Published in final edited form as:
Muscle Nerve. 2012 February ; 45(2): 250–256. doi:10.1002/mus.22275.

GENDER DIFFERENCES IN CONTRACTILE AND PASSIVE PROPERTIES OF *mdx* EXTENSOR DIGITORUM LONGUS MUSCLE

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Abstract

Introduction—Duchenne muscular dystrophy (DMD) is a severe, muscle-wasting disease caused by mutations in the dystrophin gene. The *mdx* mouse is the first and perhaps the most commonly used animal model for study of DMD. Both male and female *mdx* mice are used. However, it is not completely clear whether gender influences contraction and the passive mechanical properties of *mdx* skeletal muscle.

Methods—We compared isometric tetanic forces and passive forces of the extensor digitorum longus muscle between male and female *mdx* mice.

Results—At age 6 months, female *mdx* mice showed better-preserved specific tetanic force. Interestingly, at 20 months of age, female *mdx* muscle appeared stiffer.

Conclusions—Our results suggest that gender may profoundly influence physiological measurement outcomes in *mdx* mice. Gender should be considered when using the *mdx* model.

Keywords

dystrophin; EDL; gender; mdx; muscle force

Duchenne muscular dystrophy (DMD) is an X-linked, recessive, muscle-wasting disease. It is caused by mutations in the dystrophin gene.[1] The *mdx* mouse is the most commonly used model for studying the pathogenesis of DMD and developing new therapies.[2] Dystrophin expression is abolished in *mdx* mice due to a nonsense point mutation in exon 23 of the dystrophin gene.[3] In contrast to humans, *mdx* mice are only mildly affected. Importantly, they are fertile.[4] As a result, both male and female *mdx* mice are readily available for preclinical studies. Although it is generally accepted that gender may profoundly influence animal physiology, very few studies have explored the impact of gender on disease progression and therapeutic outcome in *mdx* mice, as reviewed by Grounds et al.[5] In fact, some studies have grouped *mdx* mice of opposite genders into one group.[6–8]

Change in muscle mechanical properties is a major clinical symptom and a critical therapeutic endpoint. However, male and female differences in muscle contractile and mechanical properties have not been studied in *mdx* mice. In this study we compared isometric tetanic force generation and passive (elastic and viscous) properties of the extensor digitorum longus (EDL) muscle between genders in *mdx* and C57Bl/10 (BL10) mice. We observed a significant difference in body weight, EDL muscle weight, and EDL muscle

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cross-sectional area (CSA) between male and female mice. Although nominal differences were detected in active and passive contractile properties between male and female BL10 mice, gender-associated changes were observed in *mdx* animals. Specifically, female *mdx* mice exhibited significantly higher specific tetanic force than male *mdx* mice at 6 months of age. Although 20-month-old male and female *mdx* mice yielded similar specific tetanic force, the absolute tetanic force was significantly reduced in the females at this age. In addition, 20-month-old female *mdx* EDL muscle was stiffer than that of aged-matched male *mdx* muscle. Interestingly, female *mdx* muscle also showed a higher hydroxyproline content than males. Taken together, our data suggest that gender may profoundly influence the physiological properties of *mdx* skeletal muscle.

METHODS

Experimental Mice

All animal experiments were approved by the animal care and use committee of the University of Missouri and were in accordance with NIH guidelines. Experimental mice (*mdx* and control BL10) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Sample size data are provided in Table 1.

Isometric Force Assay

The maximal isometric tetanic force (Po) of the EDL muscle was measured at 150 HZ, as described elsewhere.[9] Briefly, each animal was anesthetized, and the EDL muscle was gently dissected and mounted to an intact muscle test system (Aurora Scientific, Inc., Aurora, Ontario, Canada).[9] The EDL was submerged in a 30°C jacketed organ bath containing Ringer buffer oxygenated with 95% O_2 and 5% CO_2. The Po of the EDL muscle was determined at the optimal length (Lo). Data were recorded and analyzed using LabVIEW-based software (Aurora Scientific). Time to maximal force and half-relaxation time were determined from the isometric tetanic force.

Elastic Property Assay

Elastic properties of the EDL muscle were measured in the absence of electric stimulation. Briefly, the muscle was subjected to a six-step passive stretch protocol. At each step, the EDL muscle was stretched by an increment of 10% Lo at a stretch rate of 2 cm/s.[10]

Viscous Property Assay

Viscous properties of the EDL muscle were determined by measuring the stress–relaxation rate (SRR) while the muscle was stretched and held at 110% Lo.[10]

Quantification of Hydroxyproline Content

EDL hydroxyproline content was determined as described elsewhere.[10] Tendons were removed from the muscle. The EDL muscle was lyophilized overnight. The lyophilized muscle was hydrolyzed with 6N HCl and then neutralized with 10N NaOH. The lysate was oxidized with 62 mM chloramine-T and reacted with *p*-dimethylaminobenzaldehyde and 60% perchloric acid to develop a red–purple color. The color absorbance was measured at 558 nm, and the hydroxyproline content was determined from a standard curve.

Statistical Analysis

Data are shown as mean ± standard error of mean. Statistical analysis was carried out using SAS software, version 9 (SAS Institute, Inc., Cary, North Carolina). For multigroup comparison, a three-factor analysis of variance was performed that considered strain (BL10 and *mdx*), age (6 and 20 months), gender (male and female). The data show that the

variability (as measured by the standard deviation) of the responses varied across different factor combinations. Consequently, heterogeneous variances were considered. The MIXED procedure of SAS was used for analysis. The REPEATED option in this procedure allows modeling of heterogeneous variances as well as dependencies of measurements made on the same animal. The models included interaction terms and, because at least some interactions were significantly different from zero for all outcomes considered, the effects of one factor were considered while holding the other two factors fixed. To account for the multiple tests done, a more stringent significance level of 0.01 was used rather than the usual 0.05. This approach to adjusting for multiple tests was used rather than the overly conservative Bonferroni adjustment commonly used. Residuals from the fitted model were examined and, in most cases, the assumption of normality was reasonable. In some cases, there were one or two outliers (an outlier is defined as the one that has a "studentized" residual of >3), and in these situations the model was re-fit with the outliers excluded. Results were reported as significant only if they appeared to be significant with and without the outliers. Statistical significance between two groups was analyzed by the Student *t*-test ($P < 0.05$ statistically significant).

RESULTS

Body Weight and Anatomic Properties of the EDL Muscle

At 6 months of age, BL10 female weight was significantly lower than that of BL10 males (Table 1). However, in 20-month-old BL10 mice, there was no significant body weight difference between males and females. At both the 6- and 20-month time-points, the female *mdx* mice had a significantly lower body weight than the age-matched male *mdx* mice (Table 1). A significant difference in body weight was observed between males, but not females, for 6-month-old BL10 and *mdx* mice. At 20 months of age, *mdx* body weight was significantly lower than that of the BL10 animals. However, females seemed to have lost more weight (*mdx* 44% lower than BL10), with the difference in males being less dramatic (*mdx* 17% lower than BL10) (Table 1).

Irrespective of dystrophin expression or age, female EDL muscles consistently showed a lower weight and smaller CSA compared with males (Table 1). Comparisons between strains (*mdx* vs. BL10, same age and same gender) showed that *mdx* EDL muscle had a higher muscle weight and CSA (Table 1). A statistically significant difference was noted between the BL10 and *mdx* strains for 20-month-old males (Table 1). When muscle weight was compared within the same strain (either BL10 or *mdx*), only female *mdx* mice showed an age-associated muscle weight reduction, decreasing by 18% from age 6 months to 20 months.

Characterization of Isometric Tetanic Force of the EDL Muscle

We did not detect gender differences between age-matched male and female BL10 EDL muscles for total tetanic force, specific tetanic force, time to maximal force, and half-relaxation time (Fig. 1A). Interestingly, specific tetanic force of 6-month-old female *mdx* EDL muscle was significantly higher than that of age-matched male *mdx* muscle (Fig. 1B). In 20-month-old *mdx* mice, females generated significantly lower total tetanic force than males. The 20-month-old *mdx* females took significantly more time to reach maximal force, but half-relaxation time was significantly lower than that of age-matched *mdx* males (Fig. 1B).

At 6 months of age, both genders produced a similar amount of total tetanic force, irrespective of dystrophin deficiency (Fig. 1A and B). Specific tetanic force was significantly reduced in 6-month-old male *mdx* mice compared with male BL10 mice of the

same age. A similar trend was noted in 6-month-old female mice (BL10: 205 ± 9 mN/mm^2; *mdx*: 183 ± 9 mN/mm^2), but the difference did not reach statistical significance (Fig. 1A and B). Nevertheless, 6-month-old male *mdx* muscle showed a significantly lower time to maximal force and prolonged half-relaxation time compared with BL10 muscle of the same age. At 20 months of age, total tetanic force and specific tetanic force were significantly reduced in both male and female *mdx* mice compared with age-matched BL10 mice. Interestingly, a significant difference (between BL10 and *mdx*) in time to maximal force and half-relaxation time was found in males but not females at 20 months of age.

Characterization of EDL Muscle Passive Properties

Comparison of stress–strain curve profiles between genders in BL10 mice revealed minimal alteration at both 6 and 20 months of age (Fig. 2A). Consistent with our recent report,[10] *mdx* EDL muscles were much stiffer (Fig. 2B). Specifically, *mdx* muscle yielded significantly higher stress at strains of 110–130% Lo when compared with BL10 muscle (Fig. 2B).[10] Furthermore, a rapid post-peak stress drop was seen in *mdx* muscle but not in BL10 muscle (Fig. 2B).[10] Although 6-month-old male and female *mdx* mice had similar stress–strain curves, differences were observed between male and female *mdx* at 20 months of age (Fig. 2B). Twenty-month-old female *mdx* mice yielded significantly higher peak stress, and post-peak stress retention was significantly higher at strains of 150% and 160% Lo.

The viscous properties were determined by SRR (Fig. 3). Age- and gender-matched *mdx* mice showed a significantly higher SRR than that of BL10 mice (Fig. 3A and B). At 6 months of age, female *mdx* mice demonstrated a significantly lower SRR compared with male *mdx* mice within the first 100 ms post-peak. At 20 months of age, female *mdx* mice showed significantly lower SRR than age-matched male *mdx* mice across the entire measurement range (from peak to 1.5 s) (Fig. 3B). No significant difference in SSR was observed between male and female BL10 mice (Fig. 3A).

Quantification of Fibrosis by Hydroxyproline Content

In the BL10 EDL muscle, the amount of collagen did not change significantly (Fig. 4A). The hydroxyproline content was significantly increased in *mdx* mice (Fig. 4). We also noticed gender and age differences in *mdx* mice (Fig. 4). Specifically, there was a significant difference between males and females at 6 months of age and a significant difference between 6 and 20 months of age for both males and females.

DISCUSSION

X-linked DMD mainly affects boys. Although female patients are rare,[11] manifesting female carriers have been frequently reported.[12,13] Both male and female dystrophin-null subjects have been included in preclinical studies. However, a gender difference has not been fully appreciated.[6–8]

Two studies have compared the serum creatine kinase (CK) level in male and female *mdx* mice. At the peak necrotic stage (3–4 weeks of age), both genders had similar levels of CK elevation.[14,15] Interestingly, male *mdx* mice showed an approximately twofold higher CK concentration than that of female *mdx* mice at 3 months of age.[14] However, this difference disappeared in 6-month-old *mdx* mice.[14] One study addressed histopathological differences between male and female *mdx* mice.[15] By morphometric quantification of Evans blue dye (EBD) uptake in the gastrocnemius muscle, the investigators concluded that there was significantly more myofiber damage in male *mdx* mice at the age of 6 weeks. However, at 24 weeks, there was more EBD-positive (damaged muscle) in females.[15] Collectively, these studies suggest that male *mdx* mice are more severely affected at a younger age (1.5–3

months of age). However, as *mdx* mice get older (≥6 months), female mice may suffer more histopathological damage.[14,15]

We recently examined the gender difference in aged *mdx* heart, a model of Duchenne cardiomyopathy.[16] Histological examination and hydroxyproline quantification revealed similar levels of myocardial fibrosis in both genders.[16–18] However, physiological assays with electrocardiography and cardiac catheterization showed significant differences between male and female *mdx* mice.[16] Aged female *mdx* mice displayed a dilated cardiomyopathy similar to that seen in human subjects.[16] However, the heart chamber was reduced rather than enlarged in aged male *mdx* mice.[16]

To extend previous *mdx* gender comparison studies,[14,15] in the present study we compared tetanic and passive forces in the EDL muscle of male and female *mdx* mice. We have focused our analysis on the EDL muscle, because it is one of the most commonly used muscles for study of *mdx* contractility.[19–21] Young *mdx* mice are mildly affected. However, aged *mdx* mice exhibit severe muscle wasting and dystrophy.[22–24] For this reason, we included two age groups (6 months and 20 months) in our study. Age- and gender-matched BL10 mice were also included as normal controls.

It is well established that male and female skeletal muscles display considerable differences in their metabolic properties and gene expression patterns.[25,26] However, Glenmark et al. found that male and female EDL muscles generated similar levels of total tetanic force in wild-type mice.[27] We compared the EDL muscle tetanic force in male and female BL10 mice. Consistent with the results of Glenmark et al., we observed comparable tetanic contractility between male and female BL10 mice (see Fig. 1A). Gender also did not affect the elastic and viscous properties in BL10 mice (see Figs. 2A and 3A). Hydroxyproline content quantification assay showed no significant difference in the collagen content in BL10 mice. Interestingly, there was a significant gender difference in the anatomical properties of the EDL muscle (20-month-old group only) and body weight (6-month-old group only) in the BL10 mice (Table 1). Collectively, our results suggest that gender may not affect the outcome of *in vitro* physiological assays in the EDL muscle of BL10 mice.

In contrast to BL10 mice, measurements performed in *mdx* EDL muscle showed significant gender disparity in several parameters. At the age of 6 months, female *mdx* EDL muscle displayed significantly higher specific tetanic force (Fig. 1B). However, the difference disappeared at 20 months. In fact, 20-month-old *mdx* females showed significantly lower total isometric tetanic force and a shorter half-relaxation time. It also took them longer to reach maximal force. It is interesting that adult female *mdx* mice performed better than males, but aged female *mdx* mice performed worse than males. The exact mechanisms behind this observation remain to be investigated. However, sex hormones may have contributed to this difference.[28] Six-month-old female mice are at their reproductive peak, whereas at 20 months they should have reached menopause. Another point of interest is the body weight changes in 20-month-old mice. At this age, the body weight of *mdx* females was only ~50% of that of BL10 females. However, the difference was less in males (*mdx* males were ~20% of BL10 males). The gender-associated difference in body weight comparison (between *mdx* and BL10 mice) suggests that there may be global metabolic changes. These changes may have also contributed to our observations.

In addition to active muscle force, we also noticed significant gender differences in parameters of passive properties in *mdx* mice. Specifically, 20-month-old female *mdx* mice showed significantly higher peak stress than males (see Fig. 2B). In terms of viscosity, 6-month-old female *mdx* mice showed a significantly reduced SRR compared with male *mdx* mice from peak to 0.1 s. This difference extended through the entire assay time range in 20-

month-old female *mdx* mice (see Fig. 3B). It remains unclear why these differences were only observed in *mdx* mice but not in BL10 mice. One likely reason is the level of fibrosis. Female *mdx* mice had a higher collagen content, and a significant difference was reached at 6 months of age (see Fig. 4). But why were female *mdx* mice more fibrotic than male *mdx* mice? Currently, there is no clear explanation for this observation, yet we suspect that it may relate to the renin–angiotensin system (RAS). The RAS is an important regulator of fibrosis.[29] It has been shown that females seem more sensitive to perturbations of the RAS.[30,31] It is worth noting that enhanced fibrosis was also seen in the hearts of female *mdx* mice. In fact, aged female *mdx* hearts had a significantly higher hydroxyproline content compared with male hearts.[16] We recently demonstrated that the myotendinous junction (MTJ) also influences the outcome of passive property assays.[10] Future studies are needed to clarify the role of the MTJ in gender-related stiffness changes (Fig. 3).

In conclusion, we have demonstrated that gender may profoundly influence the outcomes of physiological assays in *mdx* mice. Gender should be considered in study design and data interpretation when using *mdx* mice in DMD studies.

Acknowledgments

This work was supported by grants from the National Institutes of Health (AR-49419, to D.D.), Muscular Dystrophy Association (to D.D.), and an NIH training grant (T90DK70105 to C.H.). The authors thank Dr. Virginia Huxley for helpful discussions; Dr. Dick Madsen and Dr. Youngju Pak for help with statistical analysis; and Marianne Abdo, Juveria Nayeem, and Alexandra Kellogg for technical assistance.

Abbreviations

CK	creatine kinase
CSA	cross-sectional area
DMD	Duchenne muscular dystrophy
EBD	Evans blue dye
EDL	extensor digitorum longus
Lo	optimal length
MTJ	myotendinous junction
Po	absolute isometric force
RAS	renin & angiotensin system
SPo	specific isometric force
SSR	stress–relaxation rate

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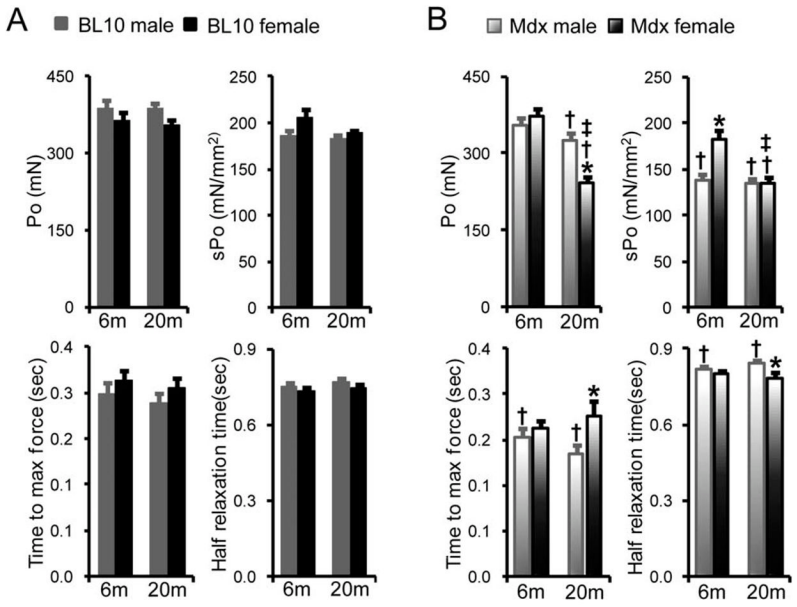

FIGURE 1.

Characterization of isometric tetanic contraction of the EDL muscle. Comparison of absolute isometric tetanic force, specific isometric tetanic force, time to maximum force, and half-relaxation time between genders in BL10 (**A**) and *mdx* (**B**) mice at 6 and 20 months of age. *Females significantly different from age- and strain-matched males; †*mdx* mice significantly different from BL10 mice within the same gender; ‡20-month-old group significantly different from 6-month-old group within the same gender and strain.

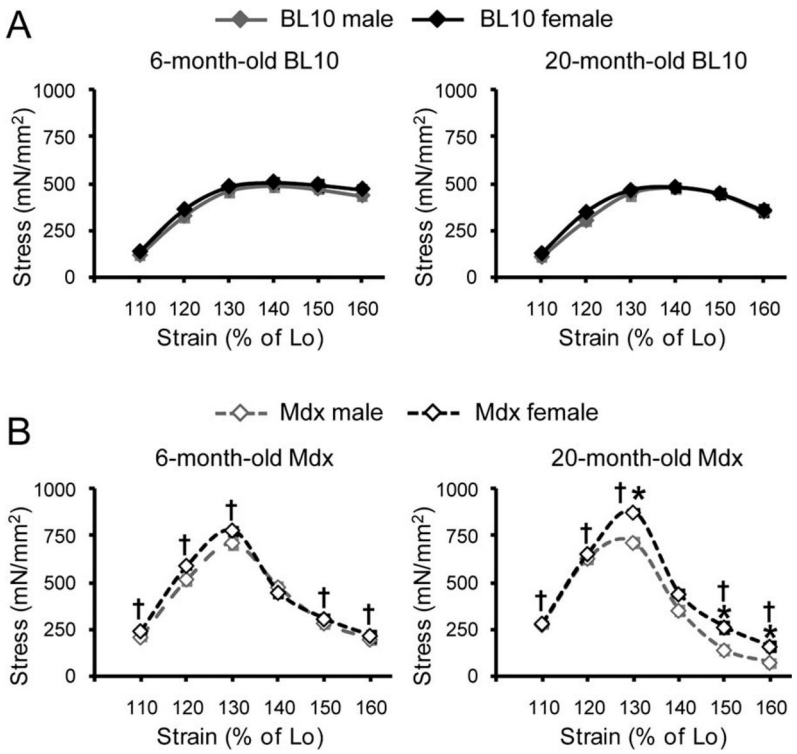

FIGURE 2.

Comparison of the stress–strain relationship between genders in BL10 (**A**) and *mdx* (**B**) mice. The elastic property of the EDL muscle at 6 and 20 months was characterized by the stress–strain relationship developed while straining the muscle until 160% Lo in an increment of 10% Lo. *Females significantly different from age- and strain-matched males; †*mdx* mice significantly different from BL10 mice within the same gender.

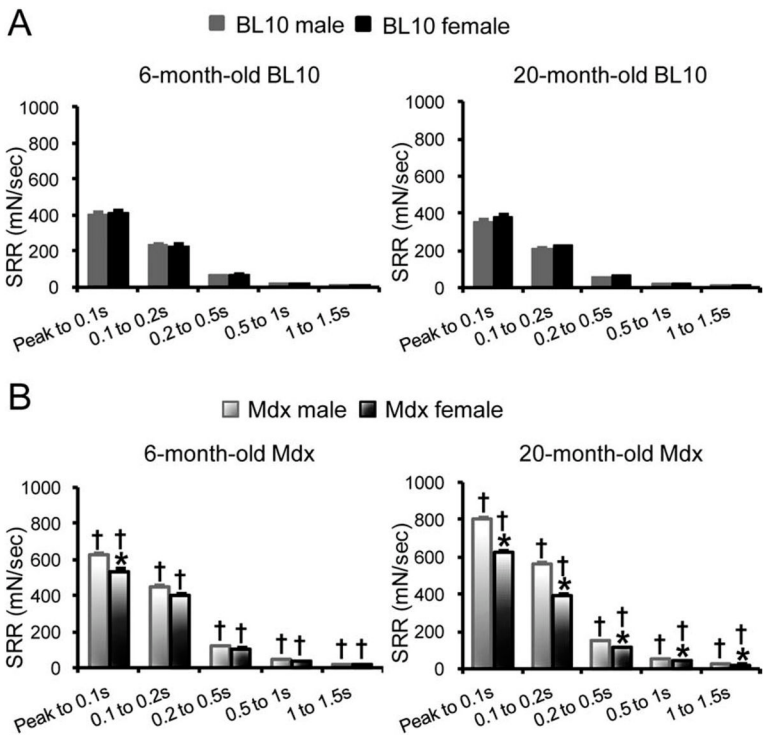

FIGURE 3.

Comparison of the stress–strain relaxation rate of the EDL muscle in BL10 (**A**) and *mdx* (**B**) mice. The viscous properties of the EDL muscle at 6 and 20 months was determined by the stress–relaxation rate. *Females significantly different from age- and strain-matched males; †*mdx* mice significantly different from BL10 mice within the same gender.

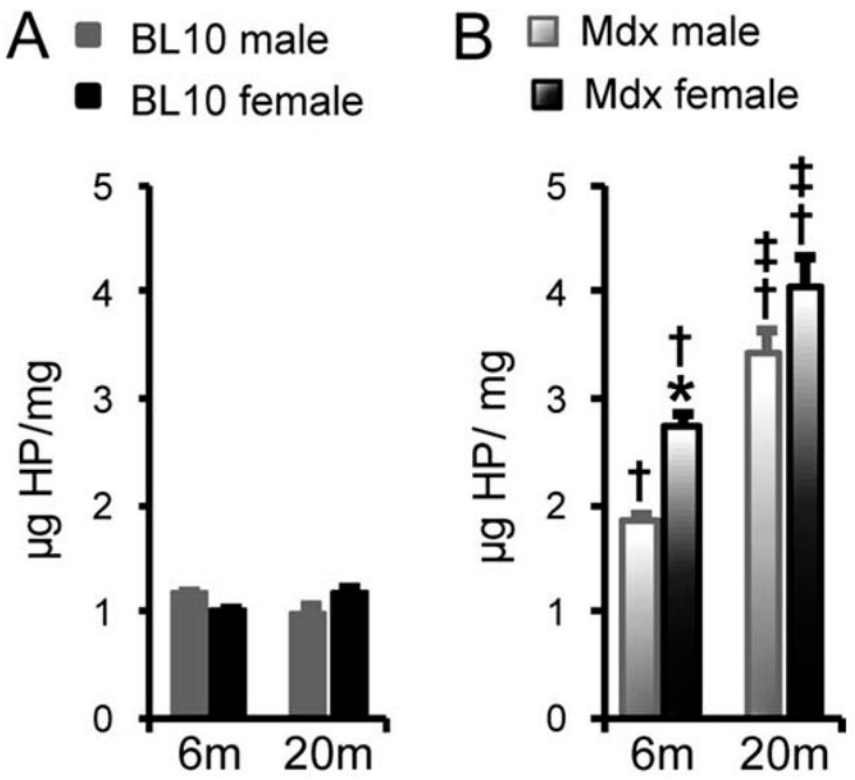

FIGURE 4.

Quantification of the hydroxyproline content in the EDL muscle of BL10 (**A**) and *mdx* (**B**) mice. *Females significantly different from age- and strain-matched males; †*mdx* mice significantly different from BL10 mice within the same gender; ‡20-month-old groups significantly different from 6-month-old groups within the same gender and strain.

Table 1

Morphometric properties of the experimental animals.

		6-month-old males	6-month-old females	20-month-old males	20-month-old females
BL10	*n*	10	9	10	10
	Body weight (g)	32.03 ± 0.57	25.67 ± 0.67 *	37.44 ± 0.64 ‡	39.96 ± 1.18 ‡
	EDL weight (mg)	13.90 ± 0.77	10.97 ± 0.40	13.00 ± 0.18	11.86 ± 0.30 *
	CSA (mm^2)	2.12 ± 0.12	1.78 ± 0.06	2.12 ± 0.03	1.88 ± 0.05 *
	Lo (mm)	14.09 ± 0.04	13.18 ± 0.05 *	13.14 ± 0.04 ‡	13.54 ± 0.04 *,‡
mdx	*n*	13	9	19	15
	Body weight (g)	35.44 ± 0.42 †	27.38 ± 0.71 *	31.12 ± 0.56 †,‡	22.48 ± 0.48 *,†,‡
	EDL weight (mg)	16.73 ± 0.42	13.56 ± 0.48 *	15.95 ± 0.33 †	11.11 ± 0.35 *,‡
	CSA (mm^2)	2.57 ± 0.07 †	2.05 ± 0.07 *	2.42 ± 0.05 †	1.79 ± 0.06 *
	Lo (mm)	13.93 ± 0.05	14.18 ± 0.16 †	14.13 ± 0.10 †	13.28 ± 0.04 *,‡

EDL, extensor digitorum longus; CSA, cross-sectional area; Lo, optimal length.

* Female significantly different from age- and strain-matched male.

† mdx is significantly different from BL10 within the same gender.

‡ Twenty-month-old group is significantly different from 6-month-old group within the same gender and strain.