect of genotype and age on body mass and extensor digitorum longus muscle properties.

	wt-7wk	mdx-7wk	wt-24mo	mdx-24mo	P-Values for Two-Way ANOVA			dko-7wk	P-Values for One-Way ANOVA
					Main effect of genotype	Main effect of age	Interaction (genotype x age)		
Body mass (g)	24.0 ^a (0.5)	26.1 ^a (0.9)	39.7 ^b (1.1)	32.2 ^c (0.5)	-	-	< 0.001	14.2 ^{*†} (1.4)	< 0.001
EDL peak isometric tetanic force (mN)	362.9 ^a (13.8)	212.6 ^b (16.5)	394.7 ^a (11.9)	313.2 ^c (17.0)	-	-	0.028	117.1 ^{*†} (11.8)	< 0.001
EDL peak eccentric force (mN)	492.1 (16.0)	325.1 (18.7)	588.0 (21.4)	460.3 (22.2)	< 0.001	< 0.001	0.336	188.3 ^{*†} (15.1)	< 0.001
EDL mass (mg)	10.4 ^a (0.4)	10.1 ^a (0.4)	12.4 ^b (0.2)	19.5 ^c (0.4)	-	-	< 0.001	6.0 ^{*†} (0.8)	0.002
EDL isometric force normalized to CSA (N/cm ²)	19.3 (0.6)	11.5 (0.8)	18.7 (0.6)	9.5 (0.5)	< 0.001	0.052	0.284	9.4 ^{*†} (0.5)	< 0.001

Values are means (SE). The main effects of genotype (wt vs *mdx mice*) and age (7wk vs 24mo) are listed in the sixth and seventh columns, respectively. When the interaction between genotype and age was significant, the p-value is given in the eighth column and the results from Holm-Sidak post hoc tests ($p < 0.05$) are indicated using the superscript lowercase letters; values with the same letter are not significantly different. The p-values in the last column pertain to the one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p < 0.05$) are indicated by:

* Significantly different from wt-7wk and

[†] *mdx*-7wk.

wt, wild-type; EDL, extensor digitorum longus muscle; CSA, cross sectional area; 7wk, 7-week old mice; 24mo, 24-month old mice.

Table 2
Effects of genotype and age on tibial bone mechanical function, cortical bone geometry and trabecular bone morphometry.

	wt-7wk	mdx-7wk	wt-24mo	mdx-24mo	P-Values for Two-Way ANOVA			dko-7wk	P-Values for One-Way ANOVA
					Main effect of genotype	Main effect of age	Interaction (genotype × age)		
Mechanical Functional Properties									
Energy absorbed to ultimate load (mJ)	3.15 ^a (0.24)	2.32 ^{a,b} (0.28)	4.17 ^a (0.57)	1.69 ^b (0.25)	-	-	0.038	1.23 ^{*†} (0.09)	<0.001
Deflection to ultimate load (mm)	0.45 ^a (0.01)	0.42 ^{a,b} (0.02)	0.51 ^a (0.06)	0.33 ^b (0.02)	-	-	0.047	0.37 [*] (0.02)	0.017
Geometric Properties: Mid-diaphysis									
Cortical cross-sectional area (mm ²)	0.69 (0.03)	0.65 (0.04)	0.75 (0.03)	0.62 (0.01)	0.003	0.499	0.101	0.43 ^{*†} (0.02)	<0.001
Cortical wall thickness (mm)	0.202 ^a (0.004)	0.210 ^a (0.007)	0.192 ^a (0.007)	0.159 ^b (0.004)	-	-	<0.001	0.145 ^{*†} (0.004)	<0.001
Periosteal diameter (mm)	1.23 (0.03)	1.15 (0.04)	1.38 (0.02)	1.29 (0.02)	0.003	<0.001	0.911	1.00 ^{*†} (0.03)	<0.001
Trabecular Morphometry: Metaphysis									
Bone Volume Fraction	0.092 (0.013)	0.059 (0.005)	0.030 (0.003)	0.022 (0.002)	0.007	<0.001	0.087	0.020 ^{*†} (0.002)	0.000
Trabecular Number (mm ⁻¹)	5.10 ^a (0.13)	4.23 ^b (0.10)	2.97 ^c (0.03)	2.76 ^c (0.04)	-	-	<0.001	3.35 ^{*†} (0.10)	0.000
Trabecular Thickness (mm)	0.041 ^a (0.002)	0.038 ^a (0.002)	0.062 ^b (0.003)	0.050 ^c (0.001)	-	-	0.036	0.032 ^{*†} (0.001)	0.002
Trabecular Spacing (mm)	0.192 (0.006)	0.237 (0.008)	0.355 (0.007)	0.377 (0.005)	<0.001	<0.001	0.090	0.305 ^{*†} (0.009)	0.000

Values are means (SE). The main effects of genotype (wt vs *mdx mice*) and age (7wk vs 24mo) are listed in the sixth and seventh columns, respectively. When the interaction between genotype and age was significant, the p-value is given in the eighth column and the results from Holm-Sidak post hoc tests (p<0.05) are indicated using the superscript lowercase letters; values with the same letter are not significantly different. The p-values in the last column pertain to the one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests (p<0.05) are indicated by:

* Significantly different from wt-7wk and

[†] *mdx*-7wk.

wt, wild-type; 7wk, 7-week old mice; 24mo, 24-month old mice.

Table 3
Effects of genotype and age on tibial bone extrinsic and intrinsic material properties.

	<i>wt-7wk</i>	<i>mdx-7wk</i>	<i>wt-24mo</i>	<i>mdx-24mo</i>	P-Values for Two-Way ANOVA			<i>dko-7wk</i>	P-Values for One-Way ANOVA
					Main effect of genotype	Main effect of age	Interaction (genotype × age)		
Extrinsic Material Properties									
Tibial wet mass (mg)	60.80 (1.71)	54.38 (2.76)	59.64 (3.52)	49.34 (0.85)	0.002	0.217	0.436	38.41 ^{**†} (2.20)	<0.001
Tibial dry mass (mg)	31.97 (1.01)	29.07 (1.12)	35.46 (1.48)	31.16 (0.51)	0.003	0.017	0.528	18.30 ^{**†} (1.23)	<0.001
Intrinsic Material Properties									
Cortical vBMD (mg/cm ³)	1346.45 (7.05)	1334.97 (8.72)	1493.03 (11.44)	1470.96 (8.62)	0.081	<0.001	0.566	1310.17 [*] (8.19)	0.013
Trabecular vBMD (mg/cm ³)	1077.11 (6.74)	1055.80 (11.51)	1184.85 (12.16)	1201.15 (7.20)	0.799	<0.001	0.063	1059.06 (15.11)	0.360
Bone hydroxyproline content (% dry bone mass)	1.96 ^a (0.05)	1.94 ^a (0.06)	1.66 ^b (0.05)	1.96 ^a (0.05)	-	-	0.004	1.92 (0.03)	0.862
Ultimate stress (MPa)	227.68 ^a (5.25)	233.34 ^a (8.01)	218.56 ^a (7.50)	170.13 ^b (7.91)	-	-	<0.001	217.26 (11.69)	0.428
Modulus of elasticity (GPa)	9.37 (0.38)	10.95 (0.81)	8.45 (0.30)	8.72 (0.31)	0.051	0.002	0.161	12.35 [*] (0.57)	0.007

Values are means (SE). The main effects of genotype (*wt* vs *mdx mice*) and age (*7wk* vs *24mo*) are listed in the sixth and seventh columns, respectively. When the interaction between genotype and age was significant, the p-value is given in the eighth column and the results from Holm-Sidak post hoc tests ($p < 0.05$) are indicated using the superscript lowercase letters; values with the same letter are not significantly different. The p-values in the last column pertain to the one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p < 0.05$) are indicated by:

* Significantly different from *wt-7wk* and

[†] *mdx-7wk*.

wt, wild-type; *7wk*, 7-week old mice; *24mo*, 24-month old mice; vBMD, volumetric bone mineral density.

Table 4

Effects of genotype and age on tibial bone to EDL muscle ratios.

	<i>wt-7wk</i>	<i>mdx-7wk</i>	<i>wt-24mo</i>	<i>mdx-24mo</i>	P-Values for Two-Way ANOVA			<i>dko-7wk</i>	P-Values for One-Way ANOVA
					Main effect of genotype	Main effect of age	Interaction (genotype x age)		
Ultimate load/peak eccentric force	24.56 ^a (1.10)	30.77 ^b (2.93)	24.98 ^{ab} (1.98)	20.38 ^a (1.05)	-	-	0.005	32.73 [*] (1.64)	0.003
Stiffness/peak isometric tetanic force, mm ⁻¹	100.37 ^a (2.44)	151.66 ^b (14.87)	116.94 ^{ab} (6.78)	119.84 ^a (7.82)	-	-	0.006	182.90 [*] (13.70)	0.001
Stiffness/peak eccentric force, mm ⁻¹	74.08 ^a (2.68)	97.59 ^b (8.32)	78.64 ^{ab} (4.60)	81.01 ^a (4.64)	-	-	0.043	111.24 [*] (5.53)	0.004

Values are means (SE). The interaction p-values are given in the eighth column and the results from Holm-Sidak post hoc tests ($p < 0.05$) are indicated using the superscript lowercase letters; values with the same letter are not significantly different. The p-values in the last column pertain to the one-way ANOVA results across the three groups of 7-wk old mice, and significant findings from Holm-Sidak post hoc tests ($p < 0.05$) are indicated by:

* Significantly different from wt-7wk.

wt, wild-type; 7wk, 7-week old mice; 24mo, 24-month old mice.