DYSTROPHIC CARDIOMYOPATHY:

THE ROLE OF MYOFILAMENTS

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by

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Dedication

This thesis is dedicated in loving memory of my father, Andrew George, who I miss everyday and was a constant inspiration throughout my studies. While he did not get to see this adventure, I know he would be proud of what I have accomplished.
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Abstract

Dystrophic cardiomyopathy is the consequence of diseases that affect the protein dystrophin. Dystrophin forms part of the dystrophin glycoprotein complex and is postulated to act as a membrane stabilizer, protecting the sarcolemma from contraction-induced damage. Duchenne muscular dystrophy (DMD) is the most severe dystrophinopathy, caused by a total absence of dystrophin. Patients with DMD present with progressive skeletal muscle weakness and due to advances in treatment, which increase life span, a cardiac component of the disease has been unmasked. Many patients with DMD now succumb to heart failure. As such this study aims to address a knowledge gap relating to the cardiac myofilament involvement in DMD. To assess the effects of DMD upon cardiomyocyte function, isolated permeabilized cardiomyocytes of wild-type and DMD$^{mdx-4cv}$ mice were attached between a force transducer and motor and subjected to a range of contractile assays. Maximal tension and force development rates (indexed by the rate constant, $k_tr$) were similar between the wild-type and DMD$^{mdx-4cv}$ cardiac myocyte preparations. Interestingly, it was found that DMD$^{mdx-4cv}$ cardiac myocytes had a greater sarcomere length dependence of peak power output when compared to the cardiac myocytes of wild-type littermates. These results suggest dystrophin mitigates length dependence of activation and augmented sarcomere length dependence of myocyte contractility which may accelerate the progression of ventricular myocyte damage in dystrophic hearts. Following this first study, the effects of mavacamten, a novel small molecule modulator of the thick filament activation, were assessed upon contractile properties in wild-type and DMD$^{mdx-4cv}$ murine permeabilized cardiomyocytes. Mavacamten decreased maximal tension and $k_tr$ in both
wild-type and DMD$^{\text{mdx-4cv}}$ cardiomyocytes, while also normalizing the length dependence of peak power between DMD$^{\text{mdx-4cv}}$ cardiac myocytes and wild-type preparations. These results highlight the potential benefits of mavacamten (i.e., reduced overall contractility while maintaining exquisite sarcomere length dependence of power output) as a treatment for dystrophic cardiomyopathy associated with DMD.
**Introduction**

The sarcomere is the fundamental contractile unit seen in striated muscle. Striated muscle functions to generate force, which results in skeletal movement and pumping of blood. A major component of striated muscle are cylindrical bundles of thick and thin filaments, called myofibrils, of which the functional unit is the sarcomere. Each sarcomere is defined at the lateral border by Z-lines, which is a line of non-contractile proteins arranged perpendicular to the thick and thin filaments. Another filament, comprised of the protein titin runs from the Z-line to the middle, or M-line, of the sarcomere. Thick filaments primarily consist of myosin molecules, but also contain myosin binding protein-C (MyBP-C), which are spaced periodically along the thick filament. Constituting the thin filament are primarily three molecules; actin, tropomyosin, and troponin. Sarcomeres are divided into several different zones, each consisting of a different arrangement of thin and thin filaments. A-bands consist of the full length of the thick filament, including thin filament overlap, while the H-zone is comprised of the area having thick filament only, i.e., there lacks overlap with the thin filament. I-bands consist thin filament regions only and span two adjacent sarcomeres. During striated muscle contraction, thick and thin filament proteins act in a highly cooperative manner to generate force and power.

**Regulation of Contraction**

**Thin filament regulation**
Muscle contraction is regulated by cytosolic $\text{Ca}^{2+}$, which activates the thin filaments. It has been postulated that the position of tropomyosin (Tm) and troponin (Tn) is the main determinant of myosin on the thick filament interacting with actin on thin filaments (Gordon et al., 2000). The thin filament is characterized by three states; blocked, closed, and open (Chalovich et al., 2022). In the blocked states myosin binding sites on actin are sterically impeded. In the closed state, myosin may weakly bind actin, while in the open state myosin strongly interacts with actin. These binding states are primarily determined by the position of Tm, which is in turn affected by $\text{Ca}^{2+}$ binding to TnC. $\text{Ca}^{2+}$ binding to TnC results in decrease in the binding between TnT and actin, which allows Tm movement exposing the myosin binding sites on actin monomers (Gordon et al., 2000). Exposure of the myosin binding sites allows the myosin head to interact with actin, resulting in force and work when thick and thin filaments slide past each other.

The Thick Filament

Myosin

Myosin is the main component of the thick filament, which makes up the A-band region of the sarcomere. Myosin consists of two inter-twined myosin heavy chain molecules each consisting of a globular head and neck motor region attached to an $\alpha$-helical tail. The tail is 1.56 $\mu$m long and has a 43 nm flexible hinged portion, known as the S2 region, which connects to the head and neck region, or S1 portion, of the myosin molecule (Rayment et al., 1993). The S2 region is referred to as the hinge region, which is thought to allow movement of the myosin head. The S1 region contains the catalytic
ATPase site and actin binding site, both of which are necessary for cross-bridge cycling during contraction. The ATPase site allows ATP hydrolysis, which provides the energy needed for contraction, while the actin binding site allows interaction between the thick and thin filaments.

Myosin molecules also consist of four light chains (MLC), which bind to the heavy chains in a two-by-two manner. MLCs are defined as either essential or regulatory light chains. Both the essential and regulatory are localized in the neck region of myosin. Therefore, it is postulated that the MLCs provide support to the neck region of myosin and may aid force production during cross-bridge cycling (Rayment et al., 1993).

Myosin is proposed to have two states when not bound to actin, they are: super-relaxed (SRX) and disordered relaxed (DRX). Myosin in the SRX state has a ten-fold decrease in the speed of ATP turnover when compared to the DRX state (Nag & Trivedi, 2021). The SRX state is characterized by its slow release of ATP hydrolysis products in comparison to other states of myosin. Additionally, SRX myosin can be found in two states, the interacting head motif (IHM) or non-IHM state. In the IHM state, the myosin heads fold into the S2 tail (Nag & Trivedi, 2021).

*Figure 1.* Schematic representation of the differing states of myosin. From (Nag & Trivedi, 2021).
Thick filament regulation

More recently a novel role for the thick filament regulation of contraction has been discovered, whereby mechano-sensing by the thick filament works in concert with thin filament regulation to tune force of contraction with workload. Myosin molecules on the thick filament sense workload and elicit a proportional myosin recruitment response (Greenberg et al., 2016). Consistent with this, myosin filament activation in cardiac muscle closely matches the stress upon the filament (Reconditi et al., 2017). Myosin heads can be recruited from the off state, or SRX, state to the on, or DRX state. As previously mentioned, the SRX state of myosin heads has a lower energetic demand when compared to the DRX state but are not thought to interact with actin. As stress is increased along the myofilaments the proportion of myosin heads in the DRX state are increased, resulting in a greater contractile capability. Consequently, the myofilament tunes its activation, and thus its contractile strength, in response to stress allowing it to better match energetics with mechanical task.

Translational significance

Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a genetic disorder caused by variants in sarcomere protein genes resulting in altered contraction and relaxation properties, elevated energy consumption, and increased patient risk for heart failure, and hypercontractility (Toepfer et al., 2020). Left ventricular hypertrophy is a prominent feature in HCM, whereby the maximal thickness of the ventricle exceeds 15mm (Palandri
et al., 2022). A majority of these mutations affect the thick filament proteins (Harris et al., 2011; Konno et al., 2010) with 70% of mutations altering the β-myosin heavy chain or the myosin binding protein C gene (Ackerman et al., 2002). A shift increasing the proportion of myosin motors in the DRX state (increased range of motion) has been postulated to account for hypercontractility and impairment of relaxation seen with HCM (Palandri et al., 2022). An increase in the proportion of DRX state myosin motors would also cause an increase in the energetic demand of the heart, due to the elevated ATP consumption rate associated with disordered cross-bridges. Consequently, therapeutic interventions are being investigated to modify the equilibrium of myosin motors to favor the SRX state, which would lower energetic demands and attenuate hypercontractility.

Clinical studies have shown the effectiveness of a small molecule modulator that acts directly upon myosin as a treatment for HCM. One such small molecule, MYK-461 (i.e., mavacamten), has been found to have positive affects in the treatment of HCM. Kansas City Cardiomyopathy Questionnaire (KCCQ) scores, a measure of health status, were significantly increased in patients with HCM treated with mavacamten when compared to those given placebo in clinical trials (Olivotto et al., 2020) over a period of 30 weeks. Other clinical trials corroborated this KCCQ score increase and showed an increase in exercise capacity in HCM patients treated with mavacamten (Tower-Rader et al., 2020).

**Mavacamten**

MYK-461 (mavacamten) is a small molecule that reduces ATP activity of the cardiac myosin heavy chain consequently leading to decreased contractility. As previously mentioned, mavacamten has shown promise as an effective treatment for
HCM in clinical trials. It has been shown that mavacamten reduces ATPase activity in a dose dependent manner in mouse cardiac myofibrils (Green et al., 2016). Additionally, mavacamten has a lower affinity for inhibiting the ATPase of skeletal myosin than cardiac myosin, and as such cardiac contractility is depressed without significant impairment in skeletal muscle function in a murine HCM model (Green et al., 2016). Mavacamten has potential as an effective therapeutic intervention for DMD due to its targeted effects in reducing cardiac contractility. It is possible that by decreasing cardiac contractility, mavacamten may be an effective treatment for mitigating contraction-induced damage which may lead to heart failure in patients with DMD.

**Duchenne Muscular Dystrophy**

Dystrophinopathies are a class of diseases that arise from mutations in the dystrophin gene. Dystrophin is a protein that connects the cytoskeleton of striated muscle cells to the extracellular matrix and forms part of the dystrophin associated glycoprotein complex (DGC). An absence of dystrophin typically presents as skeletal muscle weakness and is associated with increased susceptibility to contraction-induced damage. The most severe form of dystrophinopathy is Duchenne muscular dystrophy (DMD). DMD is X-linked recessive disease caused by a mutation in the dystrophin gene, that, in most cases, leads to its absence in the DGC of striated muscle cells. As mentioned above, dystrophin forms part of the dystrophin associated glycoprotein complex (DGC) and is postulated to function as a membrane stabilizer (Law et al., 2020) and scaffold protein, whereby an absence of dystrophin typically presents with skeletal muscle weakness and with contraction-induced damage.
DMD was first described at length in the mid-1800s in England (Meryon, 1852) and France (Duchenne & Boulogne, 1868), with the condition being named after Guillaume-Benjamin-Amand Duchenne, a French neurologist. Although little was known regarding the underlying mechanisms behind DMD at this time, the patients presented with primary symptoms that are still common in DMD patients. Later in the 1800s W.R. Gowers described the way in DMD patients compensate inherent muscle weakness to stand up. Often, they will ‘climb’ up their legs with their arms to stand and this was used as a diagnostic feature known as Gowers sign (Figure 2). Other than a terse understanding of muscular weakness seen at a young age in males, the cause of DMD was unknown until 1986, over 100 years after Duchenne had described the condition. In 1986 the DMD gene was located and a year later the mutations the result in dystrophin absence were elucidated (Hoffman et al., 1987). These advances in the understanding of the disorder allowed for a greater understanding of the condition, and thus a more targeted approach for DMD treatment.

Figure 2. Illustration of how patients with Duchenne muscular dystrophy utilize their legs to overcome muscular weakness caused by the disease.
The dystrophin gene spans greater than 2,200 kb and contains 79 exons making it the largest known human gene (Koenig et al., 1987) and is postulated to play a mechanical role in maintaining cell membrane (i.e., sarcolemmal) integrity (Valera et al., 2021). Since DMD is an X-linked recessive disease, female carriers having only one X chromosome with a dystrophin gene mutation will have a 50% chance of passing on the disease to their son. While a majority of cases of DMD are inherited, 30% of cases of DMD are the result of spontaneous mutations (Dent et al., 2005).

As previously stated, dystrophin comprises part of the DGC. The DGC is multimeric forming a structural link between the F-actin cytoskeleton proteins and the extracellular matrix in striated muscle (Ervasti & Campbell, 1993). Mechanical support is provided by the DGC to the plasma membrane of these striated muscle cells during contraction, with an absence of dystrophin causing weakness in the plasma membrane. It is thought that this absence of dystrophin may diminish sarcolemma integrity and
flexibility causing alterations in specific calcium handling and mechanical damage of the cell.

DMD affects 1 in 5,000 boys born in the United States (Nigro et al., 1990). Symptoms typically appear from the ages of 2 to 5 years, with patients presenting with a waddling gait and difficulty climbing stairs. From this, symptoms progressively worsen eventually resulting in frequent falls and a loss of ambulation, usually at ages 7 to 13 years. Symptoms continue to deteriorate with weakness progressing to the upper limbs. Historically, patient mortality results from respiratory deficits, most notably impairments of the diaphragm, in the late teen to early 20s. However, advances in treatment, including

Figure 3. A schematic representation of the location of dystrophin in relation to the myofilaments.
corticosteroid treatment and respiratory therapy, have resulted in the unmasking of a cardiac involvement in DMD (Eagle et al., 2002; Robson, 2010). Additional have confirmed that improved skeletal muscle treatment (Cheeran et al., 2017) has extended life span and continued to unmask higher prevalence of cardiomyopathy in DMD patients (Shih et al., 2020). From these cardiomyopathies heart failure can develop (Kogelman et al., 2018). As such, it is pertinent to elucidate the mechanisms by which DMD causes heart failure. It is postulated that DMD could affect the contractile properties of cardiac myofilaments.

As previously mentioned, dystrophin has been postulated to act as a membrane stabilizer. Support for the this has been shown in studies which examined the effect of contraction upon sarcolemmal stability in models of DMD. When dystrophin is absent, a greater instability of the sarcolemma was seen, shown by an increase in muscle membrane damage due to increased stress (Houang et al., 2018). Muscle membrane damage was seen in the form of micro tears, which resulted in extracellular calcium influx, which in turn activates sarcoplasmic reticulum calcium release causing calcium overload, eventually leading to myocyte death (Houang et al., 2018). Further studies on isolated working hearts of a murine DMD model showed an elevation in lactate dehydrogenase (LDH) levels in the DMD model compared to control. LDH, a marker of cardiac injury, showed elevated levels in conditions of normal preload and afterload challenge in DMD mice (Danialou et al., 2001). These increased LDH levels highlight the increased sarcolemmal damage seen in DMD models when compared to control models.
**Contractile properties in DMD**

A few studies have investigated cardiac muscle contractile properties using DMD models. Twitch force was decreased in isolated, intact trabeculae muscle of 2-month mdx mice (Janssen et al., 2005). This study also utilized a more severe double knockout (DKO) mouse, which was lacking both dystrophin and utrophin, a protein postulated to replace dystrophin in DMD (Blake et al., 1996). In the DKO mouse model a further reduction in twitch force was observed. In earlier studies, changes in contraction were seen before the presence of fibrosis or necrosis, promoting the idea that decreased function is due to a loss of dystrophin and not the result of cell death or fibrotic remodeling (Sapp et al., 1996). Additionally, the time to half-relaxation were significantly increased in an mdx murine model of 12-14 weeks (Sapp et al., 1996), further indicating the effect a loss of dystrophin may have upon contractile function. Together, these studies show how contractile properties are altered in DMD mouse models, while also implicating a loss of dystrophin as the cause for these changes.

A series of papers investigated dystrophic cardiomyopathy in a canine Golden Retriever Muscular Dystrophy (GRMD). Left ventricular fractional shortening was reduced by 11% in 9-12 month old GRMD dogs, as assessed by echocardiography (Su et al., 2012). In addition, there was ventricular remodeling with GRMD indexed by increased left ventricular end diastolic dimension. This group also investigated contractile properties of single permeabilized cardiac myocytes from the GRMD model of DMD. Cardiac myocytes preparations displayed reduced maximal Ca\(^{2+}\) activated tension and reduced length dependence of Ca\(^{2+}\) sensitivity of force (Ait Mou et al., 2018). At present,
there are no studies that investigate cardiac myofilament contractile properties (that includes force, rates of force, loaded shortening and power output and their sarcomere length dependence in the same cardiac myocyte preparation using a DMD\textsuperscript{mdx-4cv} mouse model. The DMD\textsuperscript{mdx-4cv} mouse model is a commonly used murine model which closely approximates human DMD. In this model telomere lengths are shortened to mirror the human DMD phenotype more closely as the cardiomyocytes of humans with DMD have telomeres which are 50% shorter than those without DMD. Mdx-4cv mice also have a 10-fold decrease in revertant myofibers when compared to the mdx mouse, which is another model of DMD (Yucel et al., 2018).

**Hypothesis**

We hypothesized that contractile properties and their length dependence would be depressed in cardiac myofilaments from DMD\textsuperscript{mdx-4cv} mice.

**Methods**

**Animal model**

All procedures involving animals were performed in accordance with the Animal Care and Use Committee of the University of Missouri. The DMD\textsuperscript{mdx-4cv} murine model was used for the study. DMD\textsuperscript{mdx-4cv} mice were used due to the model most closely approximating human DMD (Yucel et al., 2018). Mice were between the ages of 4-12 months. Wild-type littermate mice were used as the control group.
Solutions

Relaxing solution for permeabilized cardiac myocytes contained: 1 mM DTT, 100 mM KCl, 10 mM Imidazole, 2.0 mM EGTA, 4.0 mM ATP, 1 mM (free, 5 total) MgCl$_2$. Minimal Ca$^{2+}$ activating solution (pCa 9.0) for experimental protocol contained: 7.00 mM EGTA, 20 mM Imidazole, 5.42 mM MgCl$_2$, 72.37 mM KCl, 0.016 mM CaCl$_2$, 14.50 mM PCr, 4.7 mM ATP. Maximal Ca$^{2+}$ activating solution (pCa 4.5) for experimental protocol contained: 7.00 mM EGTA, 20 mM Imidazole, 5.26 mM MgCl$_2$, 60.25 mM KCl, 7.01 mM CaCl$_2$, 14.50 mM PCr, 4.81 mM ATP. A range of Ca$^{2+}$ concentrations for experiments was prepared by varying combinations of maximal and minimal Ca$^{2+}$ solutions. Pre-activating solution contained: 0.5 mM EGTA, 20 mM Imidazole, 5.42 mM MgCl$_2$, 98.18 mM KCl, 0.016 mM CaCl$_2$, 14.50 mM PCr, and 4.8 mM ATP.

Permeabilized cardiac myocyte preparations

Mice were weighed and then anaesthetized using isoflurane and their hearts were excised. Following excision the heart was placed into an ice-cold relaxing solution. Next, the left ventricle was cut into 2-3mm pieces and then placed into a Waring blender for 5-10s. The resulting suspension of cells was then be centrifuged for 105s at 165g. Myocytes were then permeabilized using Triton X-100. Permeabilized myocytes were attached between a force transducer and torque motor by placing myocyte preparation into stainless steel troughs (25 gauge) and secured by overlaying the ends of the myocyte with 0.5 mm length of 4-0 monofilament nylon sutures (Ethicon, Inc.), then the sutures were secured into the troughs utilizing loops of 10-0 monofilament (Ethicon, Inc.). Attachment
took place using a stereomicroscope (90x zoom). The myocyte preparation apparatus was transferred and mounted onto an inverted microscope (model IX-70, Olympus Instrument Co., Japan). A capacitance-gauge transducer and 10x amplifier (Aurora Scientific, Inc, Aurora ON, Canada) was used for mechanical measurements. Changes in length were introduced using a DC torque motor (model 308, Aurora Scientific, Inc.) by voltage commands from a personal computer using a 16-bit D/A converter (AT-MIO-16E-1, National Instruments Corp., Austin, TX, USA). Length and force signals were digitized at 1kHz and stored on a personal computer in LabView for Windows (National Instruments Corp.). The length of the sarcomere was measured using a IonOptix SarcLen system (IonOptix, Milton, MA), which uses a fast Fourier transform algorithm on the video image of the myocyte. Once attached the relaxed cardiomyocyte preparation was adjusted to a SL of ~2.25µm and passive tension was assessed by slacking the preparation in pCa 9.0 solution. Force, rate of force, and loaded shortening velocity measurements at two sarcomere lengths were made at were made at 15 ± 1°C as previously described (Hanft et al., 2017; Hinken & McDonald, 2004; McDonald, 2000).

**Data and Statistical Analysis**

Force redevelopment following a slack-restretch maneuver was fit by a single exponential equation:

\[ F = F_{\text{max}} (1 - e^{-kt_{r1}}), \]

where \( F \) is tension at time \( t \), \( F_{\text{max}} \) is maximal tension, and \( k_{r1} \) is the rate constant of force development.
Myocyte length traces were fit to a single decaying exponential equation:

\[ L = Ae^{-kt} + C \]

Where \( L \) is cell length at time \( t \), \( A \) and \( C \) are constants with dimensions of length, and the rate of shortening is \( k \) (\( k_{\text{shortening}} \)). Velocity of shortening at time, \( t \), was determined by the slope of the tangent to the fitted curve at \( t \). Loaded shortening velocities were calculated from the onset of force clamp at \( t = 0 \)ms. The Hill equation (Hill, 1938) was used to fit hyperbolic force-velocity curves to relative force-velocity data.

\[(P + a) (V + b) = (P_0 + a) b\]

\( P \) is the force during shortening at velocity \( V \); \( P_0 \) is the maximal isometric force; \( a \) is a constant with dimensions of force, while \( b \) is a constant with dimensions of velocity. Force-velocity data is normalized to isometric force. Force was multiplied by velocity to obtain power-load curves, while peak normalized power output (PNPO) values were obtained by the multiplication of relative force at optimum power by velocity at optimum power. Curves were fit using commercial software (SigmaPlot) and a custom program written using Qbasic. Wild-type and DMD\( ^{\text{mdx-4cv}} \) cardiomyocyte preparations were compared using Student’s \( t \) test. Wild-type and DMD\( ^{\text{mdx-4cv}} \) preparations were compared before versus after mavacamten treatment using paired \( t \) test. A P-value below 0.05 was considered significant. Significance was denoted by *. 
Results

Following disruption of the ventricles and permeabilization of the cardiac myocytes, immunofluorescent staining was performed to assess dystrophin content in preparations from wildtype and DMD\textsuperscript{mdx-4cv} mice. While dystrophin was present in permeabilized cardiac myocyte preparations from control hearts, dystrophin was absent myocyte preparations from DMD\textsuperscript{mdx-4cv} mice (Figure 4).

Figure 4. Immunofluorescent images show the presence and absence of dystrophin in permeabilized cardiac myocytes from wild-type littermates and DMD\textsuperscript{mdx-4cv} mouse hearts, respectively.

Contractile properties

Contractile properties were measured in permeabilized cardiac myocytes from wild-type and DMD\textsuperscript{mdx-4cv} mice. The characteristics of permeabilized cardiac myocyte preparations from both groups are provided in Table 1. The workflow for these experiments is illustrated in Figure 5. First, force and rate of force development were measured during maximal Ca\textsuperscript{2+} activation. This was followed by force, rate of force, and loaded shortening over a range of force clamps at both long (~2.25μm) and then short (~1.95μm) sarcomere length during submaximal Ca\textsuperscript{2+} activations (Figure 5).
Table 1. Control and Mdx-4cv Mouse Permeabilized Cardiac Myocyte Preparations at Long Sarcomere Length (SL).

<table>
<thead>
<tr>
<th>Control (N=7)</th>
<th>Cardiac Myocyte Preparations</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>SL (µm)</th>
<th>Passive Tension (kN·m⁻²)</th>
<th>Maximum Force (µN)</th>
<th>Maximum Tension (kN·m⁻²)</th>
<th>pCa for Half-Max. Tension</th>
<th>Relative Tension Half-Max. pCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 7</td>
<td>115 ± 9</td>
<td>21 ± 1</td>
<td>2.29 ± 0.01</td>
<td>2.07 ± 0.33</td>
<td>10.2 ± 1.2</td>
<td>46 ± 7</td>
<td>6.06 ± 0.02</td>
<td>0.56 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Mdx-4cv (N=8)</td>
<td>123 ± 4</td>
<td>25 ± 1</td>
<td>2.28 ± 0.02</td>
<td>1.31 ± 0.22</td>
<td>13.6 ± 1.6</td>
<td>45 ± 7</td>
<td>6.09 ± 0.02</td>
<td>0.50 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM.

Figure 5. Permeabilized cardiac myocyte preparation. Top, a photomicrograph of a representative mouse permeabilized cardiac myocyte preparation and a schematic showing the permeabilized myocyte attachment. The lower panel show length and force traces during maximal Ca²⁺ activation at the beginning of the experiment, followed by representative length traces during a series of force clamps at both long and short sarcomere lengths during half-maximal Ca²⁺ activation, and a final length and force trace during maximal Ca²⁺ activation.
Maximal tension was similar between the wild-type and the DMD$^{mdx-4cv}$ littermate preparations (DMD: $45 \pm 23$ kN $\cdot$ m$^{-2}$; WT: $46 \pm 17$ kN $\cdot$ m$^{-2}$) (Figure 6).

![Figure 6](image)

*Figure 6.* Maximal Tension (kN*m$^{-2}$) of permeabilized cardiac myocyte preparations from wild-type (WT) littermates and DMD$^{mdx-4cv}$ mice.

In addition, there was no difference in rates of force development (as indexed by the rate constant, $k_r$) following slack-restretch manoeuvre during maximal Ca$^{2+}$ activations between wild-type and the DMD$^{mdx-4cv}$ littermate preparations (DMD: $10.9 \pm 0.9$ s$^{-1}$, WT: $10.5 \pm 1.3$ s$^{-1}$) (Figure 7).
During submaximal Ca\(^{2+}\) activations, there was a similar sarcomere length dependence of force in permeabilized cardiac myocyte preparations from wild-type and the DMD\(^{mdx-4cv}\) littermates (Figure 8).

Submaximal force development rates (k\(_{tr}\)) were also similar between the wild-type and DMD\(^{mdx-4cv}\) preparations at long sarcomere lengths (~2.25\(\mu\)m) and short sarcomere lengths (SL) (~1.95\(\mu\)m) (DMD: Long SL: 4.96 ± 2.27 s\(^{-1}\), Short SL: 5.02 ± 2.08 s\(^{-1}\) ; Figure 8.)
WT: Long SL: 6.13 ± 3.53 s⁻¹, Short SL: 5.94 ± 2.99 s⁻¹ (Figure 9).

Figure 9. $k_v$ values showed no significant difference between groups.

Figure 10 shows representative normalized force-velocity and power-load relationships from a permeabilized cardiac myocyte preparation from a wild-type mouse (left panel) and a DMD<sup>mdx-4cv</sup> mouse (right panel). Interestingly, there was a greater sarcomere length dependence of peak normalized power output in permeabilized cardiac myocyte preparations from DMD<sup>mdx-4cv</sup> mice (DMD: ΔPNPO long SL-short SL: 0.032 ± 0.022; WT: ΔPNPO: 0.0004 ± 0.0076, p < 0.05) (Figure 11). Table 2 shows force-velocity and power-load characteristics at long and short sarcomere lengths from permeabilized cardiac myocyte preparations between groups.
Figure 10. Force-velocity and power-load curves at long sarcomere length (~2.25 µm) and short sarcomere length (~1.95 µm) in a permeabilized cardiac myocyte preparation from wild-type (WT) littermate (left) and DMD$^{mdx-4cv}$ mice (right).
Figure 12 shows sarcomere length dependence of peak absolute power of cardiac myocyte preparations between groups. Length dependence of peak power (pW) was greater in the DMD\textsuperscript{mdx-4cv} preparations when compared to the wild-type (WT) littermate preparations. From short sarcomere lengths (~1.95\textmu m) to long sarcomere lengths (~2.25\textmu m) power output increased 85.67 ± 40.58 pW in the DMD\textsuperscript{mdx-4cv} preparations but only 31.00 ± 19.52 pW in the wildtype preparations. Since the sarcomere length dependence of force was similar between groups the greater sarcomere length

![Figure 11.](image)

**Figure 11.** Change in peak normalized power output (\(\Delta PNPO\)) between long sarcomere length (~2.25\textmu m) and short sarcomere length (~1.95\textmu m) in permeabilized cardiac myocyte preparations from wild-type (WT) littermates and DMD\textsuperscript{mdx-4cv} mice. Length dependence of PNPO was significantly increased in the DMD\textsuperscript{mdx-4cv} mice (\(P=0.002\)).

**Table 2.** Permeabilized Cardiac Myocyte Preparation Force-Velocity and Power-Load Characteristics at Long and Short Sarcomere Length (SL) during Sub-Maximal Ca\textsuperscript{2+} Activation.

<table>
<thead>
<tr>
<th></th>
<th>SL ((\mu\text{m}))</th>
<th>(F_{opt}) ((P/P_0))</th>
<th>(V_{opt}) (ML·s\textsuperscript{-1})</th>
<th>(V_{max}) (ML·s\textsuperscript{-1})</th>
<th>Peak absolute power output ((\mu\text{W mg}\textsuperscript{-1}))</th>
<th>Peak normalized power output ((P/P_0\cdot\text{ML·s}\textsuperscript{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Long SL</td>
<td>2.29 ± 0.01</td>
<td>0.29 ± 0.02</td>
<td>0.53 ± 0.06</td>
<td>1.68 ± 0.17</td>
<td>3.85 ± 0.71</td>
<td>0.155 ± 0.022</td>
</tr>
<tr>
<td>Control Short SL</td>
<td>1.95 ± 0.00</td>
<td>0.30 ± 0.02</td>
<td>0.52 ± 0.05</td>
<td>1.75 ± 0.19</td>
<td>3.32 ± 0.44</td>
<td>0.156 ± 0.020</td>
</tr>
<tr>
<td>Mdx\textsuperscript{4cv} Long SL</td>
<td>2.28 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.58 ± 0.04</td>
<td>2.07 ± 0.21</td>
<td>3.32 ± 0.54</td>
<td>0.170 ± 0.014</td>
</tr>
<tr>
<td>Mdx\textsuperscript{4cv} Short SL</td>
<td>1.95 ± 0.00</td>
<td>0.30 ± 0.02</td>
<td>0.48 ± 0.03</td>
<td>1.59 ± 0.11</td>
<td>1.51 ± 0.29</td>
<td>0.138 ± 0.012</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *\(p < 0.05\) vs Control.

Figure 12 shows sarcomere length dependence of peak absolute power of cardiac myocyte preparations between groups. Length dependence of peak power (pW) was greater in the DMD\textsuperscript{mdx-4cv} preparations when compared to the wild-type littermate preparations. From short sarcomere lengths (~1.95\textmu m) to long sarcomere lengths (~2.25\textmu m) power output increased 85.67 ± 40.58 pW in the DMDmdx-4cv preparations but only 31.00 ± 19.52 pW in the wildtype preparations. Since the sarcomere length dependence of force was similar between groups the greater sarcomere length
dependence of absolute power arose from greater length dependency of loaded shortening rather than force (this is consistent with the peak normalized power output results in Figure 11).

From these results it appears that a loss of dystrophin as seen in the DMD^{mdx-4cv} alters some cardiac myofilament contractile properties. While maximal tension and rates of force development were not significantly different between groups, there was greater sarcomere length dependence of peak power output in myocyte preparations lacking dystrophin (i.e., from the DMD^{mdx-4cv} mice). These results implicate that dystrophin attenuates sarcomere length dependence of loaded shortening in mammalian cardiac myocytes. These result leads us to reject our hypothesis that the sarcomere length dependence would be decreased in the DMD mouse model.

Figure 12. Change in peak power (ΔpW) from long sarcomere length (2.25µm) to short sarcomere length (1.95µm) in cardiac myocyte preparations from wild-type (WT) littermates and DMD^{mdx-4cv} mice. Length dependence of peak power was significantly increased in the DMD^{mdx-4cv} mice when compared to wild-type (P = 0.002).
Mavacamten treatment

We next investigated the effects of mavacamten on contractile properties of cardiac myofilaments from DMD mice. Mavacamten has been shown to decrease contractility in myocardial preparations (Green et al., 2016), making it a promising drug to treat hypercontractility disorders such as HCM and it stands to reason that it may be beneficial for dystrophic cardiomyopathy, which is thought to arise, at least in part, from contraction induced damage. Our studies addressed the effects of mavacamten on contractile properties and their sarcomere length dependence in permeabilized cardiac myocyte preparations from wild-type and DMD<sup>mdx-4cv</sup> mice.

0.5 mm Mavacamten was added to pCa solutions and contractile properties were measured in permeabilized cardiac myocytes from wild-type and DMD<sup>mdx-4cv</sup> mice. The characteristics of permeabilized cardiac myocyte preparations from both treatment groups are provided in Table 3.

Table 3. Mavacamten- Control and Mdx<sup>-4cv</sup> Mouse Permeabilized Cardiac Myocyte Preparations at Long Sarcomere Length (SL).

<table>
<thead>
<tr>
<th>Cardiac Myocyte Preparations</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>SL (µm)</th>
<th>Passive Tension (kN·m&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>Maximum Force (µN)</th>
<th>Maximum Tension (kN·m&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>Maximum Tension With Mava (kN·m&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>pCa for Half-Max. Tension</th>
<th>Relative Tension Half-Max. pCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>n = 3</td>
<td>115 ± 15</td>
<td>20 ± 2</td>
<td>2.31 ± 0.01</td>
<td>1.55 ± 0.66</td>
<td>11.9 ± 1.2</td>
<td>56 ± 11</td>
<td>5.7 ± 0.1</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>Mdx&lt;sup&gt;-4cv&lt;/sup&gt;</td>
<td>n = 4</td>
<td>147 ± 21</td>
<td>29 ± 3</td>
<td>2.28 ± 0.03</td>
<td>1.24 ± 0.56</td>
<td>22.8 ± 5.3</td>
<td>48 ± 6</td>
<td>5.7 ± 0.1</td>
<td>0.54 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

Figure 13 shows that 0.5 µM mavacamten significantly decreased maximal tension (by ~45%) in cardiac myocyte preparations from both wild-type and DMD<sup>mdx-4cv</sup>
mice. This result is similar to the extent of force decline by mavacamten previously reported in permeabilized multi-cellular myocardial preparations (Awinda et al., 2020). These results indicates that mavacamten mitigates tension generation in permeabilized cardiomyocytes regardless of dystrophin content.

![Figure 13](image)

*Figure 13.* Maximal tension (kN*m⁻²) before and after mavacamten (mava) administration in wild-type (WT) and DMD\textsuperscript{mdx-4cv} mice. Maximal tension was significantly decreased in wild-type and DMD\textsuperscript{mdx-4cv} mice following treatment with mavacamten.

In addition, rates of force development were also significantly decreased by mavacamten both the wild-type and DMD\textsuperscript{mdx-4cv} preparations, highlighting the ability of mavacamten to decrease force kinetics in single permeabilized cardiomyocytes (*Figure 14*)
Figure 14. Force development ($k_r (s^{-1})$) before and after mavacamten (mava) administration in wild-type (WT) and DMD$^{mdx-4cv}$ mice. Force development was significantly decreased in wild-type and DMD$^{mdx-4cv}$ mice following treatment with mavacamten.

Mavacamten tended to steepen sarcomere length-tension relationships in cardiac myocyte preparations from both wild-type and DMD$^{mdx-4cv}$ preparations (Figure 15).
Interestingly, mavacamten normalized the sarcomere length dependence of peak absolute power in cardiac myocyte preparations between groups (Figure 16). In fact, the sarcomere length dependence of absolute peak power shifted to a higher value in the wild-type myocytes (more like the wild-type and DMD<sup>mdx-4cv</sup>) after mavacamten. Thus, mavacamten caused greater length dependence of peak power in cardiac myocyte preparations from wild-type mice (Figure 16). This increase in sarcomere length dependence of power from untreated to treated cardiac myocyte preparations was not observed in the DMD<sup>mdx-4cv</sup> preparations. These results implicate that mavacamten decreases overall contractility but maintains or even augments sarcomere length dependence of contraction in mammalian cardiac myofilaments. Table 4 shows force-velocity and power-load characteristics at long and short sarcomere lengths from permeabilized cardiac myocyte preparations treated with 0.5 mM Mavacamten.
**Figure 16.** Change in peak power (pW) between wild-type (WT), DMD<sup>mdx-4cv</sup> and mavacamten (after mava) treated permeabilized cardiomyocytes. Mavacamten treatment group included both wild-type and DMD<sup>mdx-4cv</sup> cardiomyocytes (N=3, N=4, respectively). An increase in the length dependence of peak power was seen in both DMD<sup>mdx-4cv</sup> and mavacamten treated cardiomyocytes.

**Table 4.** Mavacamten- Permeabilized Cardiac Myocyte Preparation Force-Velocity and Power load Characteristics at Long and Short Sarcomere Length (SL) during Sub-maximal Ca<sup>2+</sup> Activation.

<table>
<thead>
<tr>
<th>SL (µm)</th>
<th>F&lt;sub&gt;opt&lt;/sub&gt; (P/P&lt;sub&gt;0&lt;/sub&gt;)</th>
<th>V&lt;sub&gt;opt&lt;/sub&gt; (ML·s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (ML·s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Peak absolute power output (µW mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Peak normalized power output (P/P&lt;sub&gt;0&lt;/sub&gt;·ML·s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Long SL</td>
<td>2.31 ± 0.01</td>
<td>0.29 ± 0.02</td>
<td>0.37 ± 0.07</td>
<td>1.27 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Short SL</td>
<td>1.99 ± 0.04</td>
<td>0.28 ± 0.02</td>
<td>0.39 ± 0.06</td>
<td>1.42 ± 0.20</td>
</tr>
<tr>
<td>Mdx&lt;sup&gt;4cv&lt;/sup&gt;</td>
<td>Long SL</td>
<td>2.28 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.52 ± 0.07</td>
<td>2.03 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Short SL</td>
<td>2.00 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>0.40 ± 0.08</td>
<td>1.60 ± 0.29</td>
</tr>
</tbody>
</table>

Values are means ± SEM. * p < 0.05 vs Control.
Discussion

Murine permeabilized cardiac myocyte preparations, from DMD and wild-type (WT) littermate mice, were subjected to measures of contractile properties. Maximal tension and rates of force development were similar between the WT and DMD groups. A greater sarcomere length dependence of peak absolute and normalized power output was seen in the DMD mice, implicating greater length dependence of loaded shortening (and thus power) in cardiac myofilaments from DMD mice. This increased length dependence could potentially exacerbate hemodynamic load dependent myocyte damage in DMD hearts. As such, this potentially elucidates a mechano-transduction mechanism by which myocytes lacking dystrophin have a greater propensity for ventricular damage and consequent cardiomyopathy.

Duchenne Muscular Dystrophy (DMD) is a devastating form of dystrophinopathies. DMD is an X-linked recessive disease resulting from a complete absence of the dystrophin gene. Advances in treatment of skeletal muscle, such as corticosteroid therapy (Matthews et al., 2016), have unmasked a previously unappreciated cardiac component of the disease. As such the prevalence of patients with DMD succumbing to heart failure is increasing, causing a need to better understand the condition to allow effective, targeted treatments. While some prior research has examined the effect of DMD upon contractile properties, no work has examined the effect of DMD specifically upon power generating capacity of single permeabilized cardiac myocyte preparations.
The focus of this study was to examine how the loss of dystrophin affects contractile properties of permeabilized cardiac myocyte preparations in a murine model of DMD. Maximal tension and $k_t$ were not significantly different between the cardiomyocytes of the DMD\textsuperscript{mdx-4cv} and wild-type mice. However, a greater length dependence of loaded shortening and power was seen in the DMD\textsuperscript{mdx-4cv} cardiomyocytes when compared to the wild-type. This was contrary to our hypothesis, which postulated that these factors would be decreased in the DMD murine model. Following the initial studies, the effects of mavacamten was tested upon both groups. Mavacamten decreased maximal tension and $k_t$ to a similar extent in both wild-type and DMD\textsuperscript{mdx-4cv} cardiac myocyte preparations. It was postulated that mavacamten would attenuate any increase in length dependence power seen in the DMD\textsuperscript{mdx-4cv} cardiomyocytes. However, mavacamten treatment actually augmented the length dependence of wild-type cardiac myocyte preparations, so that they were similar when compared to the DMD\textsuperscript{mdx-4cv} cardiomyocytes, either with or without mavacamten treatment.

These findings show promise for the use of mavacamten as a potential treatment for DMD for a variety of reasons. First, since mavacamten decreased both maximal tension and force development kinetics, it is plausible that contraction-induced damage may be lessened with mavacamten treatment. As patients with DMD are more susceptible to contraction-induced damage, attenuation of tension by ventricular cardiac myocytes with mavacamten may offer a new avenue to lessen damage induce by myocyte contractile force. Additionally, slower force development rates further support the possibility that mavacamten may be effective in reducing myocardial contraction-induced
damage in DMD patient. While we predicted that that mavacamten would minimize sarcomere length dependence of contractile properties, this was not the case; in fact, mavacamten appeared to sustain or even augment sarcomere length dependence of contractile properties. Thus, mavacamten may be ideally suited to treat contraction mediated damage disorders by reducing force and kinetics yet still maintaining exquisite sarcomere length dependence of contractility, i.e., the ventricles retain the intrinsic capacity to rapidly respond to changes in metabolic demand by the Frank-Starling related mechanisms (i.e., increased end diastolic volume yields greater stroke volume).

Additional Studies

It appears that no studies have been performed directly examining the effects of DMD upon the Frank-Starling relationship in isolated or intact hearts. However, from our results it is hypothesized that that a greater end diastolic volume would elicit a greater ventricular pressure in hearts with cardiac myocytes lacking dystrophin. Due to the increased vulnerability of striated muscle cells lacking dystrophin, higher pressure generation could potentially result in more myocardial injury and chronic impairments. In fact, a recent study from our group found higher pre-loads (i.e., the load the ventricles experience before contraction) caused myocyte damage in isolated working hearts from DMD_{mdx-4cv} mice (unpublished). In parallel experiments, it was found that LDH levels (a marker of cardiac damage) were increased in the DMD_{mdx-4cv} Langendorff perfused hearts when compared to the wild-type hearts (Figure 17). Given our mavacamten results on permeabilized cardiac myocyte preparations, it stands to reason that mavacamten may reduce pre-load dependence of cardiac damage in mdx hearts. Following these initial studies observing the difference in cardiac damage between WT mice and DMD_{mdx-4cv}
mice, I performed additional preliminary studies to characterize the drug dose-response relationship of mavacamten and left ventricular pressure output in isolated mouse hearts (Figure 18). Five concentrations of mavacamten were added to isolated hearts to assess their effect upon pressure output. The effect of the drug versus pressure output was plotted with 100% drug response meaning a reduction of pressure to ~0 mmHg. As mavacamten dosage was increased, pressure decreased (Figure 18). These preliminary experiments provided a framework for future experiments to test the hypothesis that mavacamten cardioprotects against pre-load induced damage in hearts from DMD\textsuperscript{mdx-4cv} mice, which could lead to studies address the role of chronic mavacamten treatment on long-term prevention of dystrophic cardiomyopathy.
**Figure 17.** LDH levels over time in DMD\textsuperscript{mdx-4cv} and wild-type (WT) Langendorff perfused hearts. LDH levels were elevated in the DMD\textsuperscript{mdx-4cv} hearts when compared to the wild-type.

**Figure 18.** Drug response curve showing the relationship between Mavacamten concentration (μM) and the response (%). Response being the ability of the drug to decrease pressure output, with 100% response being a decrease of pressure to 0mmHg.
Overall findings

This study assessed the contractile properties of single permeabilized cardiac myocyte preparations in wild-type and DMD\textsuperscript{mdx-4cv} murine models. An increased length dependence of power generation was seen in the DMD\textsuperscript{mdx-4cv} preparations, which suggests that dystrophin has a role in regulating the contractile properties of cardiac muscle, and that a loss of dystrophin results in an augmented sarcomere length dependence of cardiac myofilament power output. Additional studies provided rationale for potential therapeutic benefits of the myosin stabilizer, mavacamten, on dystrophic hearts. In permeabilized cardiac myocyte preparations, mavacamten reduced force and rate of force but maintained sarcomere length dependence of contractile properties, which could potential attenuate contraction-induced damage while maintaining necessary beat-to-beat regulation of cardiac output. Such effects seem well-suited to assure precise balance cardiac output between the right and left ventricles (to prevent congestive heart failure) and to meet ever-changing metabolic demands of the tissues.

Limitations

Cardiac myocyte assays have several limitations. Due to the nature of selecting rod-shaped cardiac myocyte-sized preparations it is possible that the population selected is inherently biased towards selecting preparations which have an increased maximum tension-generating capacity. It is possible that myocytes that better reflect a population are not studied due to the specific requirements needed for a measurable preparation. As such it is possible that there is an increased chance of a false negative regarding tension generating capacity. Additionally, the process of measuring single cardiac myocyte
contractile properties is labor-intensive, resulting in a limited sample size. A further limitation of the study is that short sarcomere length had to be increased following mavacamten treatment, as there was insufficient tension at 1.95μm. This could have potentially altered the relationship seen between the short and long sarcomere lengths in these preparations. Finally, the mice population had a wide age range, from 4-12 months, which could potentially result in variability in the results.

**Next steps**

This study assessed the contractile properties of single permeabilized cardiac myocyte preparations in wild-type and DMD\textsuperscript{mdx-4cv} murine models. An increased length dependence of power generation was seen in the DMD\textsuperscript{mdx-4cv} preparations, which suggests that dystrophin has a role in regulating the contractile properties of cardiac muscle, and that a loss of dystrophin results in an augmented length tension relationship.

Future studies could examine the effect of a loss of dystrophin in whole heart models to examine the effect upon the Frank Starling relationship. Furthermore, if contractility is augmented in DMD, therapies which attenuate contractility in cardiac muscle could also be investigated. If contractility was attenuated, it is possible that contraction induced damage could be decreased, thus leading to more informed and targeted treatments could be provided. Preliminary data shows increased damage, measured by LDH levels, in DMD\textsuperscript{mdx-4cv} isolated whole hearts when compared to WT, and the effectiveness of mavacamten in decreasing power output in a dose dependent manner in isolated whole hearts. To build upon these studies the effect of mavacamten
upon LDH levels could be examined in isolated whole hearts of WT and DMD\textsuperscript{mdx-4cv} mice. Additionally, DMD has implications with regards to fibrosis and reactive oxygen species (ROS) metabolism, which could potentially negatively affect the myofilament function (Chung et al., 2017), as such studies could be done to examine how these factors affect cardiomyocyte function, and how to protect against disadvantageous alterations.

Overall, work from this thesis provided insight into the changes in cardiac myofilament function seen due to a loss of dystrophin in DMD, it also offers potential for therapeutic treatment for DMD via the use of a myosin specific small molecule modulator of contraction. Although the hypothesis that length dependence of power and loaded shortening would be decreased in DMD\textsuperscript{mdx-4cv} was not seen to be true, the results suggest that dystrophin acts as a modulator for length dependent activation in cardiomyocytes.
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