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A Comparison of Muscle Function Between Female and Male Mice With a Deletion to the PEVK Region of Titin

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A Comparison of Muscle Function Between Female and Male Mice with a Deletion to the PEVK Region of Titin

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Abstract

Titin, the third most abundant filament in muscle, serves many roles. It underlies muscle stiffness and functions as a signaling hub particularly during muscular growth. The PEVK region of titin contributes to muscle stiffness and is suspected to play a role in hypertrophic signaling. Most skeletal muscle studies have only used males, leaving a need to include females and investigate muscle physiology sex differences. To investigate sex differences in these functions, soleus muscles from mice with a deletion to the PEVK region ($Ttn^{\Delta 112\text{-}158}$) and wild type (WT) mice around 100 days old were extracted and attached to an apparatus that collected force, muscle length, and time under twitch and tetanus conditions. The contractile properties under twitch conditions found a significant effect on the interaction effect between the genotype and sex for the rate of force development and half relaxation time. This study also found that female $Ttn^{\Delta 112\text{-}158}$ had greater passive stress under twitch conditions, indicating sex differences in how the PEVK region modulates passive stiffness. Greater passive stiffness could inhibit cyclic movements and affect performance.

Introduction

Skeletal muscles are the motors, brakes, springs, and postural support of our bodies that are highly adaptive to different environments. Because skeletal muscle is versatile and plastic, it is highly specialized at a species, individual, and specific muscle level (Anderson & Roberts, 2019). This diversification allows for animals to succeed in their environment, and for humans, opens the door for incredible athletic performance (Dickinson et al., 2000). Muscles can be specialized to have fast contractile and relaxation properties: the fastest 100m sprint is held by Usain Bolt at 9.58 seconds with the fastest women's time held by Florence Griffith-Joyner at 10.49 seconds. Muscles can also be trained to be extremely fatigue resistant: the top men's marathon time, which was recently achieved this October, is held by Kelvin Kiptum at 2:00:35 and the women's time at 2:11:53 set by Tigst Asseffa (*Stats Zone, All Time Top Lists, Senior Outdoor*, 2023). However, as the distance of races increase, the differences between males and females decrease (Le Mat et al., 2023). In a study evaluating ultra-running events, distances longer than 195 miles, over the last 23 years, female ultra-runners were found to run faster than male ultra-runners (Ronto & Vania, 2023). In ultra-distance swimming, like the Catalina Channel Swim or Manhattan Island Marathon Swim, the average woman is faster than the average man (Melissa Dahl, 2016). The same trend has been observed in mice; female mice run longer, on average, than male mice on running wheels (Landen et al., 2023). When it comes to research on athletic performance and muscle function, many studies only include males and disregard sex differences (Costello et al., 2014). In both human and mice studies, males have been found to have a higher body mass, muscle mass, cross sectional area, and maximum power as compared with females, while female muscles typically recover faster and are more fatigue

resistant (Glenmark et al., 2004a; Landen et al., 2023). These findings suggest there may be important sex differences in muscle physiology. There are known sex differences in energy metabolism and fiber-type composition (Glenmark et al., 2004b). Different fiber-type compositions are caused by different myosin heavy chain (MyHC) isoforms, which are indicators of how fast the muscle can contract and relax and its fatigue resistance. Female muscles typically have more type I fibers which are slow and fatigue resistant and rely on oxidative metabolism, while males typically have more type II fibers, which have fast contractile properties and rely on glycolysis (Landen et al., 2023).

This study evaluates sex differences in hypertrophic signaling and passive muscle stiffness is the giant protein titin. Titin is the third most abundant filament in muscle and serves many roles. It underlies passive muscle stiffness and functions as a signaling hub particularly during muscular growth (Krüger & Kötter, 2016). The differences in muscle function are partly due to differences in protein interactions inside the sarcomere, which affect hypertrophic signaling and passive muscle stiffness (Nishikawa et al., 2020). Our hypothesis is that there are sex differences in titin's hypertrophic signaling and in the modulation of passive muscle stiffness.

Muscle Physiology

Skeletal muscles are organized into fascicles, muscle fibers, myofibrils, and sarcomeres. Fascicles contain muscle fibers which are innervated by motor neurons and contain myofibrils. Myofibrils are made up of sarcomeres, the contractile unit of skeletal muscles and the main area of interest for this study (McCuller et al., 2023) (Figure 1a).

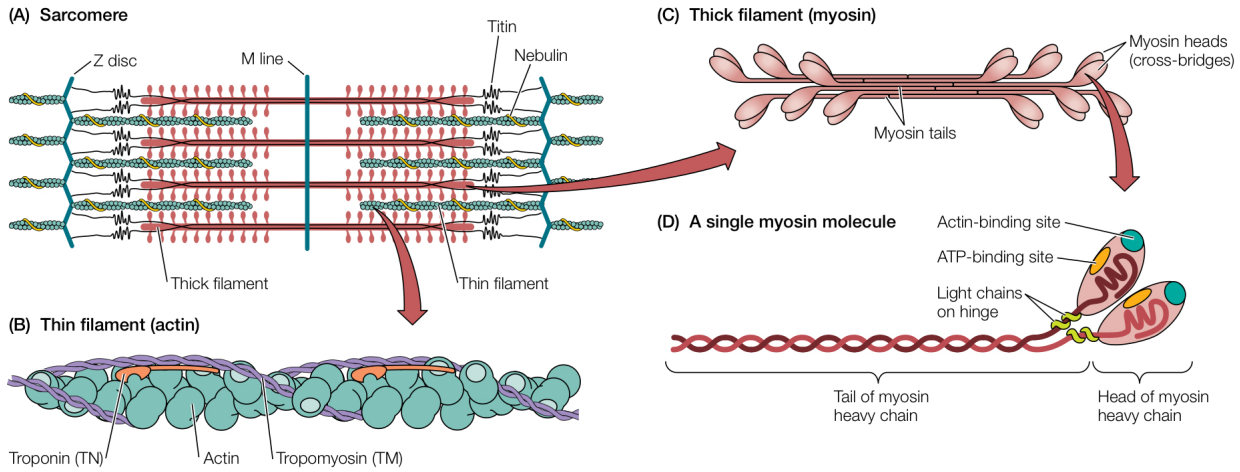


Figure 1. Structure of a sarcomere in striated muscle (R. W. Hill et al., 2016).

Sarcomeres contain three myofilaments; thin, thick, and titin (Figure 1a,b,c). These filaments work together to produce force and movement. After an action potential propagates down a motor neuron and synapses onto a muscle cell, the sarcolemma, or membrane of the muscle cell, depolarizes, causing a conformational change which allows for the release of calcium from the sarcoplasmic reticulum into the intercellular space of the muscle cell (R. W. Hill et al., 2016). Calcium binds to troponin, causing tropomyosin to move, opening up a binding site for a myosin head to bind to actin, the thin filament (Figure 1b). This binding between myosin heads and actin create cross-bridges, which are essential for active force production. Myosin heads release phosphate, initiating the powerstroke, thereby pulling actin towards the middle of the sarcomere (Figure 1c,d).

Within the sarcomere, cross-bridges undergo binding/unbinding cycles where myosin heads work asynchronously to maintain their force (R. W. Hill et al., 2016). The more cross-bridges that are formed, the more force is produced (Brynnel et al., 2018). In order for muscle

cells to relax, Sarcoplasmic Reticulum Calcium ATPase (SERCA) pumps reuptake calcium from the intracellular space back into the sarcoplasmic reticulum (R. W. Hill et al., 2016).

Muscle Properties

There are over 3,000 genes that are expressed differently in male and female skeletal muscle (Haizlip et al., 2015). There is more work to be done to see how these differences in gene expression directly impact the overall performance of an individual; however, we know that these differences influence ratios of muscle fiber-type compositions and circulating hormone levels. Starting with gene expression and muscle fiber-types, it has been shown that myosin heavy chain (MyHC) expression determines fiber-type compositions (Haizlip et al., 2015). Skeletal muscle fiber type composition is influenced by sex, species, and the muscle's location and function. There are four genes that are responsible for the majority of MyHC expression in mammalian skeletal muscles. These genes encode the four main types of skeletal muscle fiber types; I, IIA, IIB, and IIX (Haizlip et al., 2015). It is important to note that rodent skeletal muscles have a high ratio of type IIB muscle fibers, while humans do not make this fiber type (Haizlip et al., 2015). Studies evaluating muscle fiber composition found that female muscles typically have a higher ratio of type I fibers and males have a higher ratio of IIA fibers (Haizlip et al., 2015). A study looking at human MyHC gene expression in male and female vastus lateralis muscles found that the average percentage of MyHC isoforms in females were 41% of type I, 36% of IIA, and 23% of type IIX, while the average male muscles were 34% of I, 46% of IIA, and 20% of type IIX (Haizlip et al., 2015).

Fiber-types can give insight into contractile velocity, fatigability, the favored form of metabolism, and in what type of activities that muscle may excel. This variation of muscle fiber-

types is created by a multitude of factors including the number of mitochondria, number of SERCA pumps, and the types of metabolism the muscle cell favors. Type I muscle fibers are known to have slow contractile velocities but are more fatigue resistant due to their use of oxidative phosphorylation more than type II fiber types. While type I fibers typically have better endurance, type II fibers are faster at contracting and relaxing. Type IIA fibers are slower than IIB and IIX fibers. In terms of endurance, IIA fibers outperform IIB and IIX. Type IIB fibers have a fast fatigue rate because the muscle fiber relies on anaerobic glycolysis to create ATP (McCuller et al., 2023).

Information that is often measured to understand muscle kinetics and performance include shortening velocity, rate of force development, ½ relaxation time, and force at different points in time during the muscle contraction such as passive force, peak force, and active force (Figure 6). This data is typically collected while the muscle undergoes an isometric contraction, meaning that the muscle does not change length during the contraction, because the force a muscle produces is influenced by muscle length. For these studies, muscles are also typically connected to an electrode which can stimulate twitch or tetanic contractions. A twitch is a response to a single stimulus, while a tetanic contraction has a series of action potentials close together where the muscle does not fully relax before being stimulated again, causing an increase in force. The force increases through stimulation at a high frequency up to a certain threshold before it plateaus and the muscle fatigues or relaxes (R. W. Hill et al., 2016).

Connecting to overall performance, female muscles tend to be more fatigue resistant and male muscles tend to reach their maximum force faster and relax faster (Glenmark et al., 2004a). There is no significant difference in maximum force production between male and female

muscles (Haizlip et al., 2015). In a study evaluating human adductor pollicis muscle performance, they found that females had a smaller decline in force generating capacity after a minute of exercise, longer endurance time to exhaustion, and faster early recovery force than males after voluntary static contraction to muscle exhaustion (Fulco et al., 1999).

Hormones

Sex steroid hormones, especially testosterone, estrogen, and progesterone, contribute to skeletal muscle development and contractility. In both humans and mice, males typically have more muscle mass and therefore a larger cross-sectional area (CSA). Muscle mass and CSA is directly proportional to muscle force, so muscles with more mass and larger CSAs will produce more force. Hormones that play a role in skeletal muscle protein remodeling include testosterone, growth hormone, insulin, insulin-like growth factor-I and glucocorticoids. After puberty, males exceed the amount of circulating testosterone in females at any age 15 times over (Handelsman et al., 2018). Testosterone, a type of androgen, contributes toward anabolic processes and stimulates muscle growth (Figure 2).

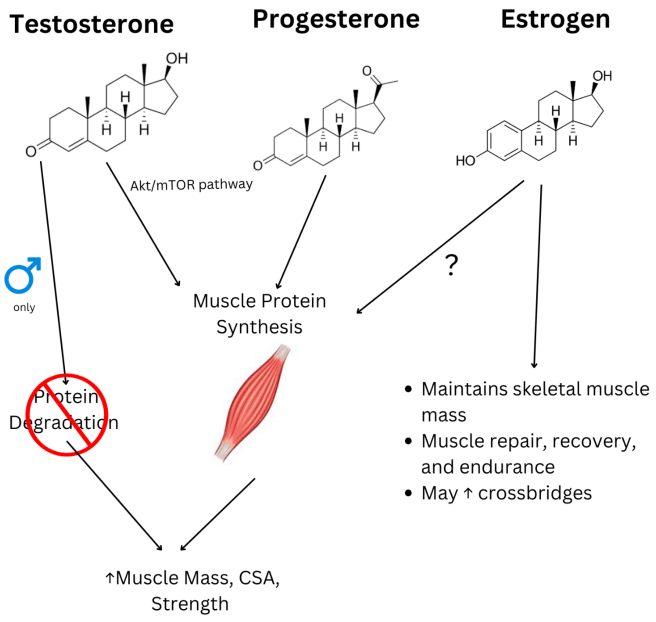


Figure 2. Summary of Sex Steroids Effects on Skeletal Muscle

Deficiency in androgens increases muscle protein breakdown and decreases muscle protein synthesis, which can cause a decrease in strength and muscle mass (Sheffieldmoore & Urban, 2004). There is a dose-response relationship of testosterone on muscle mass and strength (Handelsman et al., 2018). In both males and females, testosterone declines with age but it does not affect muscle mass in females as much as it does in males (Sheffieldmoore & Urban, 2004). Progesterone is also associated with muscle mass regulation and protein synthesis (Alexander et al., 2022; Landen et al., 2023)(Figure 2).

Estrogen plays a role in muscle repair and recovery (Figure 2). Estrogen receptors may interact and affect fiber-type composition by modulating the expression of estrogen-sensitive genes. There are two estrogen receptors (ER) that are expressed in skeletal muscle, ERα and ERβ. Mice without ERα had a decrease in the regenerative capacity of their muscles. Following

injury, male mice had higher levels of creatine kinase, a marker for injury, than the female mice (Haizlip et al., 2015). Estrogen also affects muscle size. Animal models with low estrogen levels showed an increase of body mass and muscle mass. Mice that underwent ovariectomies had an increase in fiber diameter (Haizlip et al., 2015). Another study using the ERβ null mice model was used to evaluate sex differences in force production, contraction time and relaxation time in the soleus. The female wild type mice and the ERβ null female mice had higher force production than both male groups. Contraction time and relaxation time were faster in both the male control and experimental groups than the female groups (Haizlip et al., 2015). Estrogen seems to have a role in muscle contractility (Alexander et al., 2022). Future studies should investigate interactions between sex steroids and titin.

Titin

Titin is the largest protein in the body, made up of over 33,000 amino acids, and is the third most abundant filament in muscles following actin and myosin (Bucher et al., 2010). It is located in the sarcomere with one end attached to the Z-disk and the other to the M-line, spanning half of the sarcomere (Figure 3) (Nishikawa, 2020). Titin has many roles including its function as a molecular spring, a structural scaffolding for the sarcomere, a molecular signaling hub, and as a mediator of protein quality control. Considering the size and many functions of titin, it is not well studied, especially in skeletal muscles.

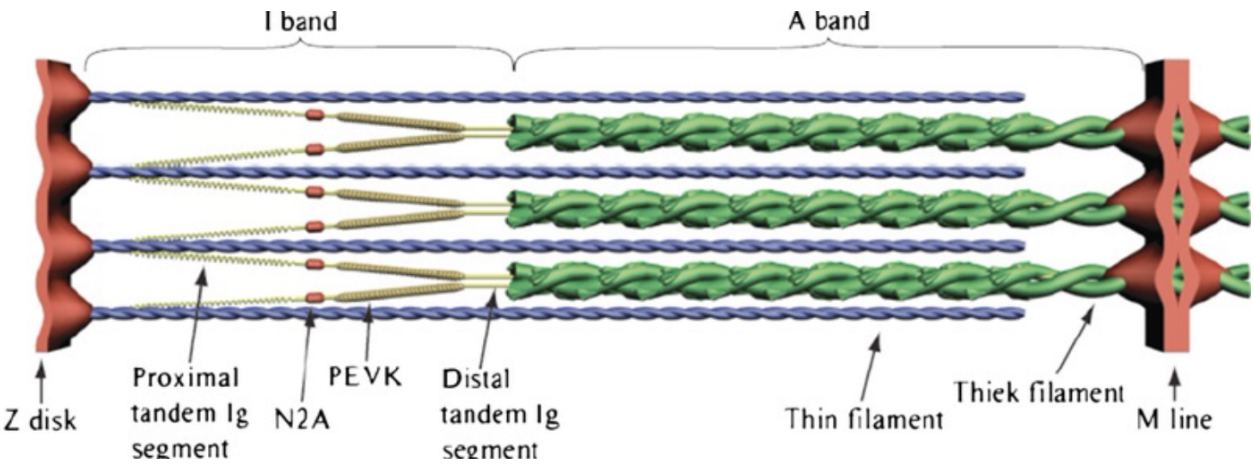


Figure 3. Half sarcomere demonstrating titin segments (Monroy et al., 2012)

The region of titin that this paper will mainly focus on resides in the I-band of the sarcomere where titin stiffness is mainly modulated. This region is what contributes most to the elastic properties of muscle and includes the tandem Ig domains, the N2A region, and most importantly for this study, the PEVK region (Figure 3). The Ig domains are compliant and straighten at low forces, while the PEVK segment is much stiffer and stretches under higher forces (Nishikawa, 2020); (Linke & Hamdani, 2014). Calcium dependent binding of the N2A region to actin increases active stiffness and decreases optimal length by limiting the stretch of the proximal Ig segment. This means that the PEVK region of titin stretches more during active stretch (Nishikawa, 2020). The PEVK region also binds to actin in the presence of calcium and increases active stiffness. There is still a need for more research about titin's role in active force and history-dependent features of muscle force production (Hahn et al., 2023).

Phosphorylation of the PEVK region can also increase passive stiffness. Calcium /calmodulin-dependent protein kinase-IIδ (CaMKIIδ) and calcium dependent protein kinase Cα (PKCα) phosphorylate the PEVK region. PKCα decreases the length of titin, increasing stiffness (Krüger & Kötter, 2016). Exercise has been shown to increase phosphorylation and the ratio of

titin molecules to myosin heavy chains, which further increases stiffness (Krüger & Kötter, 2016).

Titin not only interacts and binds with CaMKIIδ and PKCα–which modulates titin stiffness–but also associates with over 25 proteins that are involved in processes such as protein quality control and hypertrophic signaling (Linke & Hamdani, 2014). The N2A region, which is made up of four Ig domains, has been shown to be involved in hypertrophic signaling in the same mouse model this study uses, which has a portion of the N2A region deleted (Nishikawa et al., 2020). When the N2A region binds to actin in the presence of calcium, mechanical properties and hypertrophic signaling are affected (Nishikawa et al., 2020). MARP1 and MARP2 are regulators of hypertrophic signaling. MARP2 predominantly binds to titin and interferes with PKCα. An upregulation of MARP and hypertrophic signaling is also associated with increased passive stiffness (Nishikawa et al., 2020). There are also different isoforms in the PEVK region that are different lengths and therefore have different stiffness (Nishikawa et al., 2020). There is an interaction between regulating passive stiffness and hypertrophic signaling.

The PEVK segment is named after, and rich in, proline (P), glutamate (E), valine (V), and lysine (K). To further study the PEVK region and its effect on titin stiffness, a transgenic mice strain with a deletion of 47 exons, exons 112-158, was created. This reduces the PEVK segment by 75%, increasing passive stiffness at the sarcomere, muscle fiber, and muscle level (Brynnel et al., 2018). Brynnel found that the $Ttn^{\Delta 112\text{-}158}$ mice had significantly more passive tension, passive stiffness, and lower body weight compared to wild type mice. There was no significant difference in skeletal size between the mutant and wild type mice.

It was originally hypothesized that an increase in passive stiffness would hinder athletic performance, however, there were no significant differences between active tension, athletic performance in free wheel running or treadmill running, or oxygen consumption between the two groups. Brynnel hypothesized that the mice have adapted to stiffer titin by operating at a shorter sarcomere length range. This study also found that $Ttn^{\Delta 112\text{-}158}$ increased the muscle fiber length and the number of sarcomeres. $Ttn^{\Delta 112\text{-}158}$ also had more muscle mass than WT but did not have a difference in CSA. This suggests that $Ttn^{\Delta 112\text{-}158}$ mice may have lower body fat content as their body weight was lower than WT, but their skeletal size was the same. $Ttn^{\Delta 112\text{-}158}$ mice had a significantly shorter thin filament length of 1102 nm compared to 1165 nm in WT (Brynnel et al., 2018). Another strain of mice with the deletion of PEVK exons 219-225 had an increase of passive tension by 53% and an increase in maximum tension in the soleus muscle compared to WT. They also found more slow muscle fibers converting to fast muscle fibers (Van Der Pijl et al., 2020). There is very little research on male and female differences in the function of titin in skeletal muscle.

In terms of stiffness, one study looking at passive and active stiffness in the knee flexor, found that females had higher active stiffness, but lower short-range passive stiffness (Ueno et al., 2020). Another study, also looking at stiffness of the knee flexor, found that males had greater active and passive stiffness. However, after normalizing the data there were no significant differences (Blackburn et al., 2004). A study looking at leg stiffness in a 2-legged hop had similar results; males had more leg stiffness, but after normalization, there were no significant differences (Padua et al., 2005).

The goal of this study was to look at sex differences in the regulation of hypertrophic signaling and passive muscle stiffness in the PEVK region of titin. To do this, we used a titin mutant with a deletion to the PEVK region ($Ttn^{\Delta 112\text{-}158}$) and compared its muscle properties to wild type mice. Given the complexity of titin's role as a signaling hub, our hypothesis is that there are sex differences in the modulation of hypertrophic signaling and passive muscle stiffness. This would mean that the sex differences gap between $Ttn^{\Delta 112\text{-}158}$ and WT mice would be different in body weight, muscle mass, muscle area, and passive stress and force. Given the known sex differences in muscle fiber-type expression, which affect contractile speed and the predicted sex difference in passive stiffness, we also predict sex differences in twitch contractile properties. Studying sex differences in muscle function under different conditions can help us understand muscle performance and fill the gap of knowledge about sex differences in the literature.

Methods

These data were collected by previous students at the W.M. Keck Science Department. The analysis of the data mainly focuses on female and male sex differences between $Ttn^{\Delta 112\text{-}158}$ mice. Wild type comparisons were made using C57/B16 mice data collected by previous Keck science students; wild type comparisons for rate of force development were used from Haizlip (Haizlip et al., 2015).

The $Ttn^{\Delta 112\text{-}158}$ mouse model was created at The University of Arizona using homologous recombination to target the 47 exons, 112-158, in the titin PEVK region. This region of titin is only expected to be present in skeletal muscles and not affect cardiac muscle (Brynnel et al., 2018). For the $Ttn^{\Delta 112\text{-}15}$ mice, there were 8 female and 7 male mice with an average age of 104.3 ± 27.03 days (mean ± s.d.). The C57/B16 mice came from the Jackson laboratory (Ryan Nishi, 2022). Out of wild type mice, there were 7 females and 5 males with an average age of 104.1 ± 19.44 days. The sex categorization between female and male mice was made by visually examining under the tail (Figure 4).

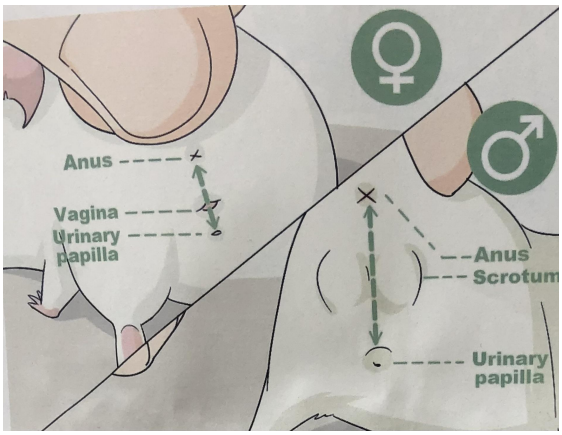


Figure 4. Female and male sex distinction

In 2021 and 2022, Dr. Monroy and other students surgically removed the soleus muscle from mutant and wild type mice and attached both ends to an Aurora Dual-Mode Muscle Lever

to collect muscle property data (Figure 5). Electrodes were connected to the muscle and isometric contractions were performed under twitch and maximal tetanic conditions. Raw data for force and length were collected in Volts which were then converted to Newtons. This procedure was done on both the right and left soleus. The muscles used in this data analysis were mainly left muscles, however, due to data availability, right muscle data was used for two females $Ttn^{\Delta 112\text{-}158}$ and one female wild type for body mass, muscle mass, and CSA, three right muscles for female $Ttn^{\Delta 112\text{-}158}$ tetanic data, one right female $Ttn^{\Delta 112\text{-}158}$ for twitch data, and one right female $Ttn^{\Delta 112\text{-}158}$ for rate of force development and half relaxation. For the $Ttn^{\Delta 112\text{-}158}$ mice data, all females and males were used. However, for wild type body mass, only five out of seven females and four out of five male mice were used because of data availability.

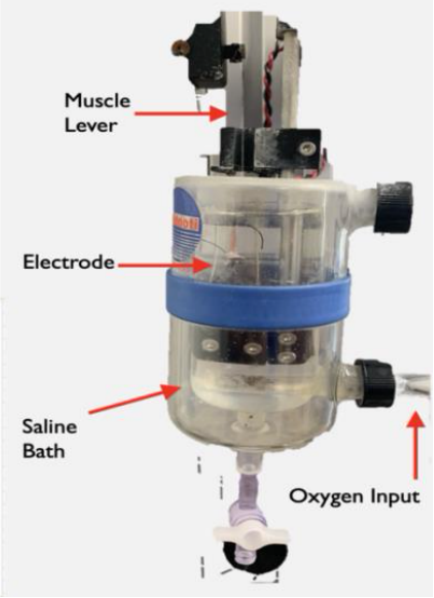


Figure 5. Aurora Dual-Mode Muscle System (Ryan Nishi, 2022)

Physiological cross-sectional area (CSA) was calculated by former students by taking the cosine of the pennation angle and dividing it by the product of muscle fiber length and

mammalian skeletal muscle density constant (Ryan Nishi, 2022). Because CSA is proportional to force production, stress was calculated by dividing maximum active force under tetanic conditions by CSA to standardized force (Figure 8).

Twitch and tetanic conditions were performed at different muscle lengths, lengthening the muscle a little each time. For each muscle, length, force and time were recorded. From this data, a force-time graph was made to determine passive force, peak force and active force (Figure 6). Optimal length (L_o) was determined for each condition at the length with the highest active force.

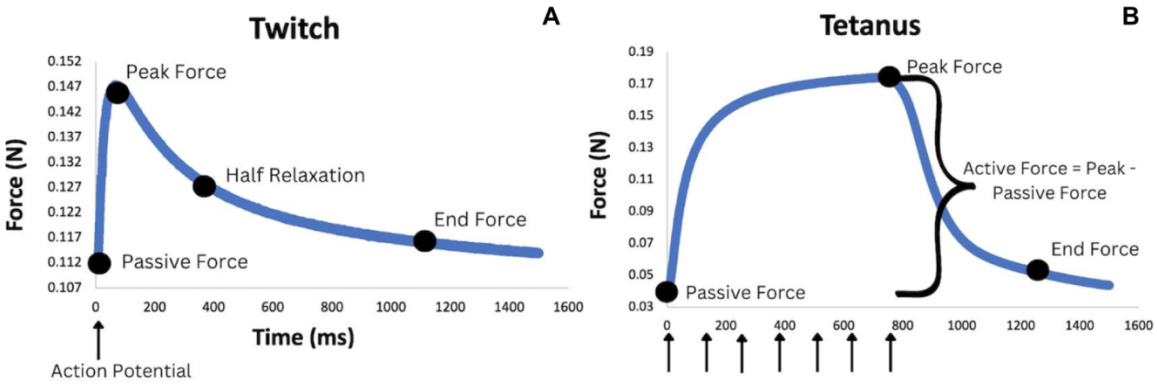


Figure 6. Data collection process for twitch and tetanic conditions using the left soleus of female $Ttn^{\Delta 112\text{-}158\ ID}$ 37. (A) Twitch. Rate of force development = time at peak force - time at passive force (B) Tetanic.

For each muscle, passive and active force were normalized by dividing each force by the maximum active force. This data was plotted against the length of the muscle divided by optimal length, creating a force-length graph. These force-length graphs were created for both twitch and tetanic conditions. The active force-length data was compared between twitch and tetanic for both female and male mice separately. To assess for passive stiffness, the passive stress-length

relationship under twitch and tetanic conditions between male and female $Ttn^{\Delta 112\text{-}158}$ and WT and mice were plotted using passive force divided by CSA and standardized length (length/optimal length). Additionally, the maximum passive force and maximum active force under twitch and tetanic conditions for each individual was taken. These values were then divided by each individual's CSA. The percent difference between female and males for each genotype was calculated by dividing the differences of the mean values for males and females by half of the sum of the mean values for males and females.

The rate of force development and half relaxation time was determined using the twitch data that produced the maximum active force and optimal length. The force-time graph was made in Microsoft Excel to collect the initial force and time of activation, peak force and time, and end force and time. The rate of force development was calculated by taking the difference between peak time and initial time. The force at half-relaxation was the peak force minus half of the difference between peak and end force. The half relaxation time was collected using the graph that was created in Excel, which represents the time from the onset of the stimulus to half relaxation.

Statistical Analysis

Microsoft Excel was used to calculate mean±standard deviations and R^2 values for lines of best fit on all of the scatter plots. Data was fitted to an exponential curve for passive data and to a polynomial order of 2 curves for active data. R studio was used to run all other statistical analysis. Levene tests were run on all dependent variables to assess if the data met assumptions of equal variance to run a two-way ANOVA. If the p values were under 0.05, the data were log transformed. The only variables that were log transformed were maximum passive force and passive stress under twitch conditions, maximum active force under tetanus conditions, and rate

of force development. A two-way ANOVA was then run, and if any p values were less than 0.05, a Tukey-Kramer test was run to evaluate differences amongst the groups.

Results

	$Ttn^{\Delta 112-158}$ Female	**$Ttn^{\Delta 112-158}$ Male**	**Wild Type Female**	**Wild Type Male**
Body Mass (g)	21.42±2.35	27.00±2.35	25.13±3.75	29.38±1.44
Muscle Mass (g)	0.007±0.0016	0.0104±0.00164	0.007514±0.001	0.01118±0.00105
PCSA (cm^2)	0.00618±0.0015	0.00834±0.0014	0.007273±0.0011	0.010800±0.00169
Initial Length (mm)	11.4411.96±0.590	11.96±0.951	10.84	10.07
TW L_o (mm)	13.78±0.94	14.70±0.65	12.14±1.42	12.19±1.04
Max Active Force (N)	0.127±0.017	0.125±0.017	0.12030±0.059	0.1583±0.029
Stress (N/cm^2)	18.35±4.62	15.26±2.66	16.304±6.89	14.75±2.12
Rate of Force Development (ms)*	99.56±48.31	62.03±11.52	76.82±18.21	112.35±43.66
Half Relaxation Force (N)	0.109±0.028	0.0831±0.0304	0.038±0.013	0.050±0.019
Half Relaxation Time (ms)*	320.41±130.49	188.93±50.18	196.93±45.5	309.25±120.17

Table 1. Summary of Results. Represented as mean±s.d. Maximum active force and stress were calculated from active force tetanus. * $P<0.05$ significant differences in the interaction effect between genotype and sex. Female $Ttn^{\Delta 112-158}$ mice (n=8), male $Ttn^{\Delta 112-158}$ mice (n=7), female WT mice (n=7), and male WT mice (n=5).

The interaction effect on size between genotype and sex was not significant; body mass ($F(1,20) = 0.31$, $p = 0.58$), muscle mass ($F(1,23) = 0.15$, $p = 0.70$), and CSA ($F(1,23) = 1.55$, $p = 0.23$) (Figure 7). $Ttn^{\Delta 112-158}$ and WT males were significantly larger across all variables, with

respect to size, as compared to females; body mass ($F(1,20) = 19.34$, $p = 0.00028$), muscle mass ($F(1,23) = 39.47$, $p = 2.7e\text{-}5$), and CSA ($F(1,23) = 25.95$, $p = 3.69e\text{-}5$) (Figure 7). The effect of genotype was significant on body mass ($F\ 1,20) = 6.34$, $p = 0.02$) and CSA ($F(1,23) = 8.23$, $p = 0.0087$), but not muscle mass ($F(1,20) = 0.39$, $p = 0.54$). The mean percent sex difference of body mass in $Ttn^{\Delta 112\text{-}158}$ mice was 23% and 15% in WT mice were. The percent sex difference in muscle mass for $Ttn^{\Delta 112\text{-}158}$ mice was 37% and 39% in WT mice. The percent difference in CSA was 30% in $Ttn^{\Delta 112\text{-}158}$ mice and was 39% in WT mice (Table 2).

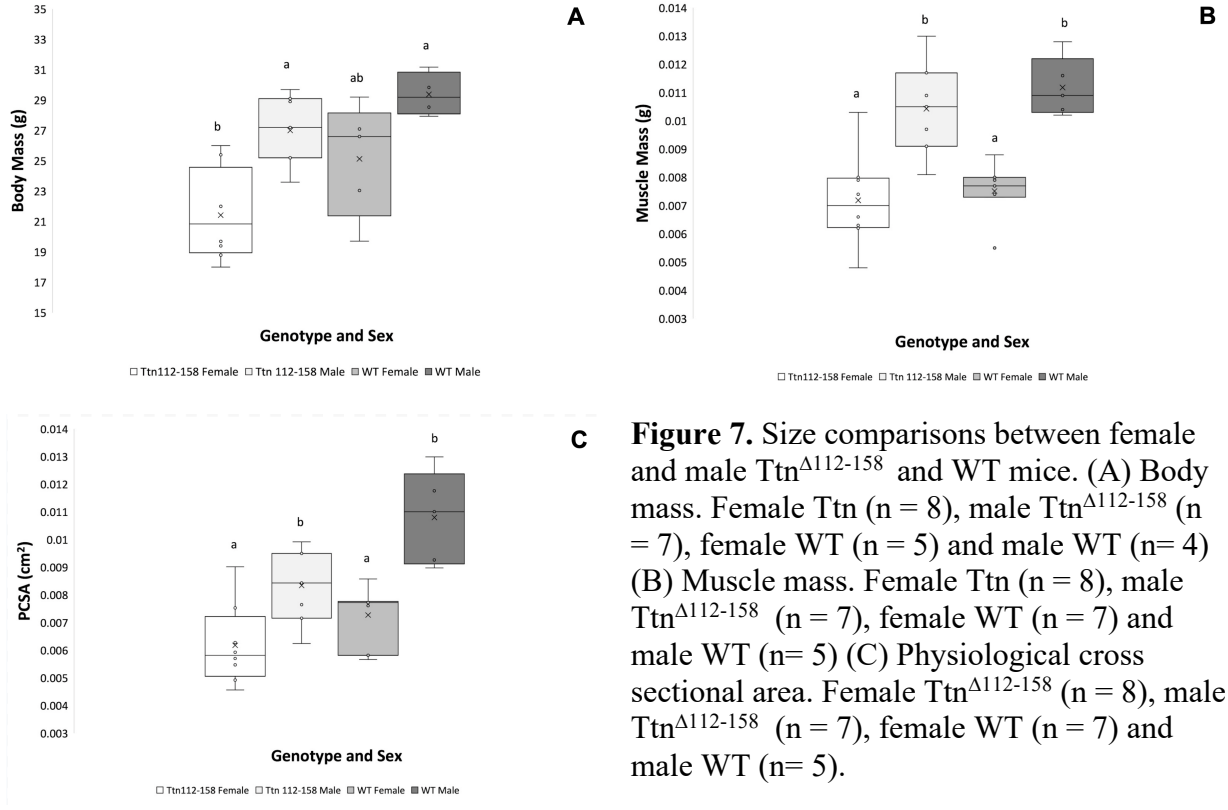


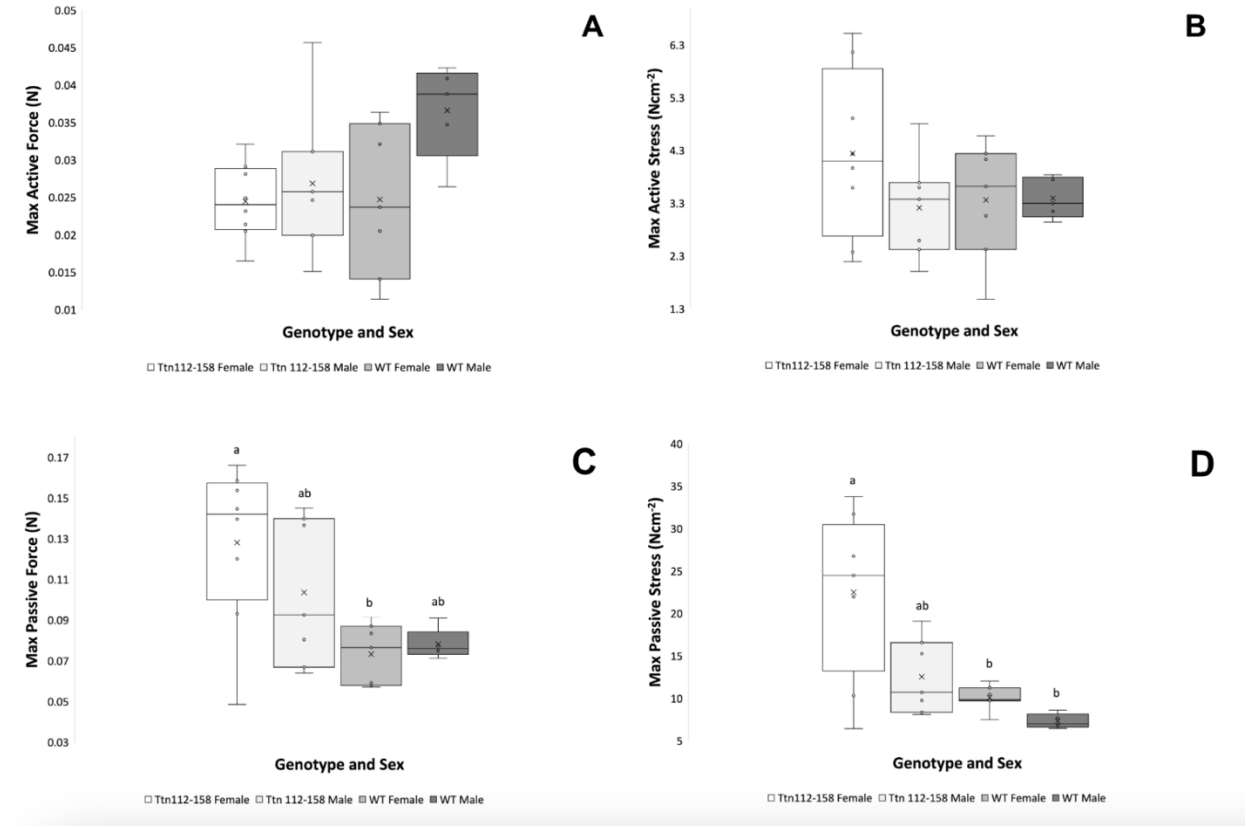
Figure 7. Size comparisons between female and male $Ttn^{\Delta 112\text{-}158}$ and WT mice. (A) Body mass. Female Ttn (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 5) and male WT (n= 4) (B) Muscle mass. Female Ttn (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 7) and male WT (n= 5) (C) Physiological cross sectional area. Female $Ttn^{\Delta 112\text{-}158}$ (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 7) and male WT (n= 5).

Muscle Property	$Ttn^{\Delta 112-158}$	Wild Type	Muscle Property	$Ttn^{\Delta 112-158}$	Wild Type
Body mass (g)	23%	15%	**TET Passive Force (N)**	9.6%	20%
Muscle Mass (g)	37%	39%	**TET Passive Stress (N/cm^2)**	42%	17%
PCSA (cm^2)	30%	39%	**TET Active Force (N)**	1.1%	27%
TW Passive Force (N)	21%	6.5%	**TET Active Stress (N/cm^2)**	33%	10%
TW Passive Stress (N/cm^2)	57%	32%			
TW Active Force (N)	9%	39%			
TW Active Stress (N/cm^2)	28%	9.8%			

Table 2. Percent Sex Differences. Percent sex difference= (Mean female - mean male)/ ((mean female + mean male)/2). TW: twitch and TET: tetanus

Under twitch conditions, maximum passive force and maximum passive stress were log transformed in order to meet the assumptions and run a two-way ANOVA (Figure 8 c,d). The interaction effect between sex and genotype was not significant; maximum passive force ($F(1,23) = 1.35$, $p = 0.26$), maximum passive stress ($F(1,23) = 0.45$, $p = 0.51$), maximum active force ($F(1,23) = 2.25$, $p = 0.15$), and maximum active stress ($F(1,23) = 1.38$, $p = 0.25$) (Figure 8 a-d). The effect of sex was significant for maximum passive stress ($F(1,23) = 8.52$, $p = 0.00059$), maximum active force ($F(1,23) = 4.35$, $p = 0.048$), but not significant for maximum passive force ($F(1,23) = 0.43$, $p = 0.52$) and for maximum active stress ($F(1,23) = 1.57$, $p = 0.40$). The effect of genotype was significant on maximum passive force ($F(1,23) = 10.27$, $p =$

0.0039) and maximum passive stress (F(1,23) = 15.85, p = 0.00059), but not significant for maximum active force (F(1,23) = 1.70, p = 0.21) and maximum active stress (F(1,23) = 74, p = 0.40). The percent sex difference for passive force was 21% for $Ttn^{\Delta 112\text{-}158}$ and 6.5% for WT (Figure 8c). For passive stress the difference was 57% in $Ttn^{\Delta 112\text{-}158}$ and 32% in WT (Figure 8d). For maximum active force, it was 9% for $Ttn^{\Delta 112\text{-}158}$ and 39% for WT (Figure 8a). For active stress, the $Ttn^{\Delta 112\text{-}158}$ difference was 28% and 9.8% for WT (Figure 8b) (Table 2).



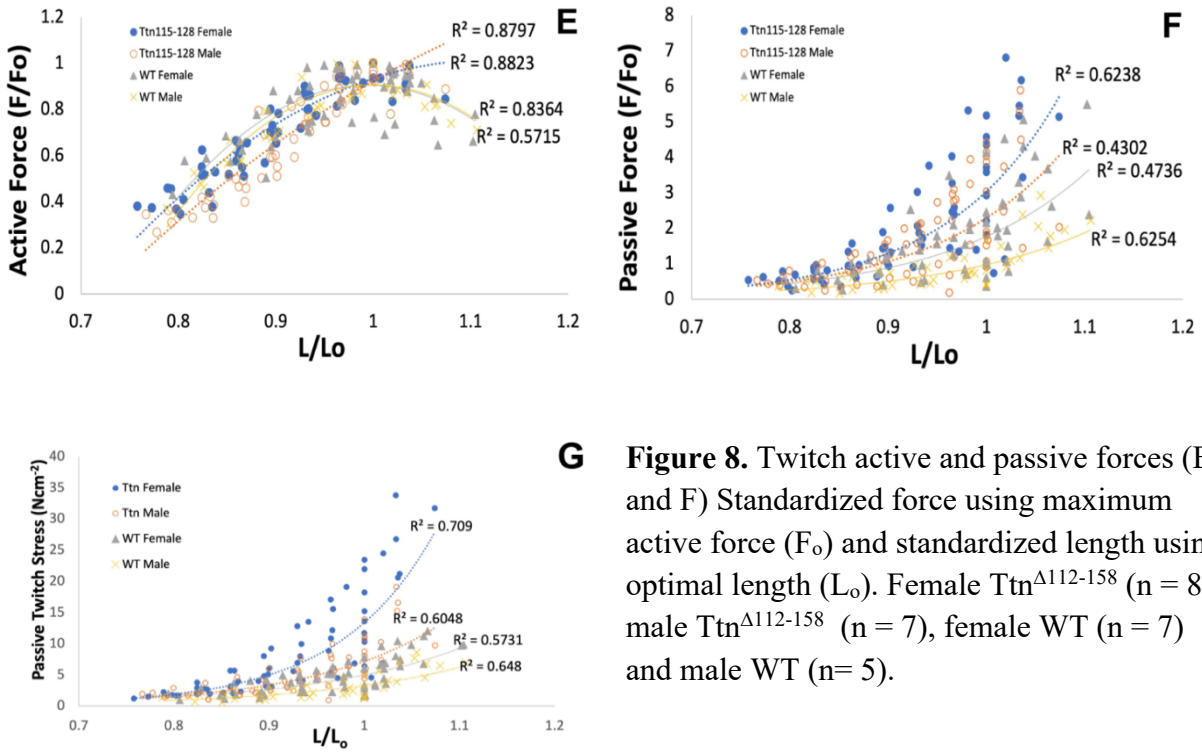
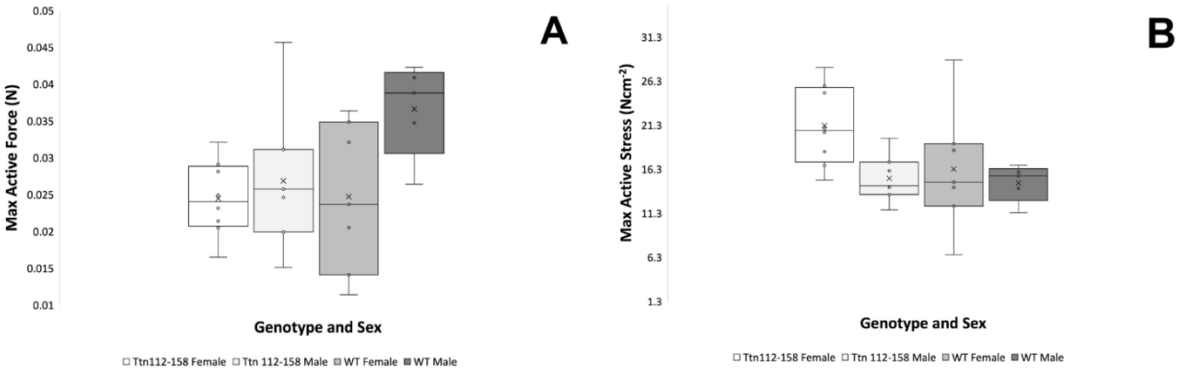


Figure 8. Twitch active and passive forces (E and F) Standardized force using maximum active force (F_o) and standardized length using optimal length (L_o). Female $Ttn^{\Delta 112\text{-}158}$ (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 7) and male WT (n= 5).

For twitch conditions, the R^2 values for the normalized active force-length relationship was R^2=0.88 for female and male $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.84 for WT females, and R^2=0.57 for WT males (Figure 8e). The normalized passive force-length R^2 values were R^2=0.62 for female $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.43 for male $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.47 for WT females, and R^2=0.63 for WT males (Figure 8f). The passive stress–normalized length R^2 values were R^2=0.71 for female $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.60 for male $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.57 for WT females, and R^2=0.65 for WT males (Figure 8g).

Under tetanus conditions, only maximum active force was log transformed (Figure 9a). There were no significant interactions between genotype and sex; maximum passive force ($F(1,23) = 0.77$, $p = 0.39$), maximum passive stress ($F(1,23) = 0.51$, $p = 0.48$), maximum active force ($F(1,23) = 2.82$, $p = 0.11$), and maximum active stress ($F(1,23) = 1.51$, $p = 0.23$) (Figure 9

a-d). The effect of sex on maximum active stress was significant ($F(1,23) = 5.10$, $p = 0.034$) with females having greater active stress than males (Figure 9b). The effect of sex was not significant for maximum passive force ($F(1,23) = 0.077$, $p = 0.78$), maximum passive stress ($F(1,23) = 3.02$, $p = 0.096$), and maximum active force ($F(1,23) = 1.88$, $p = 0.18$) (Figure 9 a,c-d). Genotype did not have a significant effect; maximum passive force ($F(1,23) = 0.95$, $p = 0.34$), maximum passive stress ($F(1,23) = 0.0012$, $p = 0.97$), maximum active force ($F(1,23) = 0.0047$, $p = 0.95$), and maximum active stress ($F(1,23) = 2.45$, $p = 0.13$). The percent sex difference for passive force was 9.6% for $Ttn^{\Delta 112\text{-}158}$ and 20% for WT (Figure 9c). The difference for passive stress was 42% for $Ttn^{\Delta 112\text{-}158}$ and 17% for WT (Figure 9d). The difference for active force was 1.1% for $Ttn^{\Delta 112\text{-}158}$ and 27% for WT mice (Figure 9a). The difference for active stress for $Ttn^{\Delta 112\text{-}158}$ was 33% and 10% for WT (Figure 9b) (Table 2).



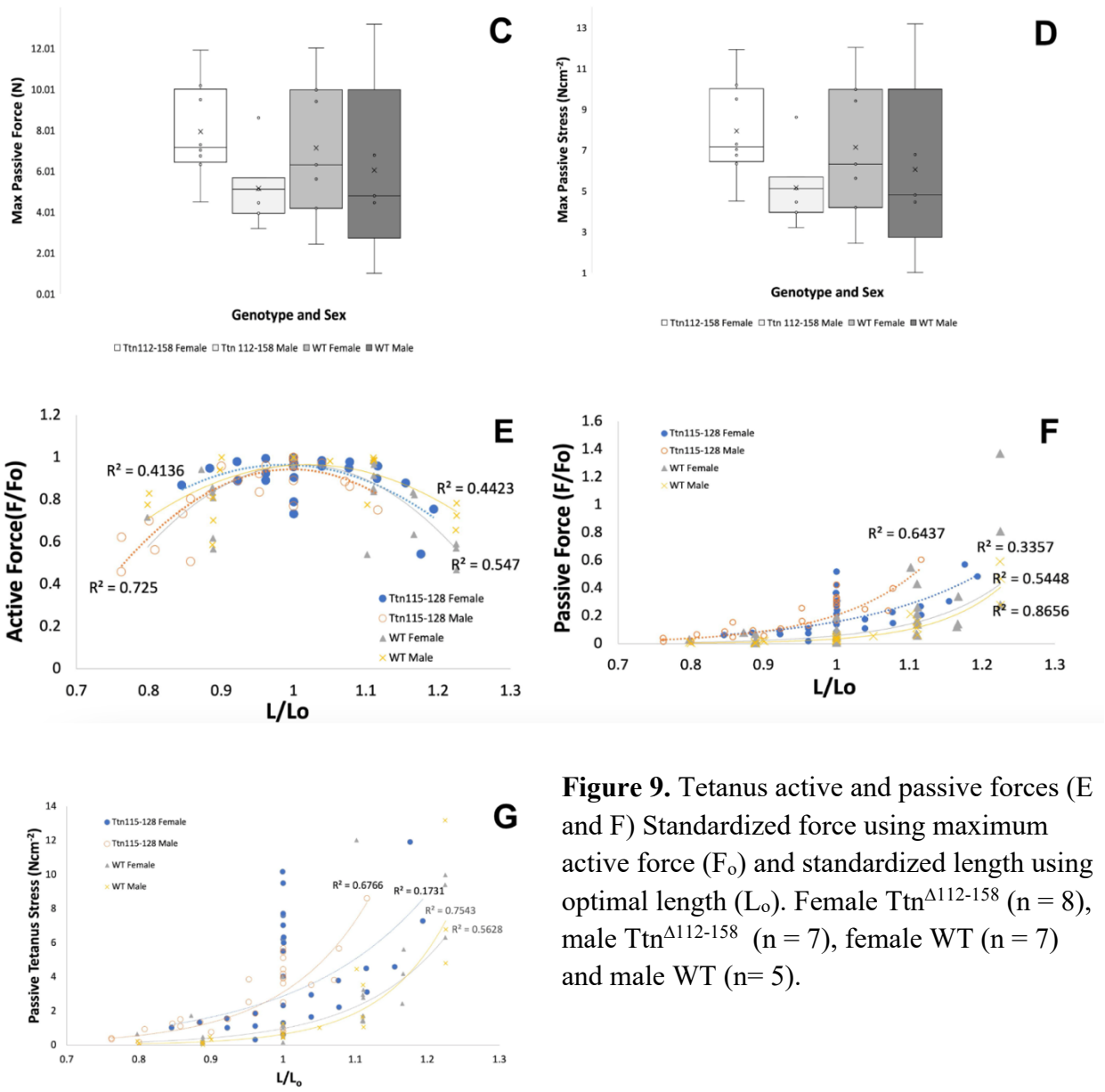


Figure 9. Tetanus active and passive forces (E and F) Standardized force using maximum active force (F_o) and standardized length using optimal length (L_o). Female $Ttn^{\Delta 112\text{-}158}$ (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 7) and male WT (n= 5).

Under tetanic conditions, the R^2 values for the normalized active force-length relationship was R^2=0.41 for female $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.73 for $Ttn^{\Delta 112\text{-}158}$ mice R^2=0.55 for WT females, and R^2=0.44 for WT males (Figure 8e). The normalized passive force-length R^2 values were R^2=0.64 for female $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.34 for male $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.54 for WT females, and R^2=0.87 for WT males (Figure 8f). The passive stress–normalized length R^2 values were

R^2=0.17 for female $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.68 for male $Ttn^{\Delta 112\text{-}158}$ mice, R^2=0.56 for WT females, and R^2=0.75 for WT males (Figure 8g).

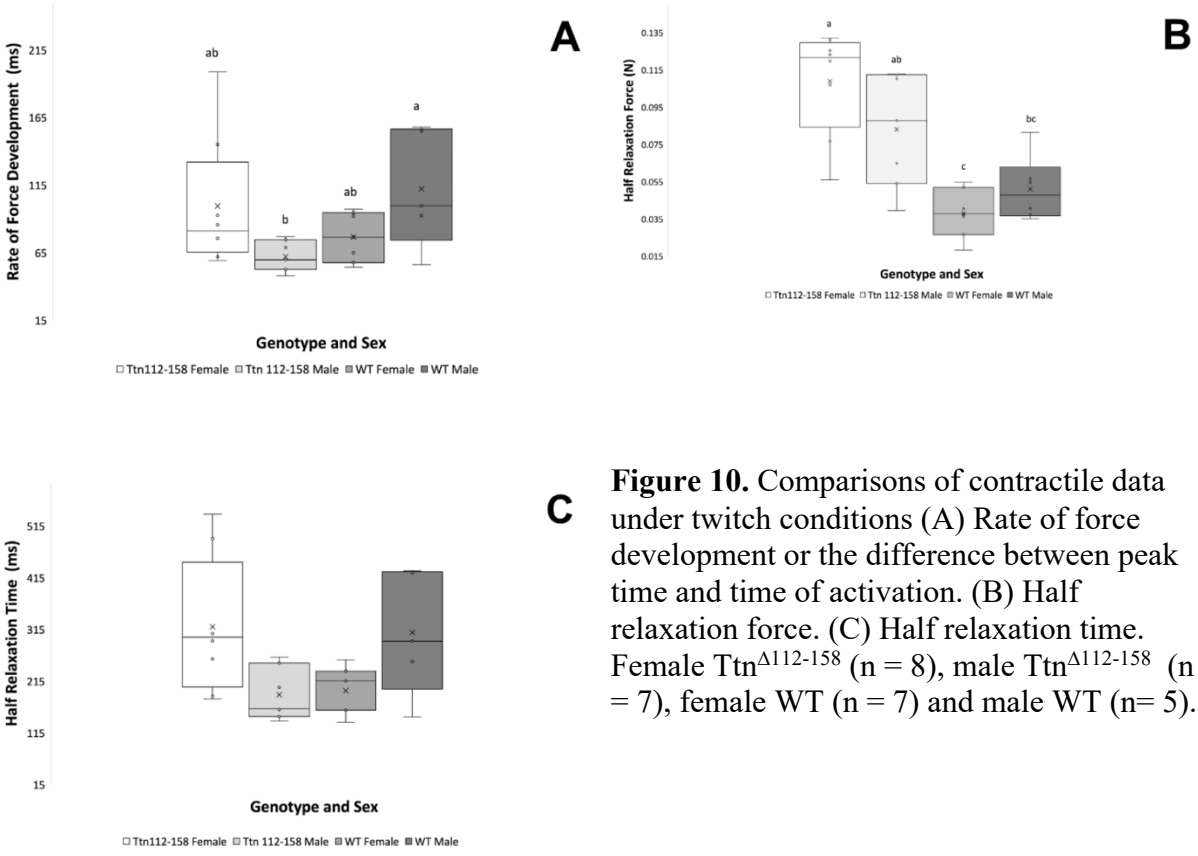


Figure 10. Comparisons of contractile data under twitch conditions (A) Rate of force development or the difference between peak time and time of activation. (B) Half relaxation force. (C) Half relaxation time. Female $Ttn^{\Delta 112\text{-}158}$ (n = 8), male $Ttn^{\Delta 112\text{-}158}$ (n = 7), female WT (n = 7) and male WT (n= 5).

For contractile properties under twitch conditions, the rate of force development was the only variable that was log transformed (Figure 10a). The interaction effect between sex and genotype was significant for rate of force development ($F(1,23) = 8.18$, $p = 0.0088$) and for half relaxation time ($F(1,23) = 10.95$, $p = 0.003$) (Figure 10 a,c). The interaction was not significant for half relaxation force ($F(1,23) = 4.02$, $p = 0.057$) (Figure 10b). The effect of sex was not significant; rate force development ($F(1,23) = 0.37$, $p = 0.55$), half relaxation force ($F(1,23) = 0.94$, $p = 0.34$), and half relaxation time ($F(1,23) = 0.45$, $p = 0.51$). The effect of genotype was

significant for half relaxation force (F(1,23) = 32.7, p = 7.97e-6), but not for rate of force development F(1,23) = 1.01, p = 0.33) and half relaxation time (F(1,23) = 0.18, p = 0.68). The rate of force development percent sex difference was 46% for $Ttn^{\Delta 112\text{-}158}$ and 38% (Figure 10a). The difference for half relaxation force was 27% for $Ttn^{\Delta 112\text{-}158}$ and 28% for WT (Figure 10b). For half relaxation time it was 52% for $Ttn^{\Delta 112\text{-}158}$ and 44% for WT (Figure 10c) (Table 2).

In the normalized active force-length graphs, the R^2 values comparing twitch and tetanus for females were R^2=0.88 for $Ttn^{\Delta 112\text{-}158}$ under twitch conditions and R^2=0.41 for tetanus conditions. For female WT mice, R^2=0.55 under twitch conditions and R^2=0.57 under tetanus conditions (Figure 11a). For $Ttn^{\Delta 112\text{-}158}$ males, R^2=0.88 under twitch conditions and R^2=0.73. under tetanus conditions. For male WT mice, R^2=0.84 under twitch conditions and R^2=0.44 (Figure 11b).

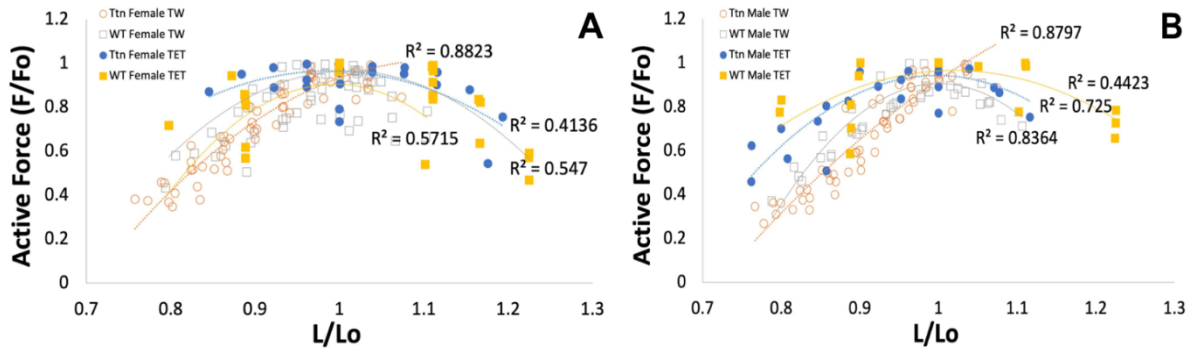


Figure 11. Comparisons of twitch and tetanic active force-length relationship (A) Female. $Ttn^{\Delta 112\text{-}158}$ mice (n = 8) and WT (n = 7). (B) Male. $Ttn^{\Delta 112\text{-}158}$ mice (n = 7) and WT (n = 5).

Discussion

This study compared female and male mutant mice with a large deletion to the PEVK region ($Ttn^{\Delta 112\text{-}158}$) and WT mice to evaluate sex differences in how the PEVK regulates hypertrophic signaling and passive muscle stiffness.

In terms of hypertrophic signaling, there were no significant interaction effects between sex and genotype. There was a general trend in which the sex difference in WT mice were greater than in $Ttn^{\Delta 112\text{-}158}$ mice in both muscle mass and CSA. For body mass, the sex difference was slightly greater in $Ttn^{\Delta 112\text{-}158}$ than WT mice. However, because our interest is in hypertrophic signaling, muscle mass and cross-sectional area (CSA) represent muscle growth better than body weight due to greater confounding factors. This could indicate that there are sex differences in PEVK hypertrophic signaling. Both $Ttn^{\Delta 112\text{-}158}$ and WT males had significantly greater body mass, muscle mass, and CSA. This was expected as males have higher circulating levels of testosterone, which stimulates muscle growth (Landen et al., 2023). This is also congruent with Hill et al. (2020) findings in ten-week-old WT mice (C. Hill et al., 2020). There was a significant effect of the PEVK deletion on body mass and CSA. Brynnel et al (2018) also found a significant difference in body weight between $Ttn^{\Delta 112\text{-}158}$ and WT mice at 180 days and older (Brynnel et al., 2018). This confirms that the PEVK region is involved with hypertrophic signaling.

Under twitch and tetanus conditions, there were no significant interactions between sex and genotype for passive and active force and stress. However, the $Ttn^{\Delta 112\text{-}158}$ female mice tended to have greater passive and active stress, while the $Ttn^{\Delta 112\text{-}158}$ male mice had similar averages to the WT. Passive stress could have negative effects on overall muscle performance,

especially in cyclic movements. Maximum active stress in both female mice was significantly higher than the male mice. The WT mice did not have as much of a sex difference than the $Ttn^{\Delta 112\text{-}158}$ mice for passive stress. This, as well as female $Ttn^{\Delta 112\text{-}158}$ mice having a steeper slope on the twitch passive stress-relative length graph, supports that there are sex differences in PEVK passive stiffness regulation. It is known that calcium/calmodulin-dependent protein kinase-IIδ (CaMKIIδ) and calcium dependent protein kinase Cα (PKCα) phosphorylate the PEVK region and modulate passive stiffness, so future research should investigate sex differences in expression of these enzymes (Krüger & Kötter, 2016).

There was an opposite trend for twitch and tetanus active force where there was a greater sex difference in the WT mice than the $Ttn^{\Delta 112\text{-}158}$ mice. This makes sense given that there was a greater sex difference in WT mice for muscle mass and CSA, which are both directly proportional to active force. Differences in maximum active force during tetanic conditions were not significant between any groups.

There were a couple of differences found between twitch and tetanus conditions. The first is passive force. Under twitch conditions, the sex difference in passive force was greater in $Ttn^{\Delta 112\text{-}158}$ mice than WT, while the opposite was true for tetanus. Additionally, the sex differences in passive stress were not seen as well in the tetanus stress-relative length graph. The same was the case when comparing twitch and tetanus best fit lines for the relative active force-length graph. For both females and males, the $Ttn^{\Delta 112\text{-}158}$ twitch mice had steeper slopes. This supports that the $Ttn^{\Delta 112\text{-}158}$ mice have greater passive stiffness because in early stages of activation, muscle stiffness increases especially in stiffer isoforms of titin (Nishikawa et al.,

2020). This was expected and was what Brynnel et al (2018) and Nishi (2020) also found (Brynnel et al., 2018; Ryan Nishi, 2022).

For contractile properties using twitch data, a significant interaction effect was found between sex and genotype for the rate of force development and the time at half relaxation. The results for contractile properties for WT mice were not as anticipated. The male WT mice had a longer rate of force development and took longer to reach half relaxation. It was hypothesized that the rate of force development and time at half relaxation would be longer in female mice because of the higher ratio of type I fibers, or slow twitch fibers, in female mice as compared to male mice. In wild type soleus muscles, Haizlip et al. (2015) and Gustafsson & Posuette (1975) found that relaxation time and contraction time was longer in females than males (Gustafsson & Pousette, 1975; Haizlip et al., 2015). Hill et al. (2020) looked for time to half-peak tetanus in wild type soleus muscles and found that female mice had significantly longer time to half-peak tetanus at 10 weeks (C. Hill et al., 2020). One study looking at sex differences in half relaxation times across fast fatigable, fatigue resistance, and slow muscle fiber types did find that males had longer half relaxation times. They found significant contractile sex differences in the type II muscle fiber types, but not in the type I, which is what the soleus is mainly made up of (Celichowski & Drzymała, 2006; Soukup et al., 2002). The $Ttn^{\Delta 112\text{-}158}$ female mice did have longer times to peak and half relaxation, which does go along with what was predicted as female $Ttn^{\Delta 112\text{-}158}$ mice tend to have greater passive muscle stiffness. An increase in passive muscle stiffness would probably increase these times even more. Because of this huge difference in rate of force development and half relaxation, sex is likely a factor in passive muscle stiffness.

Conclusion

There appears to be sex differences in hypertrophic signaling and passive muscle stiffness. There were greater sex differences in $Ttn^{\Delta 112\text{-}158}$ mice than WT mice in muscle mass, CSA, and passive and active stress. Female $Ttn^{\Delta 112\text{-}158}$ mice had the greatest amount of passive stiffness. At an organismal level, passive stiffness could negatively affect cyclic activities such as running and swimming. Future research can be done to evaluate sex differences in athletic performance with an increase in passive stiffness. Learning more about sex differences in passive stiffness and hypertrophic is important, as this distinction is overlooked in many muscle physiologies studies. This can help us get a better understanding of sex differences in muscle performance.

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