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Female Titin Mutant Mice Outperform Young and Old Males

A Thesis Presented

by

Reese Ger

To The Keck Science Department

of

Claremont McKenna, Scripps, and Pitzer Colleges

In Partial Fulfillment of

The Degree of Bachelor of Arts

Senior Thesis in Human Biology

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Table of Contents

Abstract

Exercise performance is widely believed to generally be maximal among young males. However, little research has been conducted to investigate the impact of sex or age on muscle performance. This study investigated the effects of sex and age on forelimb and all limb grip strength performance in wildtype and titin mutant mice. Titin is the largest known protein, with known involvement in muscle function. The mice in this study were Ttn$\Delta^{112\text{-}158}$ with a 75% deletion to the PEVK region of titin. Based on limited existing research, we hypothesized that wildtype mice would outperform all Ttn$\Delta^{112\text{-}158}$ mice, males would outperform females within the same genotype and age, and young mice would outperform their old counterparts. We did find that wildtype mice universally outperformed Ttn$\Delta^{112\text{-}158}$ mice, male wildtype mice demonstrated greater grip strength than females, and younger mice outperformed older mice. However, female Ttn$\Delta^{112\text{-}158}$ mice outperformed males with age-related decline disproportionately affecting males. Pairwise statistics revealed that all observed differences were sex-driven, underscoring the importance of including females in research. Possibly through some combination of changes in body mass composition, muscle quality, fiber type composition, and hormones our age and sex-based results in wildtype and Ttn$\Delta^{112\text{-}158}$ mice may be explained, although further studies are needed.

Introduction

In humans, skeletal muscle accounts for nearly 40% of body weight and contains 50-75% of all proteins (McCuller et al., 2023). The importance of skeletal muscle on our ability to complete the tasks of daily life cannot be overstated. Assessment of peak grip strength is one straightforward method to quantify muscle performance. Yet, this essential muscle function occurs through a complex process which is influenced by several factors. This study will focus on the components of muscle which are known to be modulated by the protein titin and influenced by age and sex differences, accounting for much of the variation between individuals. This makes sex and age considerations indisposable factors to better understand the role of titin from a more holistic and inclusive perspective. These factors include *muscle structure* due to the integral role of titin as one of the three major filaments of muscle, *fiber type* and *hormones* as they are known to have different levels between the sexes and with age-related changes, and the interaction of *sex* and *age* with respect to *titin*. It is very possible that age-related physiological differences may impact male and female mice differently, further complicating muscle performance among mice of different ages and sexes. As there are several diseases which are known to cause titin mutations in humans, it is crucial to gain a better understanding of its function and how patients of differing age and sex may be impacted by their disease in the hopes of one day improving their quality of life.

a. Overview of muscle structure

There are three main types of muscle: striated, smooth, and cardiac. This paper will focus on striated muscle, which is responsible for voluntary movement and generation of necessary forces. Also known as skeletal muscle, striated muscles are composed of bundles of myofibers made of numerous myofibrils, which can be further broken down into the proteins actin and myosin. Each myofiber is a muscle cell with basic cellular units termed sarcomeres (McCuller et al., 2023). Sarcomeres are the contractile units of muscle composed of three

distinct types of myofilaments: thick, thin, and titin (Figure 1). The thick filaments are found in the center of the sarcomere and contain myosin, a type of molecular motor protein which generates active force via the formation of cross-bridges between actin and myosin. The thin filaments are actin-based and are attached to the ends of the sarcomere at the Z-disks (Squire et al., 2017). Thick and thin filaments–myosin and actin–overlap to form cross-bridges. The thick filaments attach to the thin filaments and pull in a cyclic motion which repeatedly shortens the sarcomere to contract the muscle, actuating the bone around the joint resulting in force generation. Regulated by calcium, the filaments relax and slide back into place, according to the sliding filament theory; the thin filaments slide past the thick filaments to shorten the sarcomere and contract the muscle. The third myofilament type is titin, the largest known protein. Titin (Ttn) is the largest known protein in the body with up to 363 exons and 34,000 amino acids and is a ubiquitous element of all skeletal muscles (Nishikawa et al., 2020). Titin acts as a molecular spring which spans half a sarcomere from the Z-disk to the M-line maintaining the sarcomere's structural integrity (Squire et al., 2017). Spanning half a sarcomere, titin acts as a sarcomeric scaffold by anchoring titin to the thick filaments at the A-band and thin filaments at the Z-line with six titin filaments per thick filament (Figure 1, Nishikawa et al., 2020). Titin is composed of two main sub-segments, the tandem Ig segment and the PEVK segment (Figure 2, Brynnel et al., 2018). The Ig domain is more pliable and straightens at a lower force as compared to the stiffer PEVK segment (Nishikawa et al., 2020). The latter region is rich in proline (P), glutamate (E), valine (V), and lysine (K). Stiffness may be modulated by calcium. This may occur by interacting with the glutamate-rich (E) domains of the PEVK region which becomes 10-20% stiffer in the presence of calcium (Brynnel et al., 2018).

Titin is known to contribute to muscle passive stiffness (Figure 2). Passive stiffness is the tension conferred upon the muscles when they are relaxed, as opposed to active tension, in which the muscles are contracting. This can be thought of as the spring constant *k* in Hooke's Law: F=k*x. When the stiffness, *k*, increases, so too does the force. Displacement from

equilibrium, *x*, is the other factor in Hooke's Law. In mice with a large deletion to the PEVK region of the molecular spring titin, stiffness, *k*, increases with the stiffer mutated spring, while displacement, *x*, decreases with shorter sarcomeres. These mutated mice experienced an increase in passive stiffness within the sarcomere, muscle fiber, and at the whole muscle level (Brynnel et al., 2018). While increased passive stiffness due to a shorter, and therefore stiffer, molecular spring in the form of mutated titin would seemingly minimize, and therefore, hinder muscle performance, Brynnel et al. (2018) found that not to be the case. Instead, they hypothesized that their titin mutants had adapted to function at a shorter sarcomere working range, possibly as a result of tradeoffs between *k* and *x*, resulting in similar levels of force as compared with non-mutated mice in the Brynnel study.

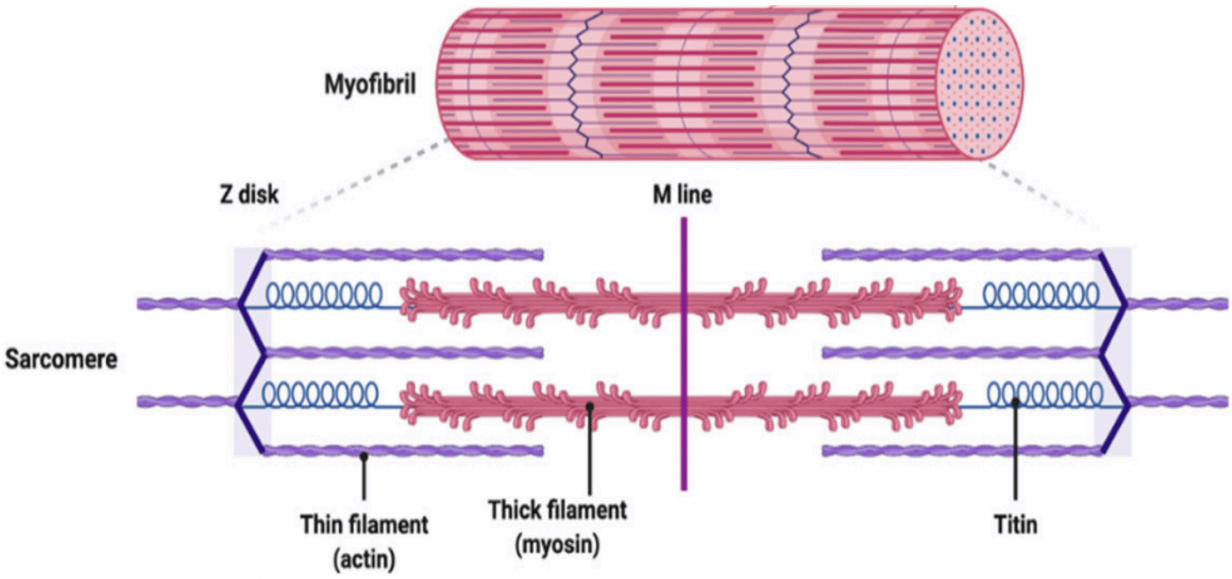

Figure 1. Schematic of the sarcomere (Tharp et al., 2020)

b. Titin

The mice investigated in the present study are Ttn$\Delta^{112\text{-}158}$ mice which have a 47 base pair deletion within the PEVK region of titin at exons 112-158 (Figure 3). This reduces the length of the PEVK segment by approximately 75%, thereby greatly increasing the passive stiffness of the sarcomeres, fibers, and muscles as shortening this highly elastic segment makes it act as a much stiffer spring (Brynnel et al., 2018). While the length of the PEVK segment in Ttn$\Delta^{112\text{-}158}$ mice was greatly reduced, their overall fiber lengths were found to increase by 13.5% as well as a 30% increase in the number of sarcomeres in series (Brynnel et al., 2018). Passive stiffness has been observed to increase with age, with precipitous increases later in life, which negatively impacts the function of muscles. It appears that Ttn$\Delta^{112\text{-}158}$ mice have managed to adapt to their stiff titin.

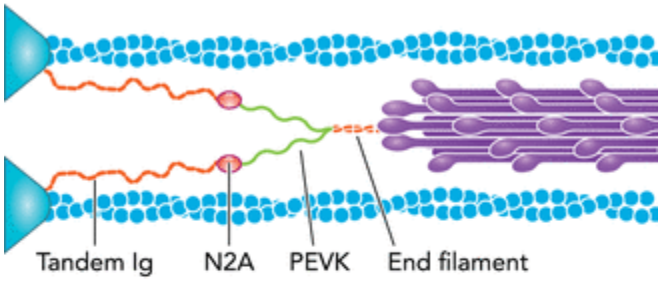

Figure 2. Schematic of the giant protein titin (Nishikawa et al., 2020)

The Ttn$\Delta^{112\text{-}158}$ model was found to have the same skeletal size as the WT mice, as revealed by comparisons of tibia length. While the two strains had comparable skeletal sizes, the Ttn$\Delta^{112\text{-}158}$ mice were found to develop a body weight deficit as compared to the WT mice as the two groups aged up to 78 weeks (Brynnel et al., 2018). Yet, most muscle groups in Ttn$\Delta^{112\text{-}158}$ mice had a significantly increased weight with no change to muscle CSA, suggesting that the increase in muscle weight can be accounted for largely by the increase in longitudinal muscle growth. The comparable skeletal size of Ttn$\Delta^{112\text{-}158}$ and WT mice, paired with the body

weight deficit and increase in muscle mass observed in the Ttn$\Delta^{112\text{-}158}$ model, suggests that the mutant mice have a greatly reduced body fat percentage (Brynnel et al., 2018). This may be due, in part, to the energetic costs of maintaining greater muscle mass.

Ttn$\Delta^{112\text{-}158}$ mice, with their 30% increase in the number of sarcomeres in series, have longitudinal hypertrophy. However, they also found that the active force generation working range length was shorter in the muscles of Ttn$\Delta^{112\text{-}158}$ mice through a shortening of thin filaments. Comparisons of Ttn$\Delta^{112\text{-}158}$ and WT EDL muscle revealed that the Ttn$\Delta^{112\text{-}158}$ mice had decreased sarcomere length and significantly greater force exerted per molecule of titin. This had particularly impactful effects on passive stiffness (Brynnel et al., 2018). This reduction to the working range through decreased sarcomere lengths theoretically reduces force production through double overlap of titin filaments. While the reduction to working range observed in Ttn$\Delta^{112\text{-}158}$ mice was quite large, minimal consequences were observed in terms of their active force development due to the reduction in thin filament length. Investigations on active-tension revealed no significant genotype effects on maximal active tension levels, but do indicate an increase in force development speed in Ttn$\Delta^{112\text{-}158}$ mice (Brynnel et al., 2018). For cyclic movements, like running, Ttn$\Delta^{112\text{-}158}$ and WT mice demonstrated very similar averages speed and distance, implying that Ttn$\Delta^{112\text{-}158}$ mice were not impeded by their stiffer muscles (Brynnel et al., 2018). While Brynnel et al. (2018) investigated both males and females with respect to age, the maximum age was only 274 days which was not long enough to see great decreases in strength.

Figure 3. The Ttn$\Delta^{112\text{-}158}$ mouse model with exons 112-158 deleted within the PEVK region of titin (Brynnel et al., 2018)

c. Sex differences (fiber types and hormones)

Skeletal muscles can be classified into different fiber types based on intrinsic properties. There are four types of muscle fibers: type I, type IIA, type IIB, type IIX. Type IIX fibers are not highly expressed in limb muscles and therefore will not be covered in the present study. Additionally, while type IIB fibers are present in mice, it is worth noting that they are not expressed in humans. Fiber types have varying presence based on muscle type, sex, and exercise. Each fiber type has a different function and contractile velocity, which is dependent upon their myosin isoform. Type I fibers are the smallest and are slow-twitch fibers, also considered to be slow oxidative fibers. While they contract slowly, they have the lowest fatigue rate and use less energy and are therefore primarily involved in actions such as maintaining posture or running great distances (McCuller et al., 2023). Type IIA, similar to type I, are considered red fibers due to their high myoglobin content. Type IIA fibers are fast-twitch, also considered fast oxidative fibers. They use more energy and have a mid-range of fatigability and are therefore suited for actions such as walking as they have better endurance (McCuller et al., 2023). Type IIB fibers are also fast-twitch fibers, but unlike type IIA, they are fast glycolytic. Being the largest fibers due to their high density of both actin and myosin, type IIB fibers are highly fatigable and are used for actions such as sprinting or weight-lifting. These fibers are considered white fibers because they are poor in myoglobin, instead getting ATP largely through anaerobic glycolysis (McCuller et al., 2023). Summarized, the relative velocities are I < IIA < IIX < IIB (Haizlip et al., 2015). While mice are good models, their myosin heavy chain (MyHC) isoforms differ from humans in richness of glycolytic enzymes and oxidative capacity.

Sexual dimorphisms vary by muscle group but exist across vertebrates in fiber type composition; generally, both males and females have the greatest abundance of IIX fibers, but males have more IIB fast-twitch fibers (IIX > IIB > IIA) whereas females have more type IIA slow-twitch fibers (IIX > IIA > IIB) (Haizlip et al., 2015). These differences in fiber-type composition improve the speed and strength of male muscles and the endurance and recovery

of female muscles through slower oxidative fibers and greater oxidative capacity. However, the prevalence of each fiber type between the sexes varies by muscle group. Type IIA muscle, for example, has deferring contributions in soleus (50% in male and 36% in female), plantaris (16% in male and 37% in female), and tibialis anterior (39% of male and 25% of female) muscles (Haizlip et al., 2015).

In part due to differences in fiber-type composition, sex-based differences are observed in fatigue recovery and endurance testing. Across most muscle groups, male muscles were found to be more easily fatigable and have a greater cross sectional area (CSA) than female muscles (Haizlip et al., 2015). The muscles of females are more fatigue-resistant, giving them better endurance including recently in ultramarathons where they excelled beyond male competitors (Le Mat at al., 2023). While males may lose an endurance contest, they are notorious for having large muscle groups–particularly in the legs–allowing them to be faster sprinters and enabling them to lift many times their body mass (Bartolomei et al., 2021). Hormones have been found to interact with both fiber-type composition and contractile function. Thyroid hormone, estrogen, and testosterone levels differ between the sexes and can regulate the abundance of each fiber-type, overall muscle size, and muscle function (Haizlip et al., 2015). Mammalian hypothyroidism has been found to produce a lower percentage of type II fibers in both males and females as compared to healthy individuals. However, hypothyroid females demonstrate both a greater proportion of type II fibers and type II fiber atrophy as compared to males (Haizlip et al., 2015).

Previous studies have found skeletal muscle to be a highly estrogen-responsive tissue. Estrogen's primary role with respect to muscle appears to be regeneration and maintenance. Additionally, estrogen-deficiency in females–which is naturally occuring with advancing age due to menopause–has been found to increase overall body mass, likely due to an increase in fat as opposed to an increase in myofilaments, and the mass of individual muscles, while simultaneously reducing time to peak tension by 50% (Haizlip et al., 2015). These findings are

further supported by estrogen's capacity for influencing muscle fiber size, overall muscle weight, capacity for contractility, muscle regeneration, and minimal ability to alter fiber-type distribution. A study considering the role of estrogen, nitric oxide, and MyHC expression in allowing female wildtype (WT) mice to have greater exercise capacity as compared to male mice found females able to run a 54% greater distance than age-matched males (Oydanich et al., 2019). When matched for body or muscle mass, females again demonstrated better performance. It was suggested that this improvement in endurance was potentially enabled by their increased type I and decreased type II MyHC expression in the soleus muscle. Following ovariectomy and subsequent decrease in estrogen, females no longer demonstrated enhanced endurance. Similarly, males treated with estrogen demonstrated exercise capacity similar to intact females (Oydanich et al., 2019). These results suggest that as far as endurance goes, estrogen may be the primary modulator or sex differences. Estrogen and progesterone may also improve strength by participating in the maintenance of muscle; hormone replacement therapy in females with progesterone and estrogen lead to an increase in vertical jump height in postmenopausal women (Haizlip et al., 2015).

Testosterone is an androgen associated with significant increases in muscle mass. Like estrogen, testosterone levels naturally drop with age. In males, decreased testosterone levels have been correlated with a decrease in both muscle mass and strength as well as increasing body fat content (Haizlip et al., 2015). Testosterone is not thought to enhance contractile function as testosterone replacement studies have not found improvements to endurance (Haizlip et al., 2015). However, testosterone supplementation is significantly associated with an increase in strength alongside observations of its anabolic effects, even in females; in postmenopausal women, testosterone supplementation led to a 50% increase in protein synthesis and in guinea pigs, testosterone injection led to an increase in muscle fiber size (Haizlip et al., 2015).

d. Age and sex

Known as sarcopenia, age-related muscle loss is a well known phenomenon that leads to a decrease in skeletal muscle function through the progressive loss of muscle mass, quality, and strength. Between WT mice of 3 and 24 months of age, transcriptional changes altered genes associated with energy metabolism, MyHC isoforms, cell proliferation, and immune function. With age, type II fibers are less numerous thereby decreasing the ratio of type II-to-type I fibers resulting in noticeable performance changes (Lin et al., 2018). These changes included decreases in peak force, reduction of muscle mass, impaired ability to regenerate myofibers, and increases in adipose-like tissues observed in muscle samples. Additionally, the energy metabolism of skeletal muscle was found to be less efficient with age (Lin et al., 2018).

A study considering the impacts of age on forelimb grip strength in male, WT mice found that the mice reached their peak force at 4 months of age, declined at both the 8 and 12 month marks, and declined robustly to 20 and 28 months when the study was terminated. Interestingly, they posited that age-related arthropathy, not lean muscle mass, potentially accounted for the observed decline in strength as exostosis–bone spurs–occurred in more that 50% of older mice (Ge et al., 2016).

Another study investigating age-related changes to mice and their muscles considered both male and female WT mice from 3 to 78 weeks. Aging was found to increase body mass between 10 and 78 weeks and, surprisingly, to increase muscle mass in nearly all muscle groups (Hill et al., 2020). Another study, however, found that WT mice decreased in body mass after 20 months–or 80 weeks–of age (Uchitomi et al., 2019). Hill et al. (2020) found that WT males have significantly greater body mass compared with females. The increase in total muscle mass observed was deemed partially attributable to age-related increases in noncontractile mass, possibly such as collagen but not fat, which would lead to an increase in body mass but not force production. Only extensor digitorum longus (EDL) muscle–a muscle located in the hind limbs–mass declined significantly and this atrophy was exclusively observed

in females between 52 and 78 weeks (Hill et al., 2020). Force was found to increase up until 30 or 52 weeks–depending on the muscle group–for both sexes, but to a higher level in males. This age-related decline was significant. Male soleus muscle demonstrated more power than female soleus muscle up until 78 weeks, at which point the females were more powerful, possibly due to the fact that the soleus is primarily composed of type I fibers. At least for WT mice, the loss of muscle quality preceded observed declines in performance as the mice aged. Muscle quality was defined as force per unit of skeletal mass and proved to be a great indicator of true strength as older adult mice with high muscle quality could produce greater force with lesser muscle mass than their low quality counterparts. Additional implications of poor muscle quality include the maintenance of large muscles increases metabolic demand. However, this method is challenging in vivo due to denervation which may mask muscle-aging (Hill et al., 2020).

No significant age-related changes in MyHC slow or fast isoforms were found in any muscle group for these 3 to 78 week old WT mice. They posited, though, that mice of very old age would have a reduction in muscle mass, a greater composition of oxidative fibers, as well as further shortening of muscle fiber length. Proposed fiber-type changes in very old mice included the atrophy of type II fibers in mice and the shift towards slow, oxidative fibers in humans with a 20-50% reduction in type II fibers generally observed in humans (Hill et al., 2020).

Aging affects the sexes differently, both hormonally and in terms of fiber type composition. In addition, a study found that the presence of dozens of metabolites and lipid species significantly differ between young and old mice (Uchitomi et al., 2019). Overall, the severity of muscle aging is dependent upon both sex and which muscle is considered. The goal of the present study is to determine how titin changes with sex and age, specifically in the muscles associated with limb strength, by investigating mice with a titin mutation.

e. Hypotheses

Prior studies consistently find males to be stronger in most muscle groups and females to demonstrate better endurance (Brynnel et al., 2018; Hill et al., 2020; Oydanich et al., 2019). Given that aging is known to affect muscle performance in the sexes differently, we hypothesized that titin properties also change with age and sex. No prior study we are aware of has considered both age and sex in Ttn$\Delta^{112\text{-}158}$ and WT mice (Table 1). Given that titin plays a role in muscle force, and that the Ttn$\Delta^{112\text{-}158}$ model has previously been shown to increase passive stiffness, we hypothesize that Ttn$\Delta^{112\text{-}158}$ mice of any age and either sex will be weaker than their WT counterparts. Within the Ttn$\Delta^{112\text{-}158}$ model, we predict that young individuals will have better all limb and forelimb performance than older mice following age-related declines observed in WT mice. In addition, we predict that male Ttn$\Delta^{112\text{-}158}$ mice will have greater peak force than females.

Table 1. Summary of Relevant Literature

Author	Genotype	Sex	Age	Findings
Brynnel	WT & Ttn	Mostly M	~8 weeks	● Shorter working range in Ttn$\Delta^{112\text{-}158}$ mice ● Longitudinal hypertrophy ● Increased passive stiffness is bad, made up for by more sarcomeres, energetically costly, therefore Ttn$\Delta^{112\text{-}158}$ mice are lighter
Ge	WT	M	16-112 weeks	● Age-related decline in grip strength
Hill	WT	M & F	3-78 weeks	● Aging resulted in increased body mass and generally increased muscle mass ● Males larger than females ● Males had lower normalized power relative to females by 78 weeks, but greater before this time ● Loss of muscle quality precedes loss of absolute performance ● Aging led to rapid deterioration in fatigue resistance ● 78 week mice potentially not old enough to demonstrate significant muscle atrophy
Oydanich	WT	M & F	Females 14-15 weeks Males 6-7 weeks	● Age-matched females ran much longer than males ● Males larger than females in body mass and muscle mass ● Experiments with estrogen astrology suggest estrogen improves exercise capacity ● Females have greater type I fiber expression than males, males have greater IIa expression ● Males not yet old enough to have reached peak sexual maturity
Present study	WT and Ttn	M & F	16-112 weeks	● Male body mass greater than females for both genotypes ● Ttn young males stronger than young females, old females stronger than old males ● Ttn mutation harms male performance more than female performance, particularly with aging

Methods

a. Study subjects

The use of mice in our study was approved by the Institutional Animal Care and Use Committee of the Claremont Colleges (CC IACUC Protocol #019-004). The Ttn$\Delta^{112\text{-}158}$ mice were genetically engineered at the University of Arizona. A breakdown of study subjects is available in Table 2. Data on old, male and female mice with a titin mutation to the PEVK region from exons 112-158 (Ttn$\Delta^{112\text{-}158}$) was collected in the lab of Professor Monroy of Keck Science Department by Susan Park and Reese Ger between September and December 2023. Additional data was consolidated from previous cohorts of Ttn$\Delta^{112\text{-}158}$ mice from the Monroy lab for young females and males. Data were compiled with previously collected data from young WT females. The age and weight of each mouse were recorded prior to testing (Table 2) and mice were given unique ID numbers that remained constant throughout the study. As mice reach peak sexual maturity around 10 weeks of age, all mice in the present study had passed this threshold (Hill et al., 2020). C57BL/6 mice are a very common strain of laboratory mice often used as the wildtype model. For this study, C57BL/6 mice will be considered wildtype (WT). WT data, including weight and forelimb grip strength, was obtained for male mice ages 4 to 28 months directly from Ge, et al. (2016). This study used very similar methods to the present study for measuring forelimb grip strength. For this reason, the authors were contacted and the data for 4 month old males was used as this study's young WT males and the 20 and 28 month olds were used as this study's old WT males. Only C57 mice were pulled and CB6 mice were excluded as the Monroy lab WT mice were exclusively C57BL/6 and high n values were available with C57BL/6 mice alone.

No data was available for old, female, WT mice. Only 3 young, male, Ttn$\Delta^{112\text{-}158}$ mice were available in this dataset; despite the small sample size in this subgroup, these individuals were included in the analysis to ensure representation across all relevant categories. Very young mice (>60 days) were excluded from the study as they were still growing with noticeably

smaller values for both body mass and force. Additionally, middle aged mice (360 days) were excluded as this study seeks to investigate sex differences only in young and old mice. Young mice were between 76 and 151 days old. Old mice were between 600 and 840 days old. The complete dataset contains 128 mice (Table 2).

b. Grip strength measurement

Measurements of fore and all limb grip strength were taken using a Bioseb Grip Strength Test and recorded in grams. These tests were chosen because of their clear indications of peak active force development. Both forelimb and all limb tests were completed because previous studies have revealed differences in muscle fiber types between muscle groups, and, consequently, differences in peak force development. The Bioseb meter was tared between each subject. The test was set up horizontally so that the mice could grab onto the bar and be pulled backwards by their tail until they released their grip. The fore limb test was defined as both front paws grasping the bar (Figure 4A) while the all limb test used a wire grate for the mice to grasp with all four paws (Figure 4B). Three trials were conducted per mouse with at least two minutes of rest between each trial for the fore limb test. About half an hour later, this protocol was repeated for the all limb test. Only the highest recorded measurements were analyzed. Body mass was recorded in grams and force was recorded in Newtons and converted to grams. Grip strength data was normalized to body mass by dividing peak force by the body mass of that mouse.

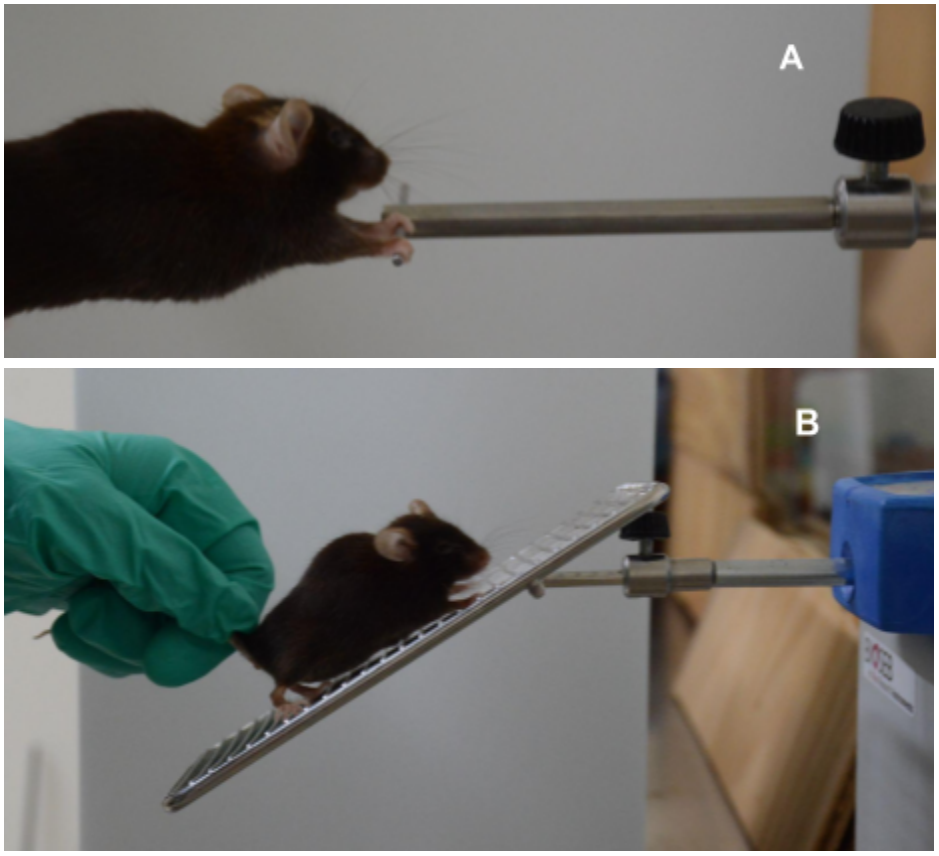

Figure 4. (**A**) Forelimb grip test, (**B**) all limb grip test

c. *Statistical analysis*

Statistical analyses were completed using Microsoft Excel version 16.56 and R-Studio version 2023.03.0+386. All plots were made on Excel as well as calculations of averages and standard error (Table 3). R-Studio was utilized to run one, two, and three-way ANOVAs to assess for variance between the means of each group as well as the interaction effects between the three independent variables: age, genotype, and sex. ANOVA tests were run for body mass, all limb force, all limb force standardized by body mass, and forelimb force standardized by body mass.

For all limb force, data on young WT females was excluded and a two-way ANOVA was run as this was the only group for which WT data existed as no data on all limb force was collected for old WT females and this grip test was not run for males of any age category by Ge,

et al., 2016. Tukey Honestly Significant Difference (HSD) full factorial analysis was run to investigate specifically which groups were significantly different from one another as broadly revealed by earlier ANOVA testing. Tukey HSD tests were run for all limb force standardized by body mass and forelimb force standardized by body mass, only three-way interactions were reported on for significant ANOVA findings. The data were tested for normality using the Shapiro-Wilks test. The test indicated a departure from normality ($p < 0.05$), suggesting that the assumption of normality was not met and the null hypothesis of normality was rejected. However, the data were not transformed as the ANOVA was deemed sufficiently robust.

Table 2: Breakdown of Study Subjects

Age Category	Genotype	Sex	Identity*	Number Indicated	Body Mass (g)	Age (days)
Young	Ttn$\Delta^{112\text{-}158}$	Male	YTM	3	27.6±0.6	79.0±3.0
Young	Ttn$\Delta^{112\text{-}158}$	Female	YTF	19	21.2±0.3	139.6±3.5
Old	Ttn$\Delta^{112\text{-}158}$	Male	OTM	10	29.4±0.5	718.6±11.4
Old	Ttn$\Delta^{112\text{-}158}$	Female	OTF	9	24.3±1.0	807.4±10.1
Young	WT	Male	YWM	20	31.8±0.5	120.0±0.0
Young	WT	Female	YWF	20	22.3±0.4	118.0±0.0
Old	WT	Male	OWM	47	34.3±0.8	737.9±17.5
Old	WT	Female	OWF	0	NA	NA

*Identity is abbreviated by age, genotype, and sex

Results

a. Quantitative values with normalized data and associated standard error

Table 3: Summary of Results

Identity*	Body Mass (g)	Forelimb Force (g)	Forelimb Force (g)/Body Mass (g)	All Limb Force (g)	All Limb Force (g)/Body Mass (g)
YTM	27.6±0.6	125.72±8.9	4.56±0.38	234.45±26.97	8.45±0.79
YTF	21.2±0.3	112.81±5.2	5.31±0.23	158.49±9.06	7.51±0.46
OTM	29.4±0.5	83.30±3.6	2.84±0.13	194.68±1.28	6.64±0.12
OTF	24.3±1.0	83.30±3.0	3.45±0.13	196.01±1.14	8.18±0.32
YWM	31.8±0.5	350.99±11.3	11.10±0.39	NA	NA
YWF	22.3±0.4	136.36±3.4	6.13±0.17	201.07±3.86	9.05±0.22
OWM	34.3±0.8	298.99±6.5	8.81±0.25	NA	NA
OWF	NA	NA	NA	NA	NA

b. Body mass

Mice of different age, sex, and genotype show significantly different body masses (Figure 5). Young mice have lower body mass as compared to old mice (ANOVA, $F = 9.55$, $df = 1$, $p < 0.0001$). Male mice have higher body mass as compared to females (ANOVA, $F = 254.18$, $df = 1$, $p = 0.0025$). WT mice have greater body mass compared to $Ttn\Delta^{112\text{-}158}$ mice (ANOVA, $F = 13.31$, $df = 1$, $p < 0.001$). The interaction effect between genotype and sex is also significant (Table 4, ANOVA, $F = 5.36$, $df = 1$, $p = 0.0223$). The difference between male and female WT mice is larger than in $Ttn\Delta^{112\text{-}158}$ mice. Subsequent Tukey post hoc pairwise testing did not yield significant findings.

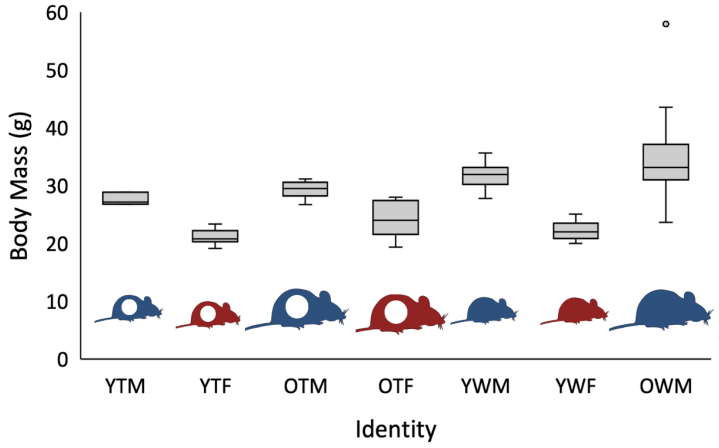

Figure 5. Body mass by age, genotype, and sex. Error bars represent standard error

Table 4: Three-way ANOVA: Body Mass (g)

Variables	df	F	p-value
Sex	1	254.18	< 2e-16***
Age	1	9.55	0.002490**
Genotype	1	13.31	0.000393***
Genotype:Sex	1	5.36	0.022305*
Genotype:Age	1	0.10	0.747319
Age:Sex	1	1.03	0.312470
Genotype:Age:Sex	3	2.17	0.0958
Meanings of ** significance: p<0.05 *, p<0.01 **, p<0.001 ***			

c. All limb force

In Ttn$\Delta^{112\text{-}158}$ mice, all limb force differs significantly by sex, but not by age (Figure 6). Males demonstrate significantly more all limb force as compared with females (ANOVA, F = 11.15, df = 1, p = 0.00193). The interaction effect between age and sex was also significant with all limb force decreasing with age, particularly in males (Table 5, ANOVA, F = 11.33, df = 1, p = 0.00179).

Standardized all limb force data shows a significant interaction effect between age and sex (Table 6, Figure 7, ANOVA, F = 4.47, df = 1, p = 0.0414).

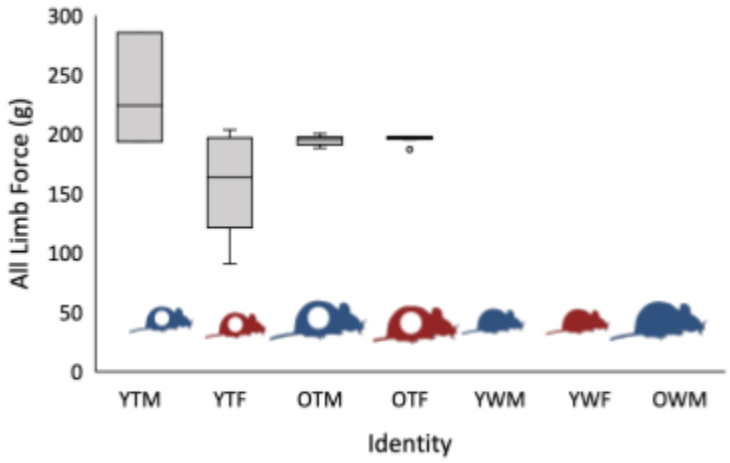

Figure 6. All limb force (g) by age and sex. Error bars represent standard error.

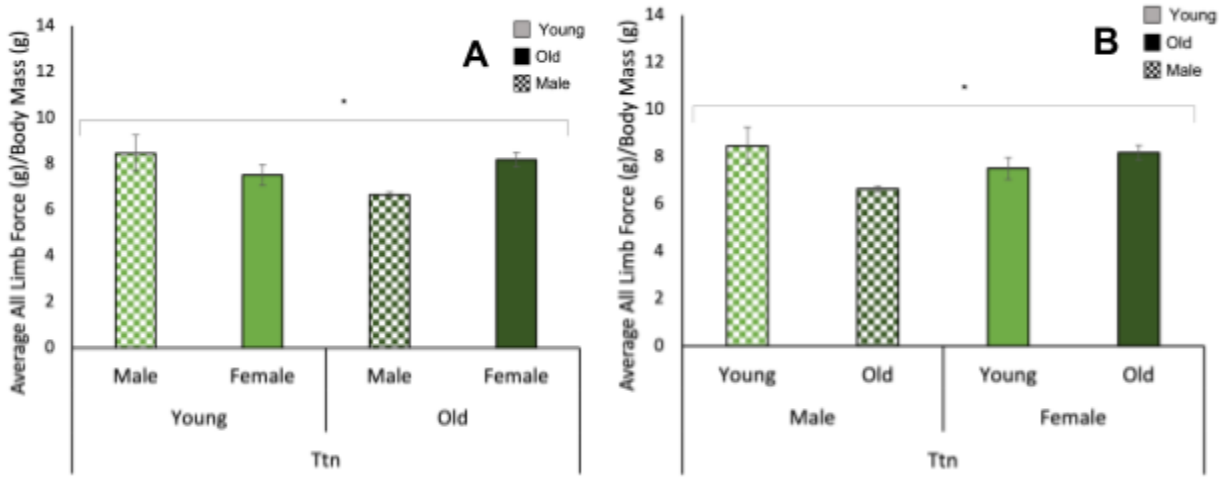

Figure 7. Standardized all limb force by age and sex. Data for **A** and **B** are the same and reorganized for visualization. * indicates p<0.05

Table 5: Two-way ANOVA: All Limb Force (g)

Variables	df	F	p-value
Age	1	2.54	0.11962
Sex	1	11.15	0.00193**
Age:Sex	1	11.33	0.00179**

Table 6: Two-way ANOVA: All Limb Force (g)/Body Mass (g)

Variables	df	F	p-value
Age	1	0.00	0.9827
Sex	1	1.69	0.2012
Age:Sex	1	4.47	0.0414*

d. Forelimb force

Forelimb force is significant for sex, age, and genotype and all interactive effects except age by genotype. WT males, both young and old, display the greatest forelimb force overall. WT mice are much stronger than Ttn$\Delta^{112\text{-}158}$ mice (Figure 8).

Forelimb force standardized by body mass data shows significant differences across sex, age, and genotype (Table 7). Ttn$\Delta^{112\text{-}158}$ females demonstrate more standardized forelimb force than males, whereas the opposite finding was observed in WT mice (ANOVA, $F = 171.88$, $df = 1$, $p < 0.0001$). Young mice demonstrate more standardized forelimb force than old mice (ANOVA, $F = 87.91$, $df = 1$, $p < 0.0001$). WT mice demonstrate more standardized forelimb force than Ttn mice (ANOVA, $F = 146.56$, $df = 1$, $p < 0.0001$).

A full factorial analysis reveals significant findings for all effects except age by genotype (Table 7). Both males and females demonstrate significantly decreased standardized forelimb force with age (Figure 9B, ANOVA, $F = 10.74$, $df = 1$, $p = 0.00139$). A 60.4% decrease in strength was observed in Ttn$\Delta^{112\text{-}158}$ males with age, while this decline was 53.7% in Ttn$\Delta^{112\text{-}158}$

females and 25.9% in WT males. Subsequent post hoc analysis finds significant differences between young male and female mice as well as old males and females (Figure 9A). The average standardized forelimb force of males and females of either age was significant with respect to genotype (Figure 9C, ANOVA, $F = 85.35$, $df = 1$, $p < 0.0001$). Subsequent post hoc analysis reveals that male WT mice have greater standardized forelimb force, while females demonstrate greater standardized forelimb force as compared with males in $Ttn\Delta^{112\text{-}158}$ mice (Figure 9C). Young female $Ttn\Delta^{112\text{-}158}$ mice were 14.1% stronger than young males. In WT mice, however, young WT males were 44.7% stronger than young WT females. The three-way interactive effect of genotype, age, and sex is also highly significant (Table 7, ANOVA, $F = 32.16$, $df = 1$, $p < 0.0001$).

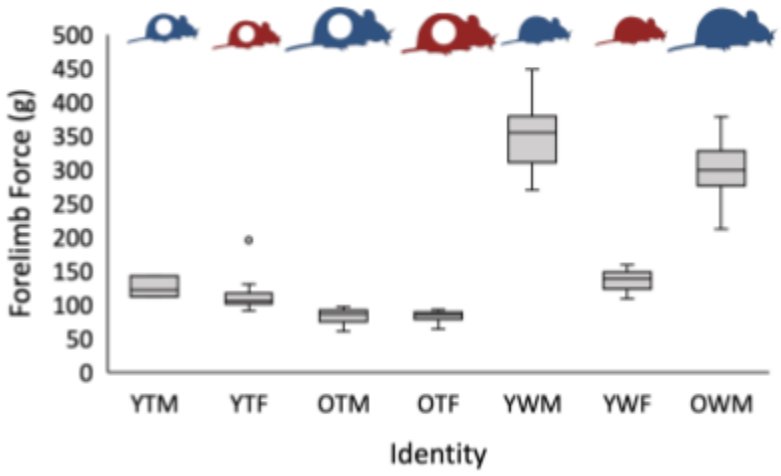

Figure 8. Forelimb force (g) by age, genotype, and sex. Error bars represent standard error

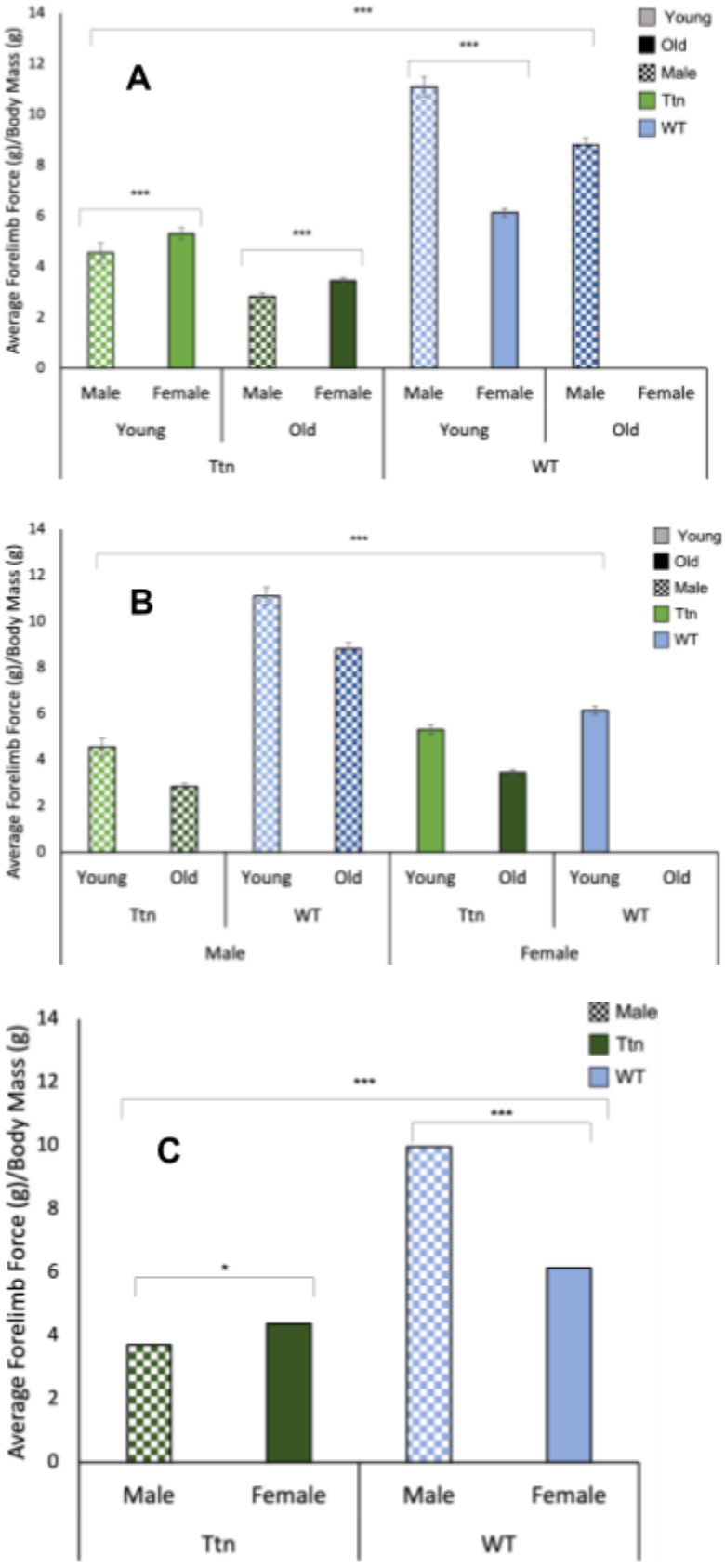

Figure 9. Standardized forelimb force by genotype, age, and sex. Data for **A** and **B** are the same and reorganized for visualization. (**C**) Standardized forelimb force by genotype and sex, values averaged for young and old mice

Table 7: Three-way ANOVA: Forelimb Force (g)/Body Mass (g)

Variables	df	F	p-value
Sex	1	171.88	<2e-16***
Age	1	87.91	7.90e-16***
Genotype	1	146.56	<2e-16***
Sex:Age	1	10.74	0.00139**
Sex:Genotype	1	85.35	1.65e-15***
Age:Genotype	1	0.38	0.5375
Genotype:Age:Sex	3	32.16	3.89e-15***

Discussion

In this study, we hoped to gain an improved understanding of the function of the titin protein by investigating the effects of aging and sex by comparing measurements of peak grip strength in mice. In line with our predictions, we found that strength declined universally with age and that WT mice outperformed Ttn$\Delta^{112\text{-}158}$ mice. Surprisingly, our results showed that female Ttn$\Delta^{112\text{-}158}$ mice were stronger than their male counterparts in the forelimb force test, whereas the opposite has repeatedly been revealed in WT mice (Hill et al., 2020).

In accordance with Ge et al. (2016), we found that body mass increases with age and grip strength decreases (Figure 10). While forelimb grip declined with age, lean muscle mass stayed constant. Therefore, they posited that in-*vivo*, pain associated with exostosis–which occurred in the carpus and digits in over 50% of older mice–could be one possible explanation for a decline in grip strength without a corresponding loss of muscle mass (Ge et al., 2016). However, Ge et al. (2016) exclusively investigated male WT mice. We can now add that Ttn$\Delta^{112\text{-}158}$ males were larger than females and that WT mice were larger than Ttn$\Delta^{112\text{-}158}$ mice.

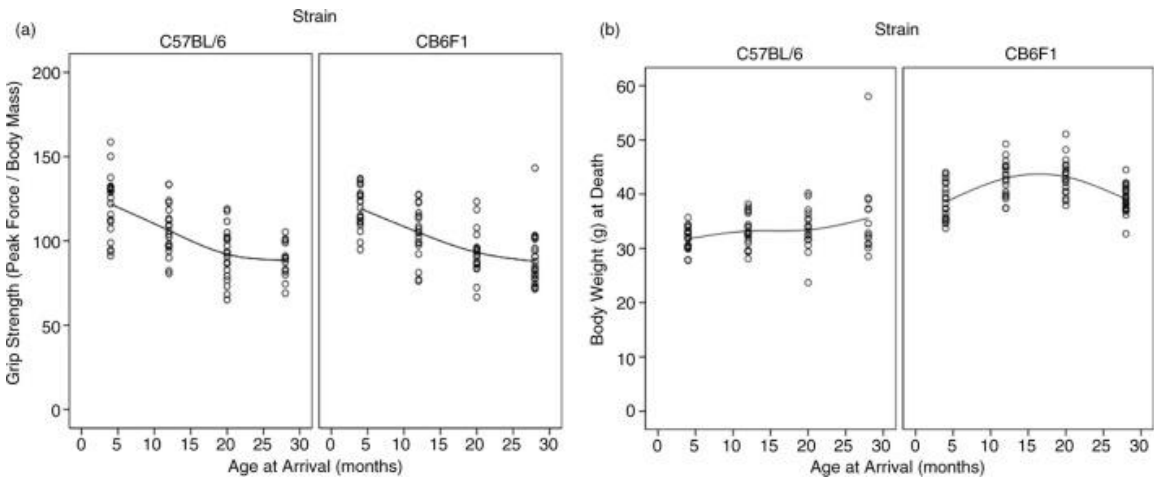

Figure 10. (**A**) C57BL/6 (WT) peak forelimb grip strength change with age. (**B**) C57BL/6 (WT) body mass change with age (Ge et al., 2016)

In the standardized all limb force test, Ttn$\Delta^{112\text{-}158}$ males declined in strength with age to a greater degree than females with a significant age and sex interaction observed. This sex-based difference has a number of possible explanations including body mass composition differences between males and females and muscle quality changes with age (Hill et al., 2020). The causes of these sex differences with aging may be due to some combination of differences in muscle fiber type composition or hormones, both of which differ between the sexes and change with age. Unfortunately, little research has been done which includes female mice, and much of that research neglected to statistically compare the sexes or use a wide enough age range, let alone within the Ttn$\Delta^{112\text{-}158}$ model or on upper extremities. Based on the results of the present study, muscle performance varies significantly between males and females. Therefore, studies exclusively considering males likely will not be relevant for females. Further, Hill et al. (2020) defined “old” mice as those at 78 weeks of age which Ge et al. (2016) found to be insufficient as age-related decline was observed starting around 80 weeks with an entire cohort surviving until 112 weeks. Halting a study at approximately 70% of lifespan does not adequately consider the effects of advanced age.

Brynnel et al. (2020), for example, did use females in a select few of their experiments, considered aging for mice 16-78 weeks, and used both Ttn$\Delta^{112\text{-}158}$ and WT mice. However, 78 weeks may not be old enough to observe females outperforming males (Ge et al., 2016). Additionally, they tested the diaphragm, plantaris, EDL, quadriceps, soleus, and gastrocnemius. Barring the diaphragm, all of those muscle groups are involved in the function of the lower limbs while the most statistically significant results of the present study were uncovered in the forelimb grip test. Brynnel et al. (2018) also used muscle weight (mg) normalized to tibia length (mm) whereas the present study considered peak forge (g) normalized to body mass (g). Finally, while both males and females were used in the Brynnel study, no statistical tests were run between the two groups. There currently exist two problems in the literature regarding the study of sex differences: the first is that including females at all in studies is surprisingly rare despite clear evidence that males and females are not the same and the second is that when females are included, the data is often split by sex with no statistical tests being run between the two groups meaning that no conclusions can be drawn with regards to sex differences (Garcia-Sifuentes and Maney, 2021). Despite the aforementioned drawbacks of the Brynnel paper, it is the only other paper to use both males and females of different ages with both Ttn$\Delta^{112\text{-}158}$ and WT mice, making their findings invaluable to the present study.

Brynnel et al. (2018) found that the males of either genotype generally outperformed the females (Figure 11). These findings are consistent with the present study which found that for the standardized all limb grip strength of young Ttn$\Delta^{112\text{-}158}$ mice, males outperform females (Figure 7). However, in older Ttn$\Delta^{112\text{-}158}$ mice, females demonstrated greater standardized all limb force than their male counterparts (Figure 7). Pairwise statistics revealed that all observed differences were sex-driven, underscoring the importance of including females in research (Table 5 and 6). In normalized forelimb force, young female Ttn$\Delta^{112\text{-}158}$ mice were 14.1% stronger than young males while in WT mice young males were 44.7% stronger than young females;

males and females are not the same, excluding either group from this study would have failed to uncover this significant difference.

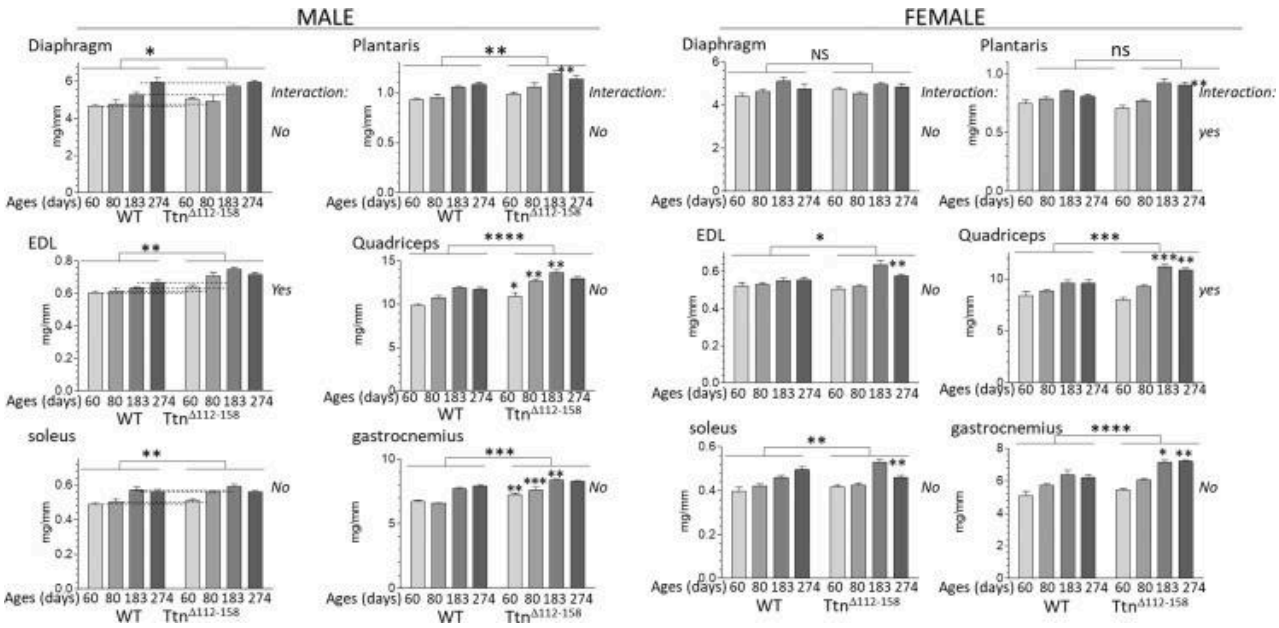

Figure 11. WT and Ttn$\Delta^{112\text{-}158}$ mice are hypertrophied, expressed as muscle weight (mg) normalized to tibia length (mm). Most muscle groups do not have a significant age effect (Brynnel et al., 2018)

For the standardized forelimb grip strength test, both Ttn$\Delta^{112\text{-}158}$ and WT mice demonstrated decreased force with age with a greater percent difference observed between mice of different sex and genotype. This finding of decreasing force with age is consistent with findings in WT mice as conducted by both Ge et al. (2016) and Hill et al. (2020). Interestingly, male WT mice were much stronger than their female counterparts, whereas the opposite was found to be true in young Ttn$\Delta^{112\text{-}158}$ mice, aligning with the findings of Brynnel et al. (2018) for standardized all limb force. Given that both the present study and the Brynnel paper uncovered this phenomenon, titin, in its role as a molecular spring, is likely responsible for these observations. With the majority of the PEVK region of titin deleted, the spring is greatly stiffened. While the exact mechanism through which this deletion impacts the phenotype of the mice is not

yet fully understood, it is clear that it hampers muscle performance, particularly in male mice who have, for some reason, poorly adjusted to their stiff titin. The WT mice were stronger than the Ttn$\Delta^{112\text{-}158}$ mice for both sex and age. However, there were no old WT female mice and both young and old WT male data was gathered from Ge et al. (2016), potentially a source of error as a different lab involved differences between experimenters, techniques, equipment, diet for the mice, and more.

The standardized forelimb grip strength data suggests that for some reason, male Ttn$\Delta^{112\text{-}158}$ mice are suffering more from the mutation than the females. Possible causes of this include the increased number of sarcomeres in Ttn$\Delta^{112\text{-}158}$ mice and, therefore, more impactful effects of sarcopenia, the role of titin as a signaling molecule, and hormones–particularly estrogen (Lin et al., 2018; Haizlip et al., 2015). Ttn$\Delta^{112\text{-}158}$ mice have been found to have a 30% increase in the number of sarcomeres in their muscles (Brynnel et al., 2018). This longitudinal hypertrophy may be compensatory as Ttn$\Delta^{112\text{-}158}$ mice have stiff titin, limiting its ability to stretch (Brynnel et al., 2018). This increase in sarcomere number and age-related sarcopenia may result in a kind of multiplicative loss, increasing the rate of loss of strength in Ttn$\Delta^{112\text{-}158}$ mice as they age. This may in part account for the 60.4% decrease with age in forelimb grip strength observed in male Ttn$\Delta^{112\text{-}158}$ mice as opposed to the 25.9% decrease in the same group of WT mice.

In addition to its role in modulating skeletal muscle stiffness mechanically as a molecular spring, titin also acts as a mechanosensor within the sarcomeric A band which is possibly responsible for the length-dependent activation of muscle (Freundt and Linke, 2019). This makes titin not just a passive element acting as a spring, but an active mechanosensor and modulator within skeletal muscle. A 2018 study found that muscle hypertrophy scaled with titin stiffness suggesting that titin acts as a mechanosensor which regulates muscle trophicity (van der Pijl et al., 2018). Titin is a signaling hub, the Ttn$\Delta^{112\text{-}158}$ mutation stiffened the muscles, resulting in hypertrophy. Perhaps, within the 47 exons deleted in this model, there existed a

receptor for a hormone predominantly present in males that impaired the function of the protein in males more so than in females.

A study investigating the exercise capacity of WT mice found that female mice have enhanced exercise capacity which they largely attributed to estrogen. Following ovariectomy, female mice no longer demonstrated enhanced running endurance. Similarly, supplementing males with estrogen resulted in exercise capacity which reflected that of intact females (Oydanich et al., 2019). Future studies may wish to measure estrogen levels in male and female Ttn$\Delta^{112\text{-}158}$ and WT mice to investigate the role of estrogen in the puzzling observed sex differences among Ttn$\Delta^{112\text{-}158}$ mice. However, the Oydanich study used a test which measured endurance, not peak strength, traits which are known to differ between the sexes. Further, mice in this study were also very young and of varying ages with males pre peak sexual maturity and females post peak sexual maturity. In the present study, only mice over 10 weeks were used as this is the approximate age at which they reach sexual maturity. At the latter end of sexual maturity is what would be considered human perimenopause, occurring around 9 months of age in WT mice. This is followed by reproductive senescence between 9 to 12 months of age, during which time they experience steroid hormone fluctuations (Brinton, 2012). Additionally, while the findings from Oydanich et al. (2019) may help to explain sex differences, they do not fully elucidate why male Ttn$\Delta^{112\text{-}158}$ mice are so much more affected by the mutation than the females that the strength trends flip for this genotype as opposed to the WT. It is also possible that type IIB fibers, which males have a greater proportion of, are more affected by the Ttn$\Delta^{112\text{-}158}$ mutation, although confirming this would require further research.

A variety of cardiomyopathies and neuromuscular diseases are known to affect humans as a result of titin mutations. Using new next generation sequencing methods, many diseases have recently been attributed to titin mutations (Savarese et al., 2016). However, further work will need to be done to uncover mutations specific to the PEVK region of titin (Figure 12).

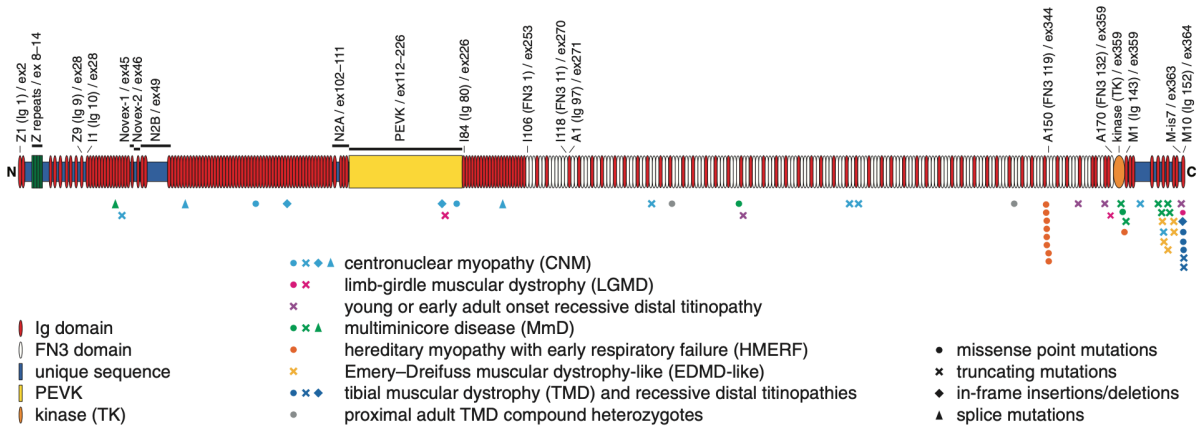

Figure 12. Mutations to the protein titin causing skeletal muscle disease (Savarese et al., 2016)

Dilated cardiomyopathy is the most common disease attributed to a titin mutation with a prevalence of up to ~1:250. This disease results in dilation of the left ventricle of the heart, leading to systolic dysfunction (Kellermayer et al., 2019). Congenital titinopathy causes early onset congenital contractures, or stiffness, consistent with what was observed in our titin mutants. Additionally, MRIs revealed that patients with congenital titinopathy had gluteal, hamstring, and calf muscle involvement–interestingly all lower extremities–in addition to cardiac involvement and respiratory insufficiency as a result of weak diaphragm (Oates et al., 2018). Udd distal myopathy – tibial muscular dystrophy (UDM-TMD) is characterized by weakness in the lower extremities, specifically minimizing ankle dorsiflexion. UDM-TMD is a progressive disease with dystrophic changes eventually leading to the replacement of the tibialis anterior muscle with adipose tissue (Udd and Hackman, 2005). Understanding sex differences and age-related progression of diseases caused by titin mutations is crucial for the patients, and their families, who struggle with these diseases.

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