THE INFLUENCE OF SEX HORMONES ON CARDIAC AND SKELETAL MUSCLE FUNCTION IN THE *MDX* MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

BY

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a fatal recessive genetic human disease affecting one in 3500 live male births. DMD is progressive, there is no cure, and patients typically die of respiratory or cardiac failure in their second decade of life. Clinical disease symptoms are exacerbated at the onset of puberty and the physiological basis of this is unknown. The *mdx* mouse is the preferred experimental animal model of DMD, although aspects of the model remain poorly understood. This dissertation characterises physiological and histological features of the dystrophic *mdx* mouse in response to manipulations of hormonal status including testosterone treatment, surgical castration, and oestrogen treatment. Sex-specific differences in the *mdx* were also examined. Furthermore, physiological and histological features of the dystrophic *mdx* mouse model throughout the *mdx* lifespan were evaluated. Cardiac muscle contractility, left atrial response to exogenous calcium, and the contractile properties of both fast-twitch (EDL) and slow-twitch (SOL) skeletal muscles were examined in male *mdx* mice ranging from 14 to 330 days of age. Testosterone treatment produced a non-significant trend towards a dose-dependent decrease in both basal and maximal left atrial contractility in the *mdx*. Surgical castration produced no significant cardiac effects within mouse strains. The *mdx* castrates had a 45% lower maximum atrial force of contraction than control castrates ($p<0.05$). Conversely, oestrogen treatment significantly improved cardiac contractility in the *mdx*. An increase in basal left atrial contractility was evident at doses of 0.08 mg/kg/day ($p<0.05$) and 0.16 mg/kg/day ($p<0.01$) and in maximum left atrial contractility at a dose of 0.16 mg/kg/day ($p<0.01$). Gender studies showed cardiac forces in *mdx* were not different between males and females at any age tested and that both sexes in *mdx* had a dampened cardiac responsiveness to exogenous calcium. Skeletal muscle function studies showed that castration produced a 25% increase in *mdx* EDL specific force generation ($p<0.01$) and no increase in SOL forces. Oestrogen treatment produced a non-significant trend towards increased EDL forces and a 29% increase in SOL specific force at a dose of 0.16 mg/kg/day ($p<0.05$). Gender studies revealed no differences between male and female *mdx* in terms of skeletal muscle force production. Further to the hormonal investigations, lifespan characterisation studies revealed that the *mdx* mouse showed reduced basal and maximal left atrial contractility specifically at ages 14 and 90 days ($p<0.05$). Skeletal muscle studies showed that specific tetanic force production was significantly lower than controls at 19 ($p<0.05$), 21, 23, 27, and 330 days of age ($p<0.01$) for EDL muscles and at 19, 21, and 23 days of age in SOL muscles ($p<0.01$). These studies further improve our understanding of the *mdx* mouse as an experimental model of DMD and emphasises that the model is most appropriate a specific ages for specific muscles. These studies further illustrate that testosterone does not improve cardiac contractility in the *mdx* mouse but that oestrogen improves both cardiac and skeletal muscle function. Further research is warranted into the potential of oestrogen as a therapeutic agent in the treatment of both cardiac and skeletal muscle manifestations of DMD.

CERTIFICATION OF DISSERTATION

This thesis is my own work, except where otherwise acknowledged, and the work is original and has not been previously submitted for any other award at any other University or Institution of Tertiary Education.

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Signature of Candidate Date

ENDORSEMENT

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Signature of Principal Supervisor Date

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"Motion is life….."

Guillaume Benjamin Amand Duchenne de Boulogne

GLOSSARY OF ABBREVIATIONS

AAV	adeno-associated virus
ACE	angiotensin converting enzyme
AO	antisense oligonucleotide
AR	androgen receptor
BW	body weight
C57	C57BL/10ScSn mouse
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
CHF	congestive heart failure
CK	creatine kinase
CNF	centronucleated fibre
CO_2	carbon dioxide
CRC	concentration response curve
CSA	cross-sectional area
CV	cardiovascular
cxmd	canine X-linked muscular dystrophy
DFZ	deflazacort
DGC	dystrophin-associated glycoprotein complex
DHT	dihydrotestosterone
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
ECG	electrocardiogram
EDL	extensor digitorum longus muscle
eNOS	endothelial nitric oxide synthase
EOM	extraocular muscles
ER	oestrogen receptor
EtOH	ethanol
FOC	force of contraction
FSH	follicle stimulating hormone
FXMD	feline X-linked muscular dystrophy
g	gram
GH	growth hormone

GI	gastrointestinal
GnRH	gonadotrophin releasing hormone
GRMD	Golden Retriever muscular dystrophy
H&E	haematoxylin and eosin
HFMD	hypertrophic feline muscular dystrophy
HR	heart rate
HW	heart weight
HW%	heart weight as a percentage of body weight
Hz	Hertz
IGF-1	insulin-like growth factor 1
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IQ	intelligence quota
K^+	potassium ion
kb	kilobase(s)
kDa	kilo Dalton(s)
KPSS	Krebs physiological salt solution
LA	left atria/left atrium
Lf	absolute muscle length
LH	leutinising hormone
LIF	leukaemia inhibitory factor
Lo	muscle length
LV	left ventricle/ventricular
LV+S	left ventricle and septum
M	molar
MDSC	muscle-derived stem cell
mdx	muscular dystrophy X-linked
mg	milligram
min	minute(s)
mL	millilitre
mM	millimolar
mN	millinewton
msec	millisecond(s)
µM	micromolar

µm	micrometre
n	number of animals in experimental group
Na^+	sodium ion
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NMJ	neuromuscular junction
O_2	oxygen
PCR	polymerase chain reaction
%	percent
PK	pyruvate kinase
Po	tetanic force
Pt	twitch force
Po:Pt	tetanus to twitch ratio
RA	right atria/right atrium
REM	rapid eye movement
rpm	revolutions per minute
RV	right ventricular free wall
RyR	ryanodine receptor
SC	subcutaneous
SEM	standard error of the mean
SERCA	sarcoplasmic reticulum calcium-ATPase
SHR	spontaneously hypertensive rat
SOL	soleus muscle
sPo	tetanic force normalised for cross-sectional area
sPt	twitch force normalised for cross-sectional area
SR	sarcoplasmic reticulum
TGF	transforming growth factor
TPSS	Tyrode physiological salt solution
U/L	units of active enzyme per litre of serum
USQ	University of Southern Queensland
VC	vital capacity
vs	versus

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1 LITERATURE REVIEW

1.1 History of Duchenne Muscular Dystrophy (DMD)

In 1858 the French physiologist Guillaume Benjamin Amand Duchenne de Boulogne (Duchenne) became interested in 13 specific clinical cases (consisting of 11 males and two young females) (Figure 1-1). Duchenne reviewed these cases, together with 15 ostensibly similar cases described in the German medical literature. Initially, the condition observed was termed *paralysie pseudo-hypertrophique* (pseudo-hypertrophic muscular paralysis). In 1868 the disease was further clinically defined and subsequently re-named Duchenne Muscular Dystrophy (DMD) in recognition of the French physiologist's work.

By 1870 Duchenne had witnessed in excess of 40 cases of DMD and proceeded to describe the disease as being characterised by progressive weakness of muscle movement (first affecting the lower limbs and eventually the upper limbs), a gradual increase in the size (hypertrophy) of many affected skeletal muscles, an increase in interstitial connective tissue within affected muscles, and the production of abundant fibrous and adipose tissues in the latter stages of the disease. The onset of DMD was determined as occurring in childhood or early puberty, was more prevalent in males than females, and potentially affected several children in the same family. Finally, and as a result of muscle biopsy techniques, Duchenne concluded that the fundamental anatomical lesion in DMD was hyperplasia of the interstitial connective tissue (Emery 1993).

Over a century passed before DMD would be defined genetically. In 1986, an historical milestone in muscular dystrophy research resulted from the isolation of the DMD gene locus (Kunkel 1986; Monaco *et al.* 1986; Koenig *et al.* 1987).

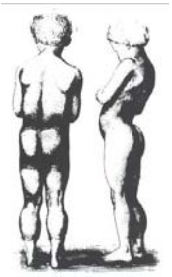
(A)  (B) 

Figure 1-1 Guillaume Benjamin Amand Duchenne de Boulogne and the first observed case of DMD

(A) Guillaume Benjamin Amand Duchenne de Boulogne (1806-1875) and (B) A sketch of the original clinical case of DMD observed by Duchenne (Reproduced from Emery 1993)

DMD was shown to be caused by mutations in the gene encoding the protein dystrophin, thus resulting in the total absence or the expression of a mutant form of dystrophin (Hoffman, Brown and Kunkel 1987).

A chronology of historical landmarks in DMD research and knowledge are summarised in Table 1-1.

Table 1-1 Landmarks in the history of DMD

1830-79	DMD emerges as a distinct clinical disease	(Meryon 1852; Duchenne 1868; Gowers 1879a; Gowers 1879b)
1867	Duchenne defines the actions of human muscles	(Duchenne 1867) (Demos *et al.* 1956)
1954-58	Serum adolase activity found to be raised in DMD boys resulting in the emergence of 'leaky membrane' theory	
1959	Serum creatine kinase (CK) levels demonstrated to be elevated in DMD patients (boys)	(Ebashi 1959)
1960	Serum CK levels found to be raised in female DMD carriers	(Schapira 1960)
1975	Evidence of plasma membrane lesions and increased sarcoplasmic reticulum (SR) Ca^{2+} levels	(Mokri and Engel 1975) (Cullen and Fulthorpe 1975)
1978-79	DMD mapped to Xp21 by X/A translocations	(Verellen 1978; Lindenbaum 1979)
1982-83	DMD mapped to Xp21 by deoxyribonucleic acid (DNA) markers	(Murray 1982; Davies 1983)
1984	*mdx* mouse discovered	(Bulfield *et al.* 1984)
1985	Gene-specific probes for DMD developed	(Kunkel 1985; Ray 1985)
	Gene deletions in DMD detected	(Monaco 1985)
1986	Isolation of the DMD gene locus	(Kunkel *et al.* 1986; Monaco *et al.* 1986)
1987-88	Cyclic deoxyribonucleic acid (cDNA) cloned and sequenced	(Koenig *et al.* 1987; Koenig, Monaco and Kunkel 1988)
	Gene specific probes developed	
1987-88	Dystrophin protein product identified	(Hoffman, Brown and Kunkel 1987)

	Dystrophin localisation functional studies commence	(Sugita 1988; Zubrzycka-Gaarn *et al.* 1988)
1989	Myoblast transfer research commences in mice	(Partridge 1989)
1990	Myoblast transfer research commences in humans	(Karpati 1990)
	Direct gene transfer therapy first attempted	(Wolff 1990)
1991	Direct gene transfer research further developed	(Dickson 1991)
	Isolation and definition of the dystrophin-glycoprotein complex (DGC)	(Ervasti and Campbell 1991; Ohlendieck and Campbell 1991)
1992	Utrophin discovered	(Tinsley *et al.* 1992)
1995	Adhalin (a protein located in the DGC) found to have a role in DMD	(Mendell *et al.* 1995)
1996	Recombinant vectors shown to be able to deliver therapeutic genes	(Petrof *et al.* 1996)
1997	DMD gene cloned	(Davies 1997)
	Potential therapeutic value of stem cells identified	(Smith and Schofield 1997)
1998	Sarcoglycans found to have a role in DMD	(Urtasun *et al.* 1998)
	Dystroglycans found to have a role in DMD	(McDearmon *et al.* 1998)
1999	Antisense oligonucleotides (AOs) shown to have the ability to facilitate dystrophin expression	(Wilton *et al.* 1999)

1.2 Genetics and inheritance

Pedigree studies, statistical methods, and evidence from affected females with X chromosome abnormalities has resulted in the accepted conclusion that DMD is an X-linked inherited recessive trait (Emery 1993) (Figure 1-2). All DMD patients are male except for a small percentage of female patients whose disease condition is a direct consequence of chromosomal abnormalities (Nonaka 1998).

The DMD gene locus is located at Xp21 on the short arm of the X chromosome (De La Porte, Morin and Koenig 1999). This 1800 kilobase (kb) gene contains 79 exons (mean size 150 base pairs (bp)) separated by introns of approximately 16 kb (Chelly *et al.* 1988) and is currently the largest identified human gene (Koenig *et al.* 1987; Koenig, Monaco and Kunkel 1988; Roberts *et al.* 1993). The dystrophin gene occupies almost two percent of the total human X chromosome (Coffey *et al.* 1992; Monaco *et al.* 1992) and accounts for between 0.05% (Brown and Hoffman 1988) and 0.1% (Hoffman and Kunkel 1989) of the total human genome.

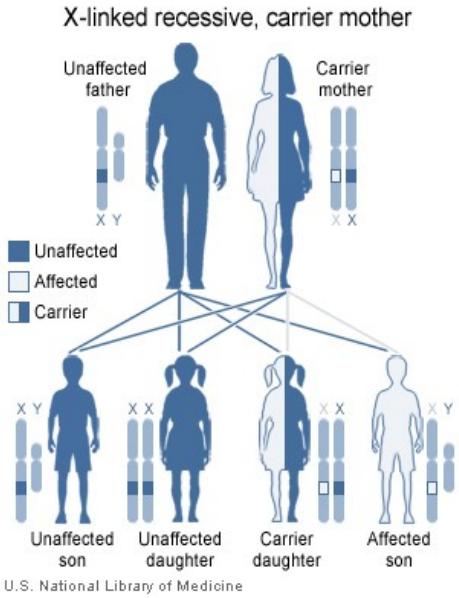


Figure 1-2 Pathways of X-linked recessive inheritance in DMD

In this example the DMD carrier woman (mother) carries one copy of the gene mutation for the X-linked recessive disorder (such as DMD). She has a DMD affected son, an unaffected daughter (who also carries one copy of the DMD mutation), and two unaffected children who do not have the mutation. Reproduced from the US National Library of Medicine. Available from http://ghr.nlm.nih.gov/info=img,inheritance_patterns/show/XlinkRecessiveMother

The 14 kb dystrophin transcript is expressed in all forms of muscle tissue and in certain neurones (Hoffman *et al.* 1988). The dystrophin protein is expressed to the greatest and equal extent in skeletal and cardiac muscles (Hoffman *et al.* 1988), lower levels are expressed in smooth muscle (Beam 1988), with the lowest levels being expressed in the brain (Monaco *et al.* 1986; Chamberlain *et al.* 1988; Nudel, Robzyk and Yaffe 1988; Nudel *et al.* 1989) (Figure 1-3).

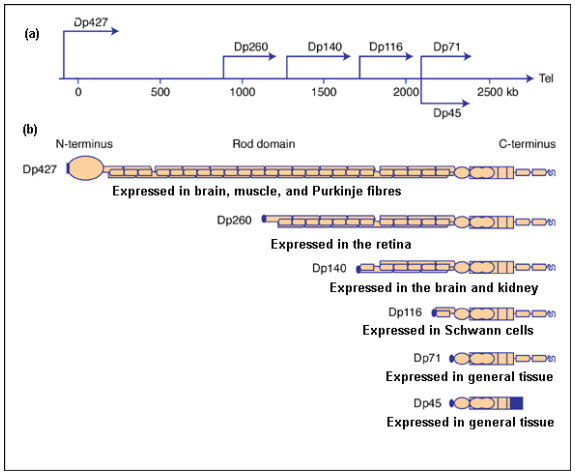


Figure 1-3 Schematic diagram of the dystrophin gene.

Schematic diagram of the dystrophin gene. (a) represents the location of the various promoters and (b) illustrates the various dystrophin isoforms and the tissues in which they are preferentially expressed (Winder 1997).

1.3 Detection, diagnosis and clinical disease features

DMD manifests in the unborn child with significant histological and histochemical abnormalities detectable in muscle tissues from DMD affected foetuses (Emery 1993). The presence of dystrophin in foetal muscle depends on the embryonic and foetal maturation of muscle cells (myocytes) (Miranda *et al.* 1988; Clerk, Strong and Sewry 1992). Dystrophin is detectable in unaffected foetuses as early as nine gestational weeks (Patel *et al.* 1988) and these foetuses develop relatively mature skeletal muscle resulting in significant dystrophin expression after 20 weeks gestation (Bieber, Hoffman and Amos 1989).

1.3.1 Genetic testing

In cases where intragenic duplication or deletion of the dystrophin gene occurs (approximately 65% of cases) (Koenig *et al.* 1987; Den Dunnen *et al.* 1989) prenatal diagnosis of DMD is feasible using DNA from chorionic villi or amniotic fluid cells (Evans *et al.* 1991). In the remaining 35% of cases, diagnosis by linkage analysis may be an option (Bakker *et al.* 1989). However, only approximately 33% of cases are inherited through a carrier mother, with the remaining 67% being sporadic, *de novo* mutations of the

dystrophin gene (Moser 1984). The dystrophin gene has a high mutation rate (one in 10,000) (Muller *et al.* 1992), which may simply be resultant of the large size of the gene (Koenig *et al.* 1987). In both *de novo* mutations and small families linkage analysis may be rendered impractical (Bieber, Hoffman and Amos 1989; Clerk *et al.* 1992). Lalic *et al.* (2005) have designed a multiplex ligation-dependent probe amplification assay (MLPA) which simultaneously screens all 79 DMD gene exons for deletions and duplications that occur in DMD patients. This assay outperforms the Beggs and Chamberlain multiplex-polymerase chain reaction (PCR) test and may currently be argued as the preferred method of choice in the initial DNA analysis of DMD patients (Lalic *et al.* 2005).

1.3.2 Serum creatine kinase (CK) and pyruvate kinase (PK) levels

Serum creatine kinase (CK) measurements are also predictors of DMD. CK is a muscle enzyme released into the blood serum in response to muscle damage, therefore an elevation in serum CK levels is observed in DMD affected neonates, DMD patients, and a percentage of DMD carriers (Emery 1993). Even at birth, and as a direct consequence of muscle degeneration, DMD neonates possess elevated serum CK levels (Bogdanovich *et al.* 2004). Serum CK levels may also be elevated as a result of skeletal muscle injury such as is induced by intramuscular (IM) injection, excessive exercise, myositis, myopathy, rhabdomyolysis, or hypothyroidism. Conversely, serum CK levels are actually lowered in females during pregnancy. In an unaffected adult, the normal range of serum CK is 22 to 198 units of active enzyme per litre of serum (U/L). In DMD serum CK levels may be 3000 to 3500 U/L and during acute episodes of muscle degeneration (rhabdomyolysis) serum CK levels may escalate to 50000 to 200000 U/L (available from http://www.mdausa.org). DMD carriers may also have elevated serum CK levels and it is estimated that approximately 67% of obligate carriers have elevated CK levels.

DMD patients may have elevated serum pyruvate kinase (PK) levels which potentially reach three to 125 times that of unaffected age matched controls. Normal PK levels are approximately 6.0 µmol/mL/h (Zatz *et al.* 1978). Researchers have also found abnormal fatty acid composition and disorganization of the erythrocyte membrane in DMD patients (Piperi *et al.* 2004).

1.3.3 Muscle biopsy

Foetal muscle biopsy *in utero* is an option when DNA molecular diagnosis is ambiguous and where linkage analysis is inconclusive or impractical (Ginjaar *et al.* 1991; Evans *et al.* 1993). Post natal detection of DMD may also be substantiated by means of muscle biopsy in which case a dystrophin level of less than 3% of normal is considered diagnostic of DMD (Brooke 2000).

1.3.4 Disease onset

DMD boys are within the reference range with respect to length and weight at birth. Delayed growth starts during the first years of life and DMD children have a mean approximate decrease of six centimetres according to the expected height of the unaffected population (Rapaport *et al.* 1991). Mothers occasionally describe that their affected son appears ‘floppy’ at birth and during infancy (Emery 1993). A perceived ‘failure to thrive’ may be associated with the development of DMD (Call and Ziter 1985), as may delay in speech and slowed progress in learning to walk. Parents often, retrospective to diagnosis, confirm a history of delays in achievement of motor milestones (Bogdanovich *et al.* 2004). Bushby, Hill and Steele (1999) report the average age at diagnosis is four years and eight months, not dissimilar to some 20 years ago (Bushby, Hill and Steele 1999).

The mean walking age in unaffected boys is 13 months of age with 97% of children walking by 18 months old (Neligan and Prudham 1969). Only 56% of DMD boys commence walking at 18 months whilst some 25% of

DMD boys do not take their first steps until at least two years of age (Emery 1993).

1.3.5 Initial clinical signs

The initial physical signs of DMD skeletal muscle degeneration typically emerge around two to five years of age (Dubowitz 1975; Jennekens *et al.* 1991). DMD affected children may exhibit a waddling gait, unsteadiness in walking, a perceived increased risk of falling, toe walking, difficulties in climbing stairs, a tendency to 'throw out a leg' when walking, turning in of the feet, and some problems in maintaining balance whilst running (Emery 1993).

1.3.6 Skeletal muscle involvement

An initial and obvious clinical sign of DMD is enlargement of the calf muscles (gastrocnemius and soleus) which, upon palpation, are often described by clinicians as feeling 'firm' or 'woody'. This skeletal muscle enlargement is characteristic of DMD and is actually *pseudohypertrophy* and not a true hypertrophy of muscle fibres (Figure 1-3). Ultrasound imaging of these pseudohypertrophic muscles reveals a bright and speckled echo pattern which confirms the deposition of adipose and connective tissue (Heckmatt, Dubowitz and Leeman 1980; Heckmatt, Pier and Dubowitz 1988). Pseudohypertrophy is progressive in the early stages of DMD but is eventually followed by atrophy and a decrease in size of most of the affected skeletal muscles. The exception to this trend is with the gastrocnemii and, to a lesser extent, the quadriceps and deltoids (De Bruin *et al.* 1997). The enlarged pseudohypertrophic muscles are weaker and more hypotonic than unaffected muscles of the equivalent size (Adams and Victor 1989) and the pseudohypertrophic changes in skeletal muscles are not correlated to either age or severity of the disease (Jones *et al.* 1983).

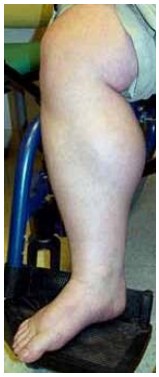
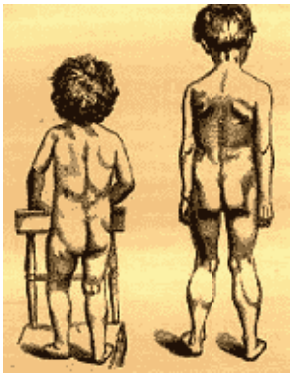
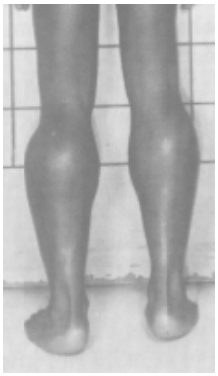
(A)  (B)  (C) 

Figure 1-4 Pseudohypertrophy of the calf muscles in DMD

Extensive pseudohypertrophy of the calf muscles in four boys with DMD. (A) available from www.neuro.wustl.edu (B) and (C) (Reproduced from Emery 1993)

DMD affected boys occasionally complain of cramps and stiffness in the gastrocnemius muscles. Skeletal muscle involvement in DMD is both bilateral and symmetrical. In the early stages the lower limbs are affected more than the upper limbs, proximal muscles to a greater extent than distal muscles, quadriceps more than the hamstrings, triceps more than biceps, wrist extensors more than flexors, neck flexors more than extensors, and feet dorsi flexors more than plantar flexors. Differential involvement also occurs within individual muscles; the sterno-costal head of the pectoralis major muscle is more affected than the clavicular head and the clavicular head of the sternomastoid muscle is more affected than the sternal head. Other affected muscles may include the deltoids and serrati anterior. DMD affected muscles are not tender to touch and there is no voluntary or percussion myotonia. Tendon reflexes become depressed with ankle jerks being the last tendon reflexes to disappear. Plantar responses always remain flexor. Finally, there is no sensory loss thus indicating that the depression of reflexes in DMD is not neuronal in origin (Emery 1993).

1.3.7 Biomechanics of movement in DMD

The resultant biomechanical effects of muscle weakness in DMD are complex (Sutherland *et al.* 1981). Prior to age four years, boys are typically unable to rise from a sitting position on the floor without the aid of the arms to assist by pushing on the thighs or on the floor (whereas

unaffected boys are able to accomplish this task at this age). At this age, DMD boys typically cannot be held up by the clinician placing their hands inside the child's upper arms as there is a tendency for the child to 'slide through' the examiners arms.

Weakness of the gluteus medius and minimus muscles result in the pelvis tilting down toward the unsupported side when the DMD child raises his leg from the ground. The DMD child subsequently inclines towards the supporting leg and as he moves forward this repeated action results in a 'waddling gait'. In addition, weakness of the gluteus maximus muscle results in a forward tilting pelvis and subsequent development of lumbar lordosis. An imbalance between development of the dorsi flexors and plantar flexors progresses and, in order to maintain their balance, DMD boys then tend to walk on their toes. As the disease further progresses, winging of the scapulae becomes apparent (Emery 1993), proximal muscles are further affected, and DMD boys become nonambulatory around 10 to 12 years of age as a result of the loss of critical lower limb strength (Bogdanovich *et al.* 2004).

1.3.8 Gowers' manoeuvre

As described, in the first three years of life, the DMD child is typically unable to rise from a sitting position on the floor with his arms folded. By four or five years of age, weakness of proximal limb muscles, the hip extensors, and the knee extensors results in the necessary use of the DMD child's arms to climb from a lying position to a standing position. The DMD child climbs up his thighs in order to extend the hips and push up the trunk (Emery 1993). This sign is known as the classical Gowers' manoeuvre (Gowers 1888) and typically further supports a clinical diagnosis of DMD (Bogdanovich *et al.* 2004) (Figure 1-5).

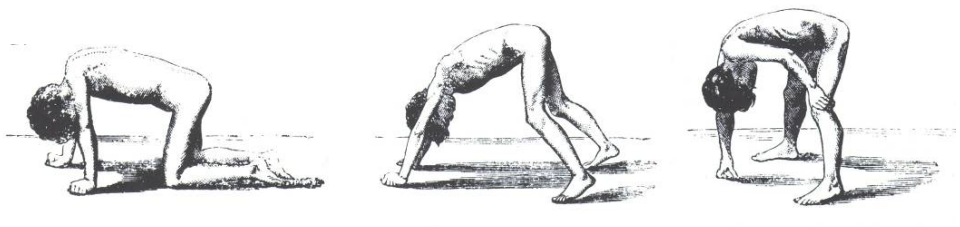


Figure 1-5 Classical Gowers' manoeuvre (sign).

Gowers noted that the boys first put the hands on the floor, then stretched their legs far apart behind them. The chief weight of the trunk then rested on the hands. By keeping their toes on the ground and pushing their body backwards, they managed to get the knees extended so that the trunk was supported by the hands and feet, all placed as widely apart as possible. Next the hands are moved alternatively along the ground backwards so as to bring a larger portion of the weight of the trunk over the legs. Then one hand is placed upon the knee and a push with this and the other hand on the ground is usually sufficient to enable the hip extensors to bring the trunk into the upright standing position (as cited in Gowers 1879a).

1.3.9 Cardiac muscle involvement

There is overwhelming clinical, physiological, and pathological evidence that the cardiac muscles are significantly affected in both DMD patients and DMD carriers (Hunter 1980; Hunsaker *et al.* 1982; Hoffman *et al.* 1992). From early childhood cardiac arrhythmias and murmurs may persist. Sinus tachycardia is present in most DMD boys after age five years and persists throughout life (Engel, Yamamoto and Fischbeck 1994). Nocturnal oxygen desaturation occurs during rapid eye movement (REM) sleep in DMD patients and this may progress to severe nocturnal hypoxaemia and resultant life threatening cardiac arrhythmias (Carroll *et al.* 1991).

General cardiac problems in DMD develops progressively from age 10 years (Cox and Kunkel 1997) with 62% of boys showing conduction changes in the electrocardiogram (ECG) consisting of a shortened PQ segment, and an increased QT:PQ ratio (prolonged QT interval). Clinically observable cardiomyopathy may become evident around 10 years of age and is progressive (Nigro *et al.* 1990). Although most DMD patients remain asymptomatic, the incidence of clinical cardiac involvement (predominantly cardiomyopathy) continues to increase over the teenage years, affecting approximately 33% of patients by age 14 years, 50% by 18 years, and arguably 100% of adult patients (Cox and Kunkel 1997). However, there is

some variation in the reported incidence of cardiac involvement between researchers with Nigro (1990) reporting clinical cardiac symptoms in 28% of patients under age 18 years and cardiac symptoms in only 57% of adult patients. From 10 to 20 years of age dilated cardiomyopathy, hypertrophic cardiomyopathy and conduction system abnormalities such as arrhythmias, bundle branch block, and heart block have also been reported (Nigro *et al.* 1990).

In DMD patients the most common ECG pattern consists of a prominent R wave in lead V_1 and the right precordial and limb leads (anterior QRS pattern) (Cox and Kunkel 1997) (Figure 1-6). This ECG pattern reflects selective atrophy and scarring of the posterobasal region and adjacent lateral wall of the left ventricle (LV) (as opposed to right ventricular (RV) hypertrophy) (Perloff *et al.* 1967). Further ECG abnormalities are often observed in DMD patients and the presence of LV systolic dysfunction detected on echocardiography is a powerful predictor of mortality in DMD (Corrado *et al.* 2002).

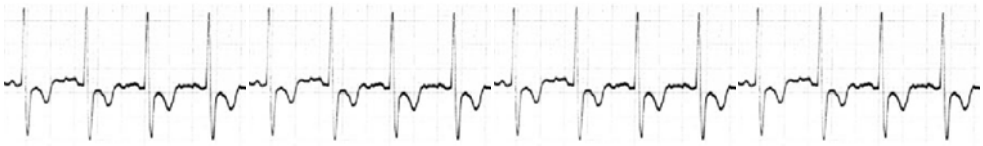


Figure 1-6 ECG pattern in a nine year old boy with DMD

The ECG shows a classical pattern seen in DMD patients consisting of a tall R wave in lead V_1 (Gulati *et al.* 2005).

X-linked dilated cardiomyopathy in DMD typically presents as congestive heart failure (CHF) in adolescent to young adult males. The disease course is rapidly progressive and results in cardiac transplantation or death within one to two years of presentation (Cox and Kunkel 1997). Khan (1994) reports an overall 10% incidence of death due to cardiac failure whilst Ishihara (2004) reports an incidence of 12.5% of cardiac related deaths in DMD (Ishihara 2004).

1.3.10 Skeletal system involvement

DMD patients may present with numerous skeletal system abnormalities including progressive narrowing of the shafts of the long bones (due to a reduction in the size of the medullary cavity and subsequent thinning of the cortices), lumbar lordosis, scoliosis, coxa valga, and impaired development of the pelvic bones and scapulae. As DMD progresses there is an absence of the normal developmental stresses imposed by muscular attachments together with the adoption of abnormal body postures and compromised limb positions (Emery 1993) (Figure 1-7).

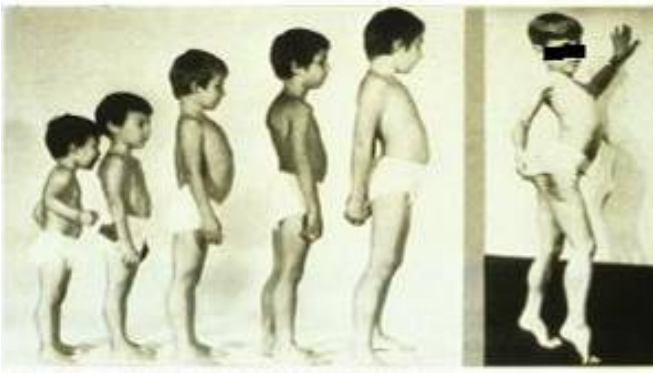


Figure 1-7 Progressive body posture changes in DMD

As DMD progresses there is an absence of the normal developmental stresses imposed by muscular attachments together with the adoption of abnormal body postures and compromised limb positions (Emery 1993).

The bones of the skeletal system undergo rarefaction and decalcification as a result of prolonged disuse and this ultimately progresses to osteoporosis (Walton and Warrick 1954). An estimated 20% to 25% of DMD boys experience a long bone fracture at some time in their lives, the risk of which increases with loss of ambulation and increased age. In the latter stages of the disease, movements of the wrists and shoulders become limited. Talipes equinovarus deformity also becomes marked with the talus bone protruding under the skin of the dorsum of the foot (Emery 1993).

Approximately 90% of DMD boys develop such severe scoliosis that is not amenable to control by nonsurgical means such as bracing or adaptive seating and 95% of scoliosis cases occur after loss of ambulation (Cambridge and Drennan 1987). Currently, it may be argued that the most effective treatment for severe scoliosis in DMD is early surgical prevention

with spinal fusion utilising segmental instrumentation as soon as significant spinal curves are ascertained and before the onset of severe pulmonary or cardiac dysfunction (Sussman 2002). Prednisone treatment potentially slows the progress of scoliosis in DMD patients (Alman, Raza and Biggar 2004).

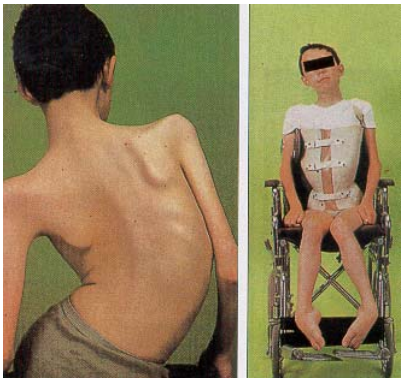


Figure 1-8 Severe scoliosis of the spine in a boy with DMD

Severe scoliosis of the spine and an equinovarus deformity of both feet in a 15 year old DMD boy (Forbes and Jackson 1997).

1.3.11 Respiratory muscle involvement

In DMD patients a characteristic pattern of progressive respiratory muscle weakness is seen during early childhood and adolescence (Smith *et al.* 1987). Respiratory vital capacity (VC) increases as predicted until around 10 years of age. A plateau of VC then occurs followed by a steady fall as respiratory muscle weakness progresses. In the majority of cases respiratory compromise is accompanied by the development of a thoracic scoliosis (see Figure 1-8). The annual decrease in VC may be as much as 250 mL in the late teenage years (Baydur *et al.* 1990). Once VC falls below 20% (one litre) predicted ventilatory failure is almost inevitable (Rideau *et al.* 1983; Rideau, Glorion and Duport 1983). In reportedly 10% (Khan and Heckmatt 1994) to 12.5% (Ishihara 2004) of DMD cases the cause of death is cardiac failure which may be exacerbated by chronic hypoxaemia and hypercapnia. Hypoventilation, at times associated with obstructive apnoeas (Khan and Heckmatt 1994), first occurs during sleep as a result of the reduction in intercostal muscle tone, lowered accessory muscle tone, and a decrease in the ventilatory drive which is most prominent in rapid eye movement (REM) sleep. Untreated, nocturnal hypoventilation progresses to daytime ventilatory

failure and ultimately *cor pulmonale*. Acute hypercapnic exacerbations complicate the clinical course of DMD and are usually exacerbated by chest infections as inspiratory muscle weakness predisposes the individual to atelectasis, and the ability to cough is impaired by expiratory muscle insufficiency. De Bruin (2001) reported a significant reduction in inspiratory flow reserve and a less negative maximum static inspiratory pressure in DMD boys compared to unaffected boys with force VC related to largest inspiratory flow during an inspiratory forced VC manoeuvre (De Bruin *et al.* 2001). In advanced DMD 'row-a-boat' phenomenon develops as a spontaneous upper-body movement displayed when sitting upright supported by a belt around the body in a wheelchair. This movement is a resultant compensation for atrophied respiratory muscles in advanced stages of the disease (Yasuma *et al.* 2001).

Analogous to the skeletal muscle pseudohypertrophy observed in DMD patients, the resting diaphragm muscle thickness is increased in DMD patients (under 12 years of age) with impaired respiratory muscle force (De Bruin *et al.* 1997). Infiltration of connective tissue (fibrosis) and the subsequent deposition of adipose tissue (as are discernable in DMD skeletal muscles) have also been observed in intercostal muscle biopsy specimens from DMD patients (Stern *et al.* 1975).

Prognosis is poor and life expectancy is generally less than 12 months once patients develop diurnal hypercapnia (Simmonds *et al.* 1998). Hypercapnic respiratory failure occurs when the inspiratory muscles fail to sustain adequate ventilation and this is the most common cause of death in patients with DMD (Inkley, Oldenburg and Vignos 1974; Braun, Arora and Rochester 1983; Cox and Kunkel 1997). From autopsy findings, Ishihara (2004) reports that the primary cause of death is respiratory failure in approximately 75% of DMD fatalities (Ishihara 2004). In addition, some 90% of episodes of respiratory failure and subsequent death in DMD patients occur during the treatment of intercurrent chest colds and death ultimately results from the DMD patient's inability to adequately cough out chest secretions (Bach, Ishikawa and Kim 1997).

1.3.12 Smooth muscle involvement

Autopsy studies have demonstrated that the smooth muscle of the gastrointestinal (GI) tract in DMD patients shows both loss and atrophy of muscles, variation in muscle fibre size, and regions of fibrosis (Huvos and Pruzanski 1967; Leon *et al.* 1986; Barohn *et al.* 1988). Involvement of the GI tract in DMD may also include clinical symptoms such as recurrent diarrhoea, malabsorption (Patterson, Ong and Drake 1964; Huvos and Pruzanski 1967), impaired colonic transit (Gottrand, Guillonneau and Carpentier 1991), oropharyngeal, oesophageal, and gastric dysfunction (Jaffe *et al.* 1990), together with delayed gastric emptying (Barohn *et al.* 1988).

Compared to cardiac and skeletal muscle involvement, smooth muscle manifestations occur relatively late in the course of DMD. Boland (1971) purports that clinical GI symptoms related to smooth muscle function most often are secondary to surgery or a respiratory illness (Boland *et al.* 1996). The functional impairment of smooth muscle in the GI tract of DMD patients can cause acute gastric dilatation and intestinal pseudo-obstruction that may prove fatal (Barohn *et al.* 1988).

1.3.13 Intellectual and behavioural impairment

DMD patients may present with varying degrees of cognitive impairment, indicating that the disease affects brain function (Engel, Yamamoto and Fischbeck 1994). Intellectual impairment reportedly occurs in approximately 19% of boys and DMD patients present a specific cognitive profile, regardless of their general level of cognitive function. Specifically, boys with DMD perform more poorly on tests requiring attention to complex verbal information than they do on other verbal or memory measures. Intellectual impairment in DMD is not progressive and does not correlate with the severity of the musculoskeletal symptoms (Hinton *et al.* 2000). The mean intelligence quota (IQ) for DMD patients is generally one standard deviation below the normal mean but ranges from IQs above 130 points to severe mental retardation (Emery 1993). One third of DMD children have an IQ below 75 points. The IQ effects associated with DMD result from a pleiotropic

effect of dystrophin mutation and the site or size of the associated gene deletion has no correlation with the severity of intellectual impairment (available from http://www.indegene.com). The basis of IQ impairment in DMD remains unclear but studies have revealed both abnormal dendritic development and the treelike branching of nerve axons (arborisation) as a possibility (Jagadha and Becker 1988). There is no difference in the IQs of boys born to carrier mothers and those who result from a spontaneous gene mutation compared to unaffected children of the equivalent age (Emery 1993). Unaffected siblings of DMD boys have normal intellect, however, there is a positive correlation between affected brothers (Kozicka, Prot and Wasilewski 1971; Ogasawara 1989) and female DMD carriers reportedly have normal IQ (Prosser, Murphy and Thompson 1969). In DMD, verbal ability appears most affected and is due to a defect in memory for numbers, patterns, and verbal labels (Karagan, Richman and Sorensen 1980). Interestingly, Miller (1985) (Miller, Tunnecliffe and Douglas 1985) reported that DMD boys with least verbal IQ depression actually survived longest.

Behavioural and emotional disturbances in DMD boys have been extensively reported (Schorer 1964; Cohen, Molnar and Taft 1968; Leibowitz and Dubowitz 1981; Pullen 1984; Smith, Sibert and Harper 1990). Allowing for age and IQ, DMD boys have a higher incidence of emotional disturbances than other physically handicapped children without cerebral involvement (Leibowitz and Dubowitz 1981).

1.4 Muscle and system sparing

In DMD, specific muscles and physiological systems may be clinically spared. For example, visual and hearing acuity are unaffected in DMD patients (Allen 1973) as are sphincter control, chewing, and swallowing (Emery 1993). Enigmatically, the extraocular muscles (EOM) appear clinically unaffected in DMD (Kaminski *et al.* 1992; Khurana *et al.* 1995).

The EOM are muscles responsible for eye movements and possess functional and morphological characteristics that set them aside from other skeletal muscles. The mechanism by which the EOM are spared the deleterious effects of DMD is unknown and it is thought that this may be due

to a broken mechanical or signalling linkage between the cytoskeleton and the extracellular matrix that uniquely occurs in EOM (Andrade, Porter and Kaminski 2000).

Specifically, dystrophic EOM lack the pathological manifestations of dystrophin deficiency such as fibrotic scarring, fatty infiltration, central nucleation, fibre splitting, fibre size variation, and hypertrophy that are witnessed in DMD skeletal and cardiac muscles (Khurana *et al.* 1995). Ringel *et al.*(1978) and Khurana *et al.*(1995) showed that haematoxylin and eosin (H&E) stained EOM tissues from dystrophin-deficient humans (DMD patients), dystrophic canines (*cxmd*), and dystrophic mice (*mdx*) were histologically indistinguishable from those of unaffected controls in each species (Ringel *et al.* 1978; Khurana *et al.* 1995). Khurana *et al.* (1995) found no upregulation of the dystrophin-associated protein in humans, utrophin in *mdx*, *cxmd*, or DMD EOM thus concluding that utrophin upregulation was not responsible for the sparing of dystrophin-deficient EOM. This is in direct contrast to the work of Matsumura *et al.* (1992) who actually found up-regulation of the above mentioned respective dystrophin-associated proteins (Matsumura *et al.* 1992). Khurana *et al.* (1995) further demonstrated that dystrophin deficiency in EOM did not result in myonecrosis or pathologically elevated levels of intracellular calcium $[Ca2+]_i$ but that dystrophin-deficient EOM are spared the pathological consequences of dystrophin deficiency due to their inherent ability to more efficiently maintain calcium (Ca^{2+}) homeostasis. Uncovering the specific mechanisms by which the EOM protect themselves (avoid or adapt to the cascade of events leading to myofibre degeneration) in DMD should further contribute to knowledge of both pathogenesis and treatment of the disease (Andrade, Porter and Kaminski 2000).

Interestingly, clinical and experimental evidence indicates that muscle fibres, whose diameter is below a certain level (estimated at approximately 20 to 25 micrometres (μm) in diameter in *mdx* mice) are not susceptible to necrosis. This apparent anatomical ‘protection against necrosis’ has been observed in other muscle fibres that are naturally of smaller diameter (including the EOM), and in fibres that have been prevented from growing normally by experimental procedures (in hamsters and mice),

or by pathological processes (in DMD patients). The molecular basis by which these relatively small diameter muscle fibres are resistant to necrosis remains elusive. Karpati (1986) proposes that, in smaller diameter muscle fibres, the normal contraction-related mechanical strains per unit surface area are relatively less than in larger fibres and leads to their relative resistance to necrosis in DMD (Karpati *et al.* 1986).

1.5 Female DMD carriers

DMD is an X-linked inherited recessive trait (Emery 1993) with the affected gene being carried from the mother to the affected son (Figure 1-2). Mothers of DMD affected males have approximately a 33% chance of carrying a dystrophin mutation (Ligon *et al.* 2000). Emery (1993) clearly defines three categories of DMD carrier status;

obligate carriers - mothers of an affected son who possess an affected brother, affected nephew by their sister, or an affected maternal uncle or other maternal male relative.

probable carriers - mothers of two or more affected sons but no other affected relatives and

possible carriers - mothers of an isolated case as well as their sisters and other female relatives

Histologically, manifesting female carriers exhibit myocyte changes consistent with those observed in DMD patients. In addition, a higher ratio of male to female stillbirths and infant deaths in DMD carriers has also been reported (Lane, Robinow and Roses 1983).

1.5.1 Serum CK and PK levels in female DMD carriers

Due to X-inactivation (the phenomena in which heterozygous females do not phenotypically express their X-linked recessive genotype or do so only randomly) DMD carriers are often clinically unaffected. A proportion of myocytes (including cardiac cells) will, however, have the mutant allele on the active X chromosome and will therefore release CK, resulting in elevated serum CK levels in DMD carriers. Dystrophin

immunostaining and Western blot analysis of muscle biopsy samples from asymptomatic carriers is also often abnormal (Clerk *et al.* 1991).

Serum CK levels may be used to give an indication as to DMD carrier status and measurement of serum CK is currently the most commonly applied test for DMD carrier detection (Lange and Zatz 1979). The use of serum CK levels in combination with mutual dystrophin immunohistochemistry and muscle biopsy significantly improves the accuracy of carrier diagnosis (Oliveira *et al.* 1992). It must be noted that whilst elevated serum CK levels are a predictive diagnostic indicator, a low CK result does not preclude carrier status (Gruemer *et al.* 1985). Serum PK levels have also been found to be elevated in DMD carriers (Falcao-Conceicao *et al.* 1983). Of obligate female DMD carriers 71% have increased serum PK levels in addition to elevated serum CK levels. Thus, when used concomitantly, the predictive value of these tests (in terms of carrier detection) improves (Zatz *et al.* 1980).

1.5.2 Female DMD carrier cardiac and skeletal muscle involvement

Female DMD carriers show an increased incidence of cardiac involvement that manifests predominantly as cardiomyopathy and progresses with age (Cox and Kunkel 1997). The progression of carrier cardiac involvement develops through a pre clinical stage, to myocardial hypertrophy or dysrhythmias, and ultimately eventuates in hypertrophic or dilated cardiomyopathy (Politano *et al.* 1996). Dilated cardiomyopathy is more common in male DMD patients whilst hypertrophic cardiomyopathy is more common in female DMD carriers.

A 10 year study by Politano *et al.* (1996) of 152 DMD carriers found preclinical and clinical cardiac involvement in 55% of carriers younger than 16 years of age and in 90% of carriers over age 16 years. Results concluded that under age 16 years, 12% of female carriers had hypertrophic cardiomyopathy whilst 3% had dilated cardiomyopathy. Over the age of 16 years, 30% of carriers had hypertrophic cardiomyopathy whilst 11% of

carriers had dilated cardiomyopathy. Carrier X-linked dilated cardiomyopathy typically presents as atypical chest pain in middle-aged females without clinical muscle weakness. The condition typically progresses over several years and frequently becomes fatal (Cox and Kunkel 1997). Politano *et al.* (1996) also found carriers showed clinical signs of skeletal muscle involvement. Skeletal muscle biopsies showed a significant number of dystrophin-negative fibres present together with a mosaic pattern of absent dystrophin immunoreactivity (Politano *et al.* 1996). Hoffman *et al.* (1992) reported that, in contrast to cardiac myocytes, skeletal myocytes in DMD female carriers actually may become progressively dystrophin-positive over time. This is potentially a result of the replacement of dystrophin-negative fibres with dystrophin-positive myoblasts through the process of genetic normalisation or the intracellular diffusion of dystrophin from dystrophin-positive nuclei along the myocyte syncytium via a biochemical normalisation pathway (Hoffman *et al.* 1992).

Adachi *et al.*(1997) reported that the levels of plasma brain natriuretic peptide (BNP) was elevated in 53% of DMD carriers and the BNP levels correlated with indices of cardiac function. In one carrier, BNP levels were elevated one year prior to the development of cardiac symptoms, increased with the display of clinical cardiac symptoms, and eventually decreased after treatment for cardiac failure, thus suggesting that BNP levels may be useful indicators of cardiac dysfunction and for evaluating the efficacy of cardiac treatment in female DMD carriers (Adachi *et al.* 1997).

A list of reported physiological manifestations in female DMD carriers are summarised in Table 1-2 below;

Table 1-2 Reported manifestations in female DMD carriers

carrier manifestation	reference
▪ clinical muscle weakness (ranging from mild clinical weakness to inability to walk)	(Moser and Emery 1974) (Kaladhar Reddy, Anandavalli and Reddi 1984)
▪ clinical cardiomyopathy	(Wiegand *et al.* 1984)
▪ abnormal muscle histology	(Bertolotto *et al.* 1981)
▪ abnormal muscle histochemistry (elevation of myonuclear Ca^{2+} levels)	(Maunder-Sewry and Dubowitz 1981)
▪ altered muscle ultrastructure	(Fisher *et al.* 1972) (Afifi, Bergman and Zellweger 1973) (Rott, Breimesser and Rodl 1985) (Stern *et al.* 1985)
▪ MLPA analysis show a deletion or duplication	(Gatta *et al.* 2005)
▪ ribosomal protein synthesis and collagen synthesis raised	(Ionasescu, Burmeister and Hanson 1980)
▪ abnormal ECG patterns	(Lane, Gardner-Medwin and Roses 1980) (Grain *et al.* 2001)
▪ abnormal echocardiogram	(Grain *et al.* 2001)
▪ defective lymphocyte capping	(Ho *et al.* 1980)
▪ elevated PK levels	(Falcao-Conceicao *et al.* 1983)
▪ elevated CK levels	(Moser and Emery 1974)
▪ elevated serum myoglobin	(Nicholson 1981)
▪ muscle provocation test (MPT) elicits ▪ an increased elevation in CK levels	(Herrmann and Spiegler 1983)

(Adapted from Emery 1993)

1.6 Life expectancy and mortality

There are conflicting opinions on life expectancy and mortality rates in DMD. As a result of the improved treatment of respiratory infections (Mukoyama *et al.* 1987), scoliosis treatment (Brooke *et al.* 1989; Galasko, Delaney and Morris 1992; Galasko and Delaney 1993), special care programs (Satoyoshi 1992; Galasko, Williamson and Delaney 1995), or the use of respirators in terminal care (Fukunaga *et al.* 1993), an improved life expectancy in DMD patients has been suggested by several authors. Some researchers maintain that no increased life expectancy is currently evident (Cambridge and Drennan 1987; Miller, Moseley and Koreska 1992; Kennedy *et al.* 1995).

With respect to mortality, several authors have reported no significant changes in mortality over the years. It is, however, generally agreed that death generally occurs between 15 and 25 years of age and most commonly from respiratory or cardiac failure (Gilroy *et al.* 1963; Inkley, Oldenburg and Vignos 1974; Newsom-Davis 1980). An extensive study of 197 DMD patients by Eagle *et al.* (2002) concluded that improved co-ordinated care improved patients' chance of survival to 25 years of age from nil in the 1960s to four percent in the 1970s and 12% in the 1980s. However, since 1990, the significant impact of nocturnal ventilation has improved the chances of DMD patients living to 25 years of age to 53% (Eagle *et al.* 2002). Patterson *et al.*(1990), in determining mode of death, concluded that boys generally die both peacefully and often unpredictably (Patterson, Morrison and Hicks 1991).

1.7 Physical therapies

The concourse of an interdisciplinary team in the physical management of DMD can facilitate a more satisfactory patient outcome. Healthcare professionals including general practitioners, physicians, orthopaedic surgeons, physiotherapists, dieticians, psychologists, social workers, and teachers have an integral role in the management of this disease for the patients, their families, and the community. Integral to primary care strategies in DMD are the adoption of physical therapies.

1.7.1 Prolongation of ambulation

In terms of physical therapy strategies, prolongation of ambulation in DMD may be achieved by the use of light-weight ankle foot orthoses or by surgically releasing contractures (Heckmatt *et al.* 1985). In contrast, Manzur *et al.* (1992) reported surgical treatment of contractures failed to substantiate prolongation of ambulation.

In a study of 144 DMD patients Vignos (1996) found contractures of the lower extremities were controlled best when patients were managed with a combination of daily passive stretching exercises, prescribed periods of

standing and walking, tenotomy of the Achilles tendon, posterior tibial-tendon transfer, and application of knee-ankle-foot orthoses. Approximately two years after bracing, the severity of the contracture of the heel cords was found to be similar in DMD patients who had had an operation and those who had not. By the fourth year after bracing, however, DMD patients who have had an operation have less severe contractures than those who had had bracing alone. Five to seven years after the operation and bracing, control of contractures was considered good, especially for the patients who had had posterior tibial-tendon transfer. Contracture of the knee was well controlled five to seven years after bracing in all patients who had had bracing, with or without an operation. This surgical program enabled the patients who had been managed with bracing to walk until a mean age of 13.6 years. After loss of the ability to walk with bracing, the ability to stand continued for an additional two years with use of orthoses. The findings of this study demonstrated the value of traditional methods of operative treatment and bracing for controlling contractures of the lower extremities in DMD patients and for prolonging their ability to walk (Vignos *et al.* 1996).

Heckmatt *et al.* (1985) concluded that the prolongation of walking (ambulation) in DMD further prevented the development of scoliosis, joint contractures and deformities together with benefiting DMD patients psychologically.

1.7.2 Orthopaedic management of scoliosis

Spinal deformity (and resultant scoliosis) is common in DMD and predictably occurs after loss of ambulation. In DMD scoliosis (in contrast to idiopathic scoliosis) there is no side preference of the convexity (Furderer *et al.* 2000). Due in part to the varying severity of the clinical course of DMD the orthopaedic management of scoliosis in the disease is not clinically standardised. Management strategies range from no treatment, to the use of customised body jackets, wheelchair inserts or braces, through to surgical spinal fusion (Colbert and Craig 1987).

The use of spinal orthoses (bracing) minimally slows the progression of scoliosis but ultimately fails to prevent a substantial curve

developing (Colbert and Craig 1987) with significant curves developing in some 94% of DMD patients (Cambridge and Drennan 1987).

Hopf (1994) argues that early surgical correction and spinal stabilisation (Cobb angle greater than 20 degrees) is the treatment of choice for scolioses in DMD. In addition, the use of multi-segmental instrumentation methods enables rapid mobilisation and postoperative care without bracing. This approach allows a prophylactic operation affording improvement of sitting position, prevention of rapid deterioration of respiratory function including assisted mechanical ventilation in late stages, and ultimately prolongation of life expectancy (Hopf *et al.* 1994). Early surgical intervention in DMD scoliosis also minimises anaesthetic, perioperative and postoperative complications (Gayet 1999).

Lumbar fixation to L5 is adequate if the scoliosis surgery is performed appropriately early and with minimal curves and pelvic obliquity. The use of pedicle screws in the lumbar spine provides a sufficient foundation to maintain the necessary surgical correction over the period of relatively short life expectancy of DMD boys (Sengupta *et al.* 2002).

Surgical pelvic fixation may be necessary in older DMD patients, who have larger spinal curves and established pelvic obliquity; however, in the presence of deteriorating respiratory function, this surgery is associated with both a greater morbidity and higher complication rate (Sengupta *et al.* 2002). In advanced DMD cases (where surgical intervention is absolutely contraindicated) orthotic treatment (such as a double plaster cast or brace) may offer an acceptable compromise (Heller *et al.* 1997)

Ultimately, nocturnal ventilation is the most important factor in the improvement in survival of patients with late stage DMD and the best prognosis remains for older DMD patients who have had both scoliosis surgery and have nocturnal ventilation (Eagle *et al.* 2004).

1.7.3 Prevention of pulmonary mortality

Respiratory failure is the primary cause of mortality in DMD, may occur suddenly, and is often precipitated by an acute respiratory infection. Researchers estimate that between 55% (Mukoyama *et al.* 1987; Brooke *et*

al. 1989) and 90% of DMD patient mortality results from respiratory failure between 16.2 and 19.0 years of age (Emery 1980; Rideau, Glorion and Duport 1983). Eagle *et al.* (2002) reports that the implementation of nocturnal ventilation has improved survival rates from 19 years of age in non-ventilated DMD patients in 1967 to 25 years of age in ventilated patients (Eagle *et al.* 2002).

The first clinical signs of respiratory insufficiency are sleep-related breathing disorders and are manifested in nocturnal hypoxia with symptoms including nightmares, restless sleep patterns, headaches, morning confusion, and daytime hypersomnolence (Smith, Edwards and Calverley 1989). In DMD patients with advanced respiratory compromise, row-a-boat phenomena is a respiratory movement adopted to compensate for atrophied respiratory muscles (Yasuma *et al.* 2001).

A respiratory vital capacity of less than one litre is currently considered to be the best single predictor of non-survival in DMD patients (Phillips *et al.* 1999). When respiratory failure becomes evident, assisted nasal ventilation is considered a paramount treatment option (Simmonds *et al.* 1998). Therefore, the provision of adequate nocturnal mechanical ventilation is the most important factor in the improvement in survival of patients with late stage DMD (Eagle *et al.* 2004).

1.7.4 Prevention of cardiac mortality

The mortality incidence of cardiac origin in DMD reportedly varies from 10% (Gulati *et al.* 2005) to 12.5% (Ishihara 2004). A 2005 retrospective study of the ECGs of 111 DMD patients under 21 years of age concluded that the progressive decline in cardiac function of patients with DMD can be positively altered by corticosteroid treatment, that the positive cardiac effects elicited by corticosteroids appear to be sustained beyond the duration of treatment, and that the benefits of corticosteroid treatment are independent of steroid type (prednisone or deflazacort) (Markham *et al.* 2005).

Jefferies *et al.* (2005) found that the early diagnosis and treatment of dilated cardiomyopathy in DMD lead to ventricular remodelling, and a subsequently improved prognosis, in patients (Jefferies *et al.* 2005). Overall it

appears that the development of improved diagnostic techniques (Ashford *et al.* 2005) and early detection of cardiac involvement leading to appropriate treatment is a major factor in preventing or delaying cardiac morbidity in DMD patients (Gulati *et al.* 2005).

1.8 Dystrophin and the dystrophin-associated glycoprotein complex (DGC)

Dystrophin is a rod-shaped cytoskeleton protein with a molecular weight of 427 kilo Daltons (kDa), making it one of the largest known human proteins (Koenig *et al.* 1987; Koenig, Monaco and Kunkel 1988). The protein consists of 4 domains; an amino-terminal actin-binding domain, a central rod domain (containing spectrin-like repeats), a cysteine-rich domain, and a unique carboxy-terminal domain (Figure 1-3). Dystrophin is located on the inner surface of the plasma membrane in cardiac, skeletal, and smooth muscle cells.

Ervasti and Campbell (1991) demonstrated that dystrophin is tightly linked to a 156 kDa extracellular glycoprotein by way of a complex of transmembrane proteins known as the dystrophin-associated glycoprotein complex (DGC). The cysteine-rich and C-terminal domains of dystrophin interact with the DGC which is divided into two protein groups; sarcoglycans and dystroglycans. The DGC thus provides a link between the cytoskeleton and laminin-2 which is the extracellular matrix protein linking dystroglycan (Ervasti and Campbell 1991) (Figure 1-12).

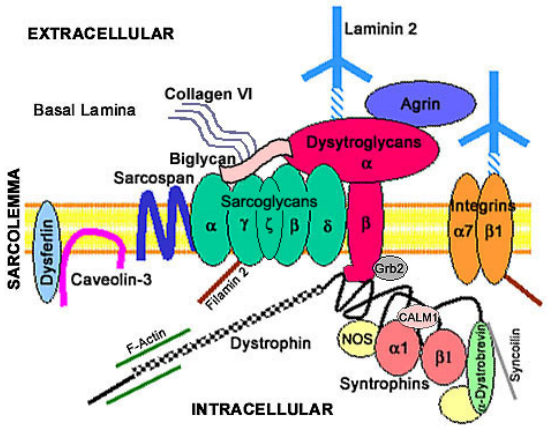


Figure 1-9 Dystrophin and the dystrophin-associated glycoprotein complex (DGC)

The DGC forms a transmembrane link between the extracellular matrix (laminin α-2 chain) and the intracellular skeleton (actin filaments), which is thought to mechanically stabilise the plasma membrane of muscle cells. Available from http://www.neuro.wustl.edu/ neuromuscular

The currently understood roles of the DGC are listed in Table 1-3 below;

Table 1-3 Currently understood roles of the DGC

Role of the DGC	Reference
▪ stabilisation of the plasma membrane against mechanical stresses	(Petrof *et al.* 1993)
▪ organisation of membrane proteins	(Brenman *et al.* 1995)
▪ signal transduction	(Brenman *et al.* 1995)
▪ Ca^{2+} regulation	(Song *et al.* 1996)

In DMD patients the DGC appears drastically reduced at the membrane (Sakuraba *et al.* 1993). The absence of dystrophin thus leads to a disruption of the DGC, a loss in the integrity of the plasmalemma, and subsequent myocyte necrosis (Hoffman 1996). Exactly how the disruption of the DGC leads to myocyte necrosis is not clearly understood, but several lines of evidence suggest that oxidative stress may play an important role in the process (Brown 1995). Mendell, Engel, and Derrer (1971) first proposed that an imbalance of free radicals contributes to the necrotic processes in DMD. This theory was further substantiated by Mizuno (1984) who reported elevated levels of free radical-scavenging enzymes in dystrophic muscle (Mizuno 1984).

1.8.1 Neuronal nitric oxide synthase and nitric oxide synthase

Neuronal nitric oxide synthase (nNOS) in skeletal muscle is anchored to the sarcolemma via the DGC and produces free radicals (Brenman *et al.* 1995; Brenman *et al.* 1996). The PDZ domain of dystrophin interacts directly with syntrophin which, in turn, is directly associated with dystrophin (Brenman *et al.* 1996) (Figure 1-12). The absence of dystrophin subsequently leads to a cytosolic mislocalisation of nNOS to the interior of the myocyte where it continues to produce nitric oxide (NO) and maintains some of its enzymatic activity (Brenman *et al.* 1995; Chang *et al.* 1996), namely the conversion of arginine to citrulline and NO (Crosbie *et al.* 1998).

In addition, there is insufficient counterbalance by NO of α-adrenergic vasoconstriction in DMD patients and the *mdx* mouse model further suggesting that the mechanical stress in DMD is worsened by the existing functional ischaemia (Thomas *et al.* 1998; Sander *et al.* 2000; Hoffman and Dressman 2001; Rando 2001). NO is a highly reactive molecule that can modify lipids, proteins, and DNA through various biochemical mechanisms (Beckman 1991; Moncada and Higgs 1993; Marletta 1994; Nathan and Xie 1994; Yun, Dawson and Dawson 1996). This knowledge has led to the hypothesis that free radical toxicity resulting from mislocalised nNOS may significantly contribute to DMD muscle pathology (Taylor *et al.* 1980; Crosbie *et al.* 1998). Regulation of nNOS activity, which normally occurs via Ca^{2+}-calmodulin, may be altered by elevated levels of intracellular Ca^{2+} in *mdx* mouse muscles (Bertorini *et al.* 1982; Jackson, Jones and Edwards 1985; Turner 1991).

Damage to the sarcolemma, an increase in cytosolic Ca^{2+} concentrations, and ischaemia can all lead to an inflammatory response and the subsequent activation of inflammatory cells and increased production of inflammatory mediators (Spencer and Tidball 2001). Wehling, Spencer and Tidball (2001) (Wehling, Spencer and Tidball 2001) concluded that the over expression of NOS in *mdx* mouse muscles reduces the concentration of

cytotoxic macrophages. This mechanism had been shown to be disease-specific as nNOS is linked directly (via α-syntrophin) to dystrophin (Thomas *et al.* 1998; Sander *et al.* 2000; Hoffman and Dressman 2001; Rando 2001). A chronic inflammatory state exists in DMD which further contributes to progressive tissue degeneration and a vicious cycle of muscle degeneration is repeated as shown in Figure 1-13.

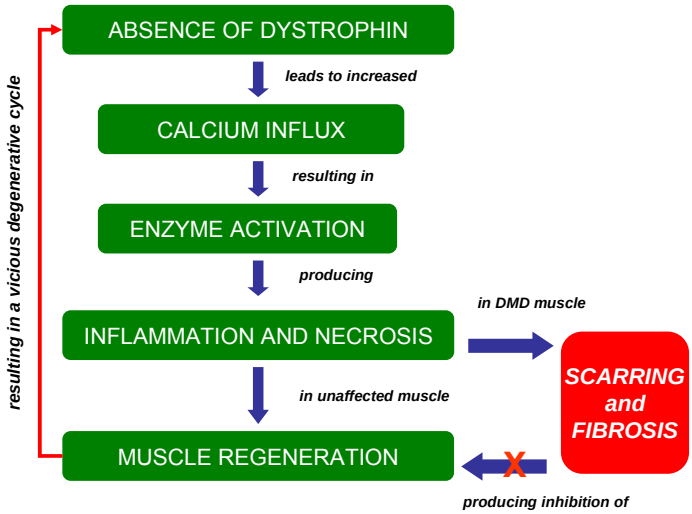


Figure 1-10 Vicious cycle of muscle degeneration in DMD

1.9 Animal models of DMD

Various animal models of DMD, both naturally occurring and genetically engineered, are available to muscular dystrophy researchers (Cooper 1989; Allamand and Campbell 2000; Bogdanovich *et al.* 2004). The utilisation of these animal models constitutes an invaluable asset, allowing extensive pre-clinical studies on the efficacy, safety, and functionality of therapeutic strategies (Nonaka 1998). However, although these experimental models possess mutations in the dystrophin gene and may present similarly to the biochemical dystrophinopathy observed in humans, there is marked disparity in terms of pathological severity and phenotype between species. Animal models of DMD may differ considerably in terms of their suitability as experimental models of the human disease. Collins and Morgan (2003) maintain that it is only possible that a successful treatment will eventually be

identified through the integration of studies in multiple species differentially suited to addressing particular research questions (Collins and Morgan 2003). Therefore, it is imperative that researchers thoroughly determine the suitability of specific animal models when determining their applicability as valid research tools in DMD studies.

1.9.1 The X-linked muscular dystrophy (*mdx*) mouse

In 1984, Bulfield *et al.* identified the naturally occurring *mdx* mouse. The *mdx* mouse is a spontaneous mutation of the C57BL/10ScSn (C57) control mouse strain with elevated serum CK levels and dystrophic muscle lesions. The *mdx* mouse was described as a murine glycolytic mutation with histological myopathy (Bulfield *et al.* 1984).

The external appearance of the *mdx* mouse is similar to the non-mutant C57 mouse strain, with no muscle wasting evident visibly, and minimal muscular weakness (Nonaka 1998) (Figure 1-14). Molecular features of the *mdx* mouse were investigated by Sicinski *et al.* (1989) who determined that a replacement of a cytosine by a thymine at position 3185 of the sequence coding of the gene produced a stop codon that permanently terminates the translation of dystrophin. The truncated protein comprises the N-terminal domain and seven repetitive sequences of the central domain. The rest is absent, and it is unknown as to whether the residual protein is actually stable and functional (Sicinski *et al.* 1989).

Points of similarity with the *mdx* mouse model and human DMD are the X-linked mode of inheritance, mutations in the dystrophin gene, elevated serum CK levels, primary skeletal muscle involvement, cardiac muscle involvement, smooth muscle involvement, and the absence of dystrophin (Emery 1993).

Figure 1-11 C57BL/10ScSn (C57) and X-linked muscular dystrophy (*mdx*) mice. Visual classification of *mdx* mutant mice is difficult due to the mild phenotype. The mouse shown lower left is a control (C57) mouse with the *mdx* mouse positioned upper right. Available from http://jaxmice.jax.org/library/notes/446c.html

However, there are significant discrepancies between the *mdx* mouse model and the human disease form. Muscle histology differs between humans and *mdx* mice in that, although muscle degeneration is evident early on in the *mdx* mouse, regeneration subsequently occurs (Dangain and Vrbova 1984). Furthermore, there is no evidence of fibre loss or progressive necrosis in the *mdx* mouse as there is in DMD (Emery 1993), and despite elevated serum CK levels, the *mdx* mouse expresses a mild phenotype (Bogdanovich *et al.* 2004).

In the *mdx* mouse, onset of the first necrotic wave is reported at around 14 to 21 days of age and continues for approximately one month. Active and efficient regeneration of skeletal muscle appears to then compensate for the repeated cycles of muscle degeneration. This progression is in distinct contrast to DMD in humans in which skeletal and cardiac muscle is progressively damaged and replaced by fibrotic tissue and scarring (Dangain and Vrbova 1984). This disparity extends through the lifecycle of the *mdx* mouse (Bogdanovich *et al.* 2004).

In aged *mdx* mice the respiratory diaphragm muscle pathology resembles muscle from DMD patients (Stedman *et al.* 1991). *Mdx* muscle exhibits susceptibility to damage by eccentric contractions and has lower specific force (Sacco *et al.* 1992; Moens, Baatsen and Marechal 1993; Petrof *et al.* 1993). The skeletal muscle of the *mdx* mouse shows dystrophic changes with active myocyte necrosis and regeneration. Necrotic fibres are frequently aggregated with tens to hundreds of fibres simultaneously undergoing necrosis (Tanabe, Esaki and Nomura 1986; Torres and Duchen 1987; Woo *et al.* 1987).

Further similarities and anomalies exist between DMD and the *mdx* mouse model. Similarities include the absence of dystrophin, a reduction in the complex of associated glycoproteins, and the presence of utrophin along the muscular membrane (not restricted to the neuromuscular junction (NMJ) as in unaffected muscle). The most significant anomaly lies in the fact that in the *mdx* mouse regeneration compensates for the degeneration of skeletal myocytes, whereas, in DMD the regeneration process is diminished and myocytes are progressively replaced with fatty and connective tissues (De La Porte, Morin and Koenig 1999).

However, due to the wide availability, low mouse breeding costs, speed of reproduction, the fact that the original mouse homozygous/hemizygous cross in this X-linked condition means that all the off spring are homozygous or hemizygous for the *mdx* mutation, and despite the obvious differences between the *mdx* mouse and the human disease, the *mdx* mouse remains the preferred DMD experimental animal model (Emery 1993; Bogdanovich *et al.* 2004)

Currently all studies involving *mdx* mice use mice from a separate wild type colony of controls such as the C57 mouse strain. This leaves researchers on the *mdx* mouse open to the criticism that after over 20 years of separate mouse straining that the experimental controls and *mdx* mice may have a different genetic background and that any reported differences may be the result of a genetic mutation other than the one on the dystrophin gene.

In order to solve this problem Kueh, Head, and Morley (2004) generated a new line of *mdx* mice (N1F1 *mdx* mice). Male C57BL/10ScSn-DMD (*mdx*) mice were bred with female C57BL/10ScSn mice, the offspring produced were mated to produce the following offspring: 50% females homozygous (*mdx*/*mdx*); 50% females heterozygous (*mdx*/+); 50% males hemizygous (*mdx*/Y) and 50% males wild type (+/Y). Kueh (2004) also showed that the litter mates can be conveniently phenotyped by serum CK measurements, skeletal muscle histology, and/or western blots for dystrophin using a polyclonal dystrophin antibody. N1F1 *mdx* mice provide an inexpensive and convenient method of generating male *mdx* mice with male litter mate controls on a genetically identical background, thus improving the

appropriateness of the *mdx* as an experimental model of DMD (Kueh, Head and Morley 2004).

1.9.2 Alternative mouse models

Other mutations in the dystrophin gene have been found in mice including the *mdx*2-5^{cv} mouse that develops a dystrophic phenotype (Chapman *et al.* 1989; Cox *et al.* 1993). Araki *et al.* (1997) observed an exon 52 knockout (52KO) mouse with high serum CK activity, an absence of dystrophin in myocytes, skeletal muscles of hypertrophic appearance, and yet only mild muscular weakness at age 12 months (Araki *et al.* 1997). The overall pathological findings in the skeletal muscles of the 52KO mouse are similar to the *mdx* mouse with marked variation in fibre size, active fibre necrosis, and active fibre regeneration. In the 52KO mouse there is no interstitial fibrosis, regeneration appears to compensate for necrosis, and the diaphragm is substantially involved (Nonaka 1998). The dystrophic (dy/dy) mouse and DMD patients both possess a calmitine deficiency (the absence of an inhibitor of calmitine-specific mitochondrial protease). This inhibitor is believed to prevent protein degradation (Lucas-Heron 1997). A double mutant lacking both dystrophin and utrophin, the *mdx/utn*$^{-}$ mouse, has also been generated and displays a phenotype closer to that of DMD patients (including cardiomyopathy) thus appearing to be an improved mouse model of the disease (Deconinck *et al.* 1997; Grady *et al.* 1997).

Mouse models only partially resemble human disease with currently no naturally occurring mouse models associated with loss of other DGC components available to researchers. This has encouraged the generation of genetically engineered mouse models for DGC-linked muscular dystrophy. Not only have analyses of these mouse models led to a significant improvement in our understanding of the pathogenetic mechanisms for the development of DMD, but they are potentially valuable tools for the augmentation of novel therapeutic approaches for the disease (Durbeej and Campbell 2002).

1.9.3 Feline disease models

In 1989, Carpenter *et al.* described a pair of male, two year old, sibling cats that presented with walking difficulties. Despite the absence of progressive muscular weakness, serum CK levels were raised, dystrophic muscular changes were apparent, dystrophin was not expressed, and yet no genomic deletion could be found (Caron *et al.* 1999). As two female siblings and the queen were unaffected, the disorder appeared to be X-linked. The condition described was progressive and histopathologically resembled DMD in displaying hypercontraction of myocytes, necrosis, phagocytosis, and a regenerative process. The cats differed from DMD in showing only a mild increase in endomysial connective tissue together with a lack of fatty infiltration. Marked muscle hypertrophy associated with hypertrophy and splitting of myocytes was present. Ultrastructurally there was distension of the SR and T-tubule system, swelling of the mitochondria, disorganisation and disarray of the myofilaments, and perforation of the plasma membrane. The histopathology was remarkable for hypertrophy and splitting of fibres, with progressive accumulation of Ca^{2+} deposits within the muscle. Unfortunately, this lineage of cats could not be further investigated as a potential DMD model as the entire family was neutered prior to original diagnosis (Cullen and Watkins 1993). The disease was termed feline X-linked muscular dystrophy (FXMD). FXMD is characterised by marked dystrophin deficiency, muscle hypertrophy, conservation of strength, moderate fibrosis, and an absence of fatty infiltration (Caron *et al.* 1999).

Subsequently, two male cats with markedly reduced dystrophin expression were investigated. They exhibited reduced level of activity, stiff gait, and general skeletal muscle hypertrophy involving the tongue and diaphragm. Due to the presence of extensive muscle hypertrophy, the diagnosis for this disease in felines was proposed as hypertrophic feline muscular dystrophy (HFMD) (Gaschen *et al.* 1992). The dystrophin gene involving the muscle and Purkinje neuronal promoters and their respective first exons was found to be deleted in the HFMD (Winand *et al.* 1994). HFMD cats are characterised by hypertrophy of the axial and appendicular musculature (Gaschen and Burgunder 2001). HFMD exhibits similar clinical

symptoms to human DMD (Carpenter *et al.* 1989). Histopathology shows muscle hypertrophy with progressive Ca^{2+} accumulation. Clinically, the diaphragmatic and glossal hypertrophies are predominant features. However, in the dystrophic cat, there is no progressive loss of myocytes, fibrosis, or muscle weakness (Hoffman and Gorospe 1991) thus limiting the applicability of the feline DMD model for experimental purposes.

1.9.4 Canine disease models

In 1958, Meier described a degenerative muscle disease in golden retriever dogs (Meier 1958). The disease was suspected as being homologous with DMD, however, a precise diagnosis of canine muscular dystrophy could not be made until the dystrophin gene was isolated in 1987 (Hoffman, Brown and Kunkel 1987; Koenig *et al.* 1987). Canine X-linked muscular dystrophy models include the Golden Retriever muscular dystrophy dog (GRMD) (Cooper *et al.* 1988; Kornegay *et al.* 1988), the German shorthaired pointer (Schatzberg *et al.* 1999), the wire-haired fox terrier (Gorospe *et al.* 1991), the Belgian shepherd, Irish terrier, miniature schnauzer, Samoyed, and Rottweiler. Specifically, in the GRMD a point mutation within the splice acceptor site of intron 6 leads to deletion of exon 7 from the dystrophin mRNA, and the consequent frameshift causes early termination of translation and resultant muscular dystrophy (Bartlett *et al.* 2000). Western blot analysis of skeletal muscle from GRMD dogs reveals a slightly truncated 390 kDa protein that is approximately 91% the size of normal dystrophin (Schatzberg *et al.* 1998).

Kornegay (1990) (Kornegay *et al.* 1990) used an affected golden retriever male dog to establish two colonies of GRMD canines. One colony was sent to Western Australia (Howell *et al.* 1997) and the first Australian carrier bitches were born in 1992. Specifically, GRMD has been shown to be an excellent model for the study of gene therapy in dystrophin deficient myopathies (Howell *et al.* 1997). GRMD disease features are tabulated in Table 1-4.

Estimating the serum CK activity tentatively identifies affected canines within the first 48 hours of life but carrier detection cannot be

determined by serum CK activity. For definitive carrier diagnosis, an exon seven specific genomic PCR product is used (Bartlett *et al.* 1996). Diagnosis of affected and non-affected canines may also be determined immunocytochemically in muscle (Howell *et al.* 1997). GRMD pups usually suckle less well and are weaker than non-affected pups. They tend to have decreased weight gain, with more specific clinical signs appearing at six to eight weeks of age (Table 1-4).

Table 1-4 Disease features of the GRMD canine model of DMD

GRMD disease features	reference
▪ elevated serum CK levels (>10,000 U/L compared with 100 U/L in controls)	(Cullen and Watkins 1993)
▪ cardiomyopathy	(Emery 1993)
▪ contractures	(Kornegay *et al.* 1994)
▪ death commonly as a result of cardiac failure	(Valentine, Cummings and Cooper 1989)
▪ difficulty in swallowing due to hypertrophy of the tongue and temporal muscle atrophy	
▪ functional benefits but histopathological adverse effects with prednisone treatment	(Liu *et al.* 2004) (Beltran *et al.* 2001)
▪ electroretinogram abnormalities not the same as in DMD	(Childers *et al.* 2002)
▪ eccentric muscle contractions induce greater muscle injury in GRMD than in controls	
▪ gross cardiac muscle lesions as the disease progresses	(Howell *et al.* 1997).
▪ histologically prominent muscle necrosis	(Emery 1993)
▪ necrosis and calcification of muscle	(Hoffman *et al.* 1994)
▪ necrosis and calcification of the diaphragm muscle	(Howell, Kakulas and Pass 1994)
▪ progressive muscle atrophy	(Kornegay *et al.* 1994)
▪ regeneration of myocytes	(Hoffman *et al.* 1994)
▪ revertant myocyte formation	(Howell *et al.* 1997)
▪ severely reduced transcripts from the dystrophin gene	(Sharp *et al.* 1992)
▪ shortened lifespan	(Valentine, Cummings and Cooper 1989).

Gait abnormalities are displayed as early as 10 weeks, which worsen gradually. Initial clinical signs include a progressive stiffness of the limb muscles, with the hind limbs affected more severely than the fore limbs, and as the pup walks or runs the hind limbs often advance simultaneously (bunny hopping). During the first year the gait becomes progressively more

restricted with the canines tiring easily. Overextension at the carpus, overflexion at the tarsus, and abduction of the paws occurs. Affected animals may die within days, months, or years of birth (with some canines living for six years or more). Pups may actually die within days of birth with a fulminating neonatal form of GRMD (Cullen and Watkins 1993). Mature GRMD canines usually die as a result of degeneration of the cardiac muscle (Valentine, Cummings and Cooper 1989).

Currently, the GRMD canine disease model appears the most appropriate due to the progressive nature of the disease and the clinical, pathological, and histological similarities to human DMD (Emery 1993). However, individual variation in phenotype, shorter lifespan than unaffected siblings, cost, and size prohibit large-scale canine straining and widespread research use. Subsequently, the use of canine models is largely restricted to comparative pathophysiological experiments and evaluation of DMD treatments that have previously been successful in amelioration of the *mdx* phenotype (Bogdanovich *et al.* 2004).

1.9.5 Zebrafish model

The zebrafish is rapidly emerging as an excellent model for specific human disease traits (Dooley and Zon 2000). Bassett *et al.* (2003) demonstrated that the *sapje* class of recessive lethal zebrafish mutations disrupts the zebrafish orthologue of the dystrophin gene. The progressive muscle degeneration phenotype of *sapje* mutant zebrafish embryos was found to result from the failure of embryonic muscle end attachments, thus potentially providing a model for pathological mechanisms in DMD muscle. Guyon *et al.* (2003) concluded that the zebrafish was an appropriate model for the study of dystrophin and its associated proteins, in that zebrafish; are small vertebrates (thus beneficial for genetic studies requiring large numbers of animals), possess a body of predominantly skeletal muscle (making the model attractive for studying muscle protein function), have potential orthologs to human DGC proteins, express sarcoglycans, dystroglycan, and dystrophin, and can be manipulated from diploid to haploid thus making the

model suitable for screening recessive genetic traits (Postlethwait and Talbot 1997).

1.9.6 The *Caenhorhabiditis elegans* worm

Invertebrate DMD animal models, including the *Caenhorhabiditis elegans* worm (*C. elegans*), are inexpensive, rapidly growing, and amenable to large genetic studies (Gaud *et al.* 2004). *C. elegans* possesses a dystrophin-like gene (*dys-1*) encoding a protein displaying the equivalent structural features as human dystrophin (Bessou *et al.* 1998; Roberts and Bobrow 1998) together with body-wall muscles with sarcometric structure and protein composition closely related to mammalian striated muscles (Schnabel *et al.* 1997).

In addition, the *C. elegans* model has been used to identify genes specific to counteracting the absence of dystrophin (Gieseler, Grisoni and Segalat 2000; Mariol and Segalat 2001; Gieseler *et al.* 2002). In a sensitised *MyoD* genetic background, *dys-1* mutations result in progressive locomotion impairment with widespread degeneration of body-wall muscles (Gieseler, Grisoni and Segalat 2000). The *C. elegans* model has limitations in that the *C. elegans* striated muscles differ in two ways to mammalian striated muscle in that they do not fuse and do not regenerate. Gaud *et al.* (1963) maintained that the *C. elegans* model was appropriate for performing large compound screens as a first pass with a view to eventually testing selected compounds on the *mdx* mouse. Luca (2004) argues that drug screening using the *C. elegans* model cannot have pre-clinical implications due to the lack of mechanistic support the model offers due to the significant differences between nematodes and mammals.

1.10 Pathology and histology of DMD

Significant abnormalities in muscle pathology are evident in DMD skeletal muscle prior to the observation of clinical symptoms. At the preclinical stage of the disease, histological changes may simply include increased variation in myocyte diameter and an increase in the number of

eosinophilic fibres (identified as densely eosin stained rounded fibres containing increased levels of Ca^{2+}_i), which are ordinarily absent or artefactual in unaffected skeletal muscle. Emery (1993) maintains that the accumulation of Ca^{2+}_i in DMD affected skeletal muscle typically precludes muscle necrosis. This increase in Ca^{2+}_i in DMD muscle has been demonstrated by a number of researchers using several methods including X-ray microanalysis (Maunder-Sewry and Dubowitz 1979), biochemical (Bertorini *et al.* 1982), and histochemical techniques (Bodensteiner and Engel 1978; Emery and Burt 1980).

In the preclinical stages of DMD, regenerating myocytes are observed as possessing small cell diameter, basophilic cytoplasm, increased ribonucleic acid levels, and large vesicular nuclei with prominent nucleoli (Emery 1993). Then, as the disease progresses, regenerating fibres are observed less frequently, necrotic myocytes become more obvious, and invasion by macrophages occurs (Cullen and Fulthorpe 1975). The pathological changes observed in DMD patients may also present in manifesting female DMD carriers (Emery 1993).

In cases of clinically established DMD, the histological changes in the quadriceps and gastrocnemius muscles include increased variation in fibre diameter, fibre necrosis, invasion by macrophages, and the eventual infiltration of adipose and connective tissue (Emery 1993) (Figure 1-12).

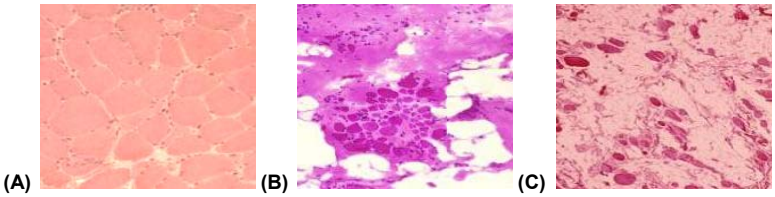


Figure 1-12 Human skeletal muscle biopsies of unaffected and DMD affected individuals.

Skeletal muscle biopsies of (A) unaffected individual at three years (b) DMD patient three years old (C) DMD patient nineteen years old (post mortem). The haematoxylin and eosin stained muscle biopsy shows marked variation in myocyte diameter, large hyaline fibres, and increased connective tissue in DMD affected muscle tissues (B) (C) compared to that of the unaffected individual (A). Available from http://www.rehabinfo.net

Figure 1-13 illustrates examples of typical pathological features of DMD affected muscles including the presence of numerous immature muscle fibres (Figure 1-13A), intermediate sized regenerating muscle fibres (Figure 1-13B), and the occurrence of elevated numbers of small regenerating fibres (Figure 1-13C). Pathological features of late stage DMD (Figure 1-13D) include an increase in endomysial connective tissue, variable myocyte size, elevated numbers of small rounded myocytes, and an abundance of hyper contracted myocytes.

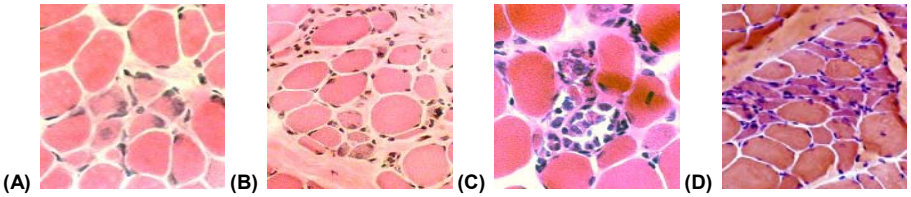


Figure 1-13 H&E stained sections of DMD affected human skeletal muscles.

The H&E stained tissues show features of (A) basophilic fibres (B) macrophage invasion (C) regenerating fibres and (D) late pathology in DMD. Available from www.neuro.wustl.edu

Interestingly, Karpati, Carpenter and Prescott (1988) concluded that smaller diameter myocytes appear more resistant to necrosis than myocytes of larger diameter, however, the cellular or molecular basis by which smaller calibre muscle fibres are resistant to the necrotizing effect of the dystrophic gene expression remains unknown.

It must be noted that muscle histology differs between DMD patients and *mdx* mice in that, although muscle degeneration is evident early on in the *mdx* mouse, regeneration subsequently occurs (Dangain and Vrbova 1984) that does not occur in humans. Therefore, it is imperative that this difference be born in mind when extrapolating experimental data between the species.

1.10.1 Calcium homeostasis

Bodensteiner and Engel (1978) reported that the muscle biopsies of DMD patients revealed 43 times the level of non-necrotic Ca^{2+} positive fibres of controls (Bodensteiner and Engel 1978). It is widely accepted that myocyte degeneration in DMD is related to the mechanical role of dystrophin, with the consequence that its absence would enhance contraction-induced focal

sarcolemmal damage. This, in turn, can increase cytosolic Ca^{2+} levels, leading (via a positive feedback loop) to activation of mechano-sensitive and leak Ca^{2+} channels, of Ca^{2+} dependent proteases, and ultimately necrosis in DMD muscle (Ruegg and Gillis 1999; Alderton and Steinhardt 2000; Mallouk, Jacquemond and Allard 2000; Imbert *et al.* 2001; Robert *et al.* 2001).

1.10.2 Centronucleated fibres

Centronucleated fibres (CNFs) are myofibres with their nuclei located in the central region of the cytoplasm (not peripherally) and are generally recognised as being regenerated myofibres. CNFs are commonly observed in the histopathology of both DMD patients and animal models of the disease (Narita and Yorifuji 1999). In DMD patients the population of CNFs is considered to be lower than in milder forms of muscular dystrophy (Tohgi *et al.* 1994).

In the histopathology of the *mdx* mouse (Figure 1-14), muscle degeneration occurs at three to five weeks of age, after which myofibres regenerate (presenting predominantly as CNFs) and the mouse lives a relatively normal life compared with the control strain. In contrast, the muscles of the *mdx* mouse diaphragm show progressive myofibre degeneration and interstitial fibrosis throughout the animal's lifespan (Stedman *et al.* 1991; Dupont-Versteegden and McCarter 1992; Louboutin *et al.* 1993; Gillis 1996; Carter *et al.* 1997; Anderson *et al.* 1998; Yang, Luo and Petrof 1998). Narita (1999) concluded that the failed CNF formation in the diaphragm muscles of *mdx* mice may actually be the equivalent phenomena as observed in human DMD patients (Narita and Yorifuji 1999).

Vital staining with Evans Blue (EB) dye shows that the sarcolemmal strength of CNFs compensates for the fragility of muscle tissue (Matsuda, Nishikawa and Tanaka 1995; Straub *et al.* 1997). CNFs are larger than non-CNFs (possibly further explaining their resistance to mechanical stresses) and have been shown to clearly be able to compensate for the fragility of muscle tissue. However, the mechanism by which CNFs acquire resistance to mechanical stress and grow into peripherally nucleated normal muscle fibres is still not known (Narita and Yorifuji 1999).

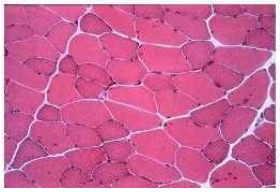
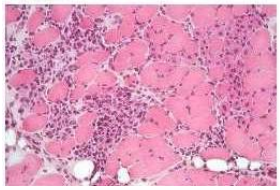
(A)  (B) 

Figure 1-14 H&E stained SOL muscles in a C57 and an *mdx* mouse.

Histological slides of H&E stained skeletal muscles in (A) a control C57 mouse and (B) an *mdx* mouse at four weeks of age. Note the presence of peripheral nuclei in unaffected muscle (A), whereas (B) possesses necrotic and regenerating regions which are characterised by infiltrating lymphocytes (inflammatory/necrotic areas) and areas of regenerating myofibres with central nucleation (CNFs). Available from http://www.unige.ch/sciences/pharm/fagie/research/necrosis.html

1.11 Therapeutic agents and strategies in DMD treatment and research

Current therapeutic strategies for the treatment of DMD specifically target components of the disease pathology with a specific view to prolonging ambulation, improving quality of life, and extending longevity. Five dominant approaches currently exist with respect to DMD therapeutics and research; dampening the inflammatory response, improving protein synthesis, inhibiting proteolysis, increasing muscle cell proliferation and regeneration, and gene based therapies. Typically the pharmacological strategies contrastingly involve either (a) the promotion of muscle growth, regeneration, and anabolism or (b) the inhibition of muscle lysis, cell death, and catabolism. The gene based therapies focus on repairing the primary genetic defect in DMD.

1.11.1 Dampening the inflammatory response

Inflammatory cells including mast cells (Gorospe *et al.* 1994), T cells, and macrophages (Spencer *et al.* 2001; Wehling, Spencer and Tidball 2001) have all been demonstrated to have a role in the inflammatory responses exhibited in *mdx* and DMD pathology. Therapeutics aimed at decreasing the inflammatory response in DMD focus primarily on eliciting immunosuppression through corticosteroids (prednisone, prednisolone,

dexamethasone, azathioprine, and deflazacort). In addition, natural remedies (Chinese herbal medicine and green tea extract) and cytokine modulation (TNF-α transgene, infliximab, nNOS transgene, and L-arginine) have also been investigated as anti-inflammatory strategies and treatments.

1.11.2 Corticosteroids

The administration of corticosteroids to ameliorate the progression of DMD was initiated in the 1970s (Demos *et al.* 1976). Over the past 30 years research studies (involving over 1000 DMD patients) have reported positive effects of corticosteroids in DMD (Emery 2001). Significant functional improvement in DMD patients has been observed as early as seven to 10 days after treatment (Sansome, Royston and Dubowitz 1993; Dubowitz *et al.* 2002; Emery 2002). Multiple, randomised trials have found improved function and strength in patients treated with prednisone (Mendell *et al.* 1989; Fenichel, Florence *et al.* 1991; Griggs *et al.* 1991; Griggs *et al.* 1993) and it has been estimated that the progress of the disease can be slowed by at least 3 years with long-term prednisone treatment (Fenichel, Florence *et al.* 1991). It should be noted that high dose of corticosteroids can, in fact, stimulate proteolysis & inhibit protein synthesis in skeletal muscle (Odedra, Bates & Millward 1983). Furthermore, prednisone was shown not to improve muscle repair and fibre growth in the *mdx* mouse model of DMD (Anderson, McIntosh and Poettcker 1996) and Burrow (1991) found no difference in dystrophin expression when the number of somatic dystrophin reverent fibres were compared from muscle biopsies of DMD patients treated with prednisone and controls (Burrow *et al.* 1991).

However, a methyloxazoline derivative of prednisone, deflazacort (DFZ), improves muscle repair and fibre growth in *mdx* mice (Anderson, McIntosh and Poettcker 1996). Clinically, DFZ has demonstrated similar positive effects to prednisone with an improved side effect profile when used in several clinical conditions (Aicardi *et al.* 1991; Gray *et al.* 1991; Loftus *et al.* 1991; Arizon, Anguita, Valles, Lopez-Rubio *et al.* 1993; Arizon, Anguita, Valles, Montero *et al.* 1993; Broyer *et al.* 1997) and in DMD (Biggar *et al.* 2001). Biggar *et al.* (2001) concluded that DFZ can preserve gross motor and

pulmonary function in boys with DMD with limited side effects. Angelini *et al.* (1994) further reported a significantly longer period of ambulation in DFZ-treated patients. DFZ is not currently commercially available in Australia.

The specific mechanisms through which corticosteroids produce a clinical benefit in DMD remain elusive. Systemic side effects are significant and include weight gain, cataracts, hypertension, diabetes, behavioural changes (Bogdanovich *et al.* 2004), and an increased risk of vertebral fractures as a result of bone demineralisation (Bachrach 2005). Glucocorticoid therapy further provokes bone fragility by increasing urinary Ca^{2+} loss, altering vitamin D metabolism, and impairing osteoclasts (Bachrach 2005). The side effect profile and risks involved in long-term use of steroids precludes their administration by some clinicians (Dubrovsky *et al.* 1998) and for those who opt to prescribe corticosteroids there remain controversial aspects to the treatment. Debate continues as to the specific age at which to commence treatment, the correct dose of corticosteroid to administer, the appropriate length of treatment and regime to adopt, when withdrawal is appropriate, and realistically what functional benefits can be expected (Dubrovsky *et al.* 1998; Muntoni *et al.* 2002). A comparatively small group of DMD patients (usually the most severely affected) fail to respond to corticosteroid therapy at all (Dubrovsky *et al.* 1998). However, despite the side effect profile, the controversy surrounding the specific therapeutic regimen, and the fact that a percentage of DMD patients fail to positively respond to treatment, corticosteroids (specifically prednisone) continue to be the first-line drugs of choice in the palliative treatment of DMD. A list of reported therapeutic benefits and proposed mechanisms of action of various corticosteroids in DMD research and treatment are presented in Table 1-5.

Table 1-5 Therapeutic benefits and theoretical mechanism(s) of action of corticosteroids in the treatment of DMD.

▪ Preservation of muscle strength (prednisone)	(Mendell *et al.* 1989) (Fenichel, Pestronk *et al.* 1991) (Fenichel, Florence *et al.* 1991) (Fenichel, Mendell *et al.* 1991)
▪ Preservation of muscle strength (DFZ)	(Bonifati *et al.* 2000)
▪ Anabolic effect on muscle (prednisone)	(Rifai *et al.* 1995)
▪ Stabilisation of myocyte membranes (prednisone)	(Jacobs *et al.* 1996)
▪ Attenuation of muscle necrosis (prednisolone)	(Takagi, Watanabe and Kojima 1998)
▪ Improved Ca^{2+} homeostasis (prednisolone)	(Metzinger *et al.* 1995) (Vandebrouck *et al.* 1999)
▪ Positive immunosuppressive effect with a reduction in cytotoxic $CD8^{+}$ cells (prednisone)	(Kissel, Burrow and Rammohan 1991)
▪ Reduction of infiltration of inflammatory cells into muscle (prednisolone)	(Wehling-Henricks, Lee and Tidball 2004)
▪ No immunosuppressive effects (azathioprine)	(Kissel *et al.* 1993) (Griggs *et al.* 1993)
▪ Positive effect on myogenesis (dexamethasone and prednisolone)	(Guerriero and Florini 1980) (Passaquin *et al.* 1993) (Sklar and Brown 1991) (Hardiman, Sklar and Brown 1993)
▪ Utrophin accumulation (dexamethasone)	(Pasquini *et al.* 1995)
▪ Increase in utrophin expression (prednisone)	(Courdier-Fruh *et al.* 2002)
▪ No increase in utrophin expression (prednisone)	(Kinali *et al.* 2002)
▪ Myoblast proliferation and differentiation	(Ball and Sanwal 1980)
▪ Inhibition of muscle proteolysis (prednisone)	(Rifai *et al.* 1995)
▪ Slowed progression of scoliosis	(Alman, Raza and Biggar 2004)
▪ Preservation of ventilatory function (prednisone)	(Yilmaz, Karaduman and Topaloglu 2004)
▪ Down-regulation of the expression of genes involved in the immune response (prednisone)	(Gosselin and McCormick 2004) (Muntoni *et al.* 2002)

1.11.3 Natural remedies

Both Chinese herbal medicines and green tea extract have been proposed as potential therapeutic agents in the treatment of DMD. Experimental results (in *mdx* mice) were mixed and inconclusive for green tea extract (Buetler *et al.* 2002). Chinese herbal treatments (including oral medications, decoctions, therapeutic massages and acupuncture) elicited minimal functional improvements with significant side effects including shortness of stature, hirsutism, cushingoid appearance, and weight gain (Urtizberea *et al.* 2003).

1.11.4 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a cytokine that is up-regulated in DMD (Porreca *et al.* 1999) and enhances the inflammatory response via direct toxicity. Research strategies attempting to modulate TNF-α levels in *mdx* mice (thus controlling cell-mediated damage to dystrophic muscle) to date have produced unpredictable and conflicting results (Tidball and Wehling-Henricks 2004) (Table 2-6).

1.12 Nitric oxide based therapeutics

The loss of NOS from dystrophic muscle significantly contributes to muscle pathology. This effect has been demonstrated to be a result of processes including the loss of both NO regulation of the inflammatory response (Wehling, Spencer and Tidball 2001) and NO-mediated vasodilation (Thomas *et al.* 1998; Sander *et al.* 2000). NO has been shown to have roles in both the scavenging of cytotoxic free radicals (Clancy, Leszczynska-Pizia and Abramson 1992; Kubes and Granger 1992; Wink *et al.* 1995) and the activation of satellite cells (Tatsumi *et al.* 2002). Wehling *et al* .(2001) demonstrated an anti-inflammatory effect, the normalisation of NO production in *mdx* mice, reduced concentration of macrophages, and reduction in muscle damage with NOS transgene expression. NO based therapeutics thereby has the potential to both decrease damage and promote

repair of DMD muscle (Table 1-6). NO also plays a role in signalling pathways in cardiac myocytes (Massion and Balligand 2003).

1.12.1 L-arginine

L-arginine is the substrate for NOS and research has indicated its potential use in DMD treatment via mechanism of utrophin upregulation. Researchers have demonstrated that L-arginine causes an increase in levels of utrophin in *mdx* mice and controls that is specifically muscle membrane localised to the sarcolemma (Chaubourt *et al.* 1999; Chaubourt *et al.* 2000; Voisin *et al.* 2005). Most recently, Voisin (2005) reported an up to three fold increase in utrophin levels, a 57% reduction in CK levels, a 35% reduction in lower limb muscle fibre necrosis, lowered collagen levels, and decreased lipid overloading in *mdx* mice treated with L-arginine (Voisin *et al.* 2005). Exceeding normal NO levels in muscles may be cytotoxic and this needs be born in mind when considering the potential use of NO upregulation in DMD treatment (Zhuang *et al.* 2001) (Table 1-6).

1.12.2 Utrophin upregulation

Utrophin is a cytoskeleton protein with over 80% homology with dystrophin and is expressed, at the NMJ, in normal and DMD muscles (Chaubourt *et al.* 1999). Dystrophin and utrophin are believed to have distinct roles in muscle with dystrophin responsible for a predominantly mechanical function and utrophin a predominantly architectural function (Rivier *et al.* 1997). The finding that the over expression (upregulation) of utrophin can functionally replace dystrophin in transgenic *mdx* mice has fuelled interest in developing pharmacological therapies that will increase endogenous utrophin expression in DMD patients (Tinsley *et al.* 1996). Increased levels of utrophin have been observed previously in DMD patients albeit in levels insufficiently raised to produce a positive clinical effect (Vainzof *et al.* 1995). The identification of molecules or drugs that potentially up regulate utrophin provides an opportunity for DMD researchers (Table 1-6).

1.13 Improving protein synthesis

Loss of functional muscle mass ultimately reduces both quality of life and longevity in DMD patients. Various therapeutic strategies aim to maintain the integrity of muscle mass through augmenting protein synthesis. The conservation of muscle mass in DMD also protects the potential for gene or stem cell therapies as the primary target muscle cells are preserved (Tidball and Wehling-Henricks 2004) (Table 1-6).

1.13.1 Anabolic steroids

The therapeutic use of anabolic steroids (such as norethandrolone, methandrostenolone, and oxandrolone) in the treatment of DMD has been extensively investigated (Charash 1965, Dowben and Perlstein 1961, Gamstorp 1964) and the challenge remains in isolating agents producing desirable therapeutic outcomes with minimal androgenic side-effects (Table 1-6).

1.13.2 Growth factors

Results with growth hormone (GH) therapy have been mixed and unpromising. However, insulin-like growth factor 1 (IGF-1) has been demonstrated to have a potent anabolic effect in mouse skeletal muscle *in vivo* (Bark *et al.* 1998; Musaro *et al.* 2001; Musaro *et al.* 2004) via the promotion of cell regeneration (Barton *et al.* 2002) and increased protein synthesis (Adams and McCue 1998; Barton-Davis *et al.* 1998), presenting promise as a potential pharmacological treatment in DMD (Table 1-6).

1.14 Decreasing proteolysis through reducing calpain activity

Proteolysis may be decreased via inhibition of Ca^{2+}-dependent proteases (calpains) or inhibition of the ubiquitin/proteasome system. Anti-proteolytic strategies potentially slow catabolic processes and reduce the

degradation of substrates integral to maintaining muscle function (Tidball and Wehling-Henricks 2004).

Ca^{2+} influx into dystrophic muscles is elevated (Bodensteiner and Engel 1978; Turner *et al.* 1988; Turner 1991; McArdle, Edwards and Jackson 1992). A 1995 study by Spencer, Croall and Tidball demonstrated that calpains in *mdx* mouse muscles have greater activity levels than in the C57 control strain and further work by Spencer and colleagues has shown that the over expression of a calpastatin (endogenous inhibitors of calpain) transgene in *mdx* mice reduced dystrophic pathology (Spencer and Mellgren 2002; Tidball and Spencer 2002) (Table 1-6).

1.15 β_2-adrenergic agonists

On the basis that β_2-adrenergic agonists potentially increase calpastatin expression in muscle, Spencer *et al.* (2002) investigated albuterol as a potential therapeutic agent. Results indicated an increase in muscle strength despite no improvement in muscle function tests. β_2-adrenergic agonists may not mediate their effects exclusively via calpains as it has also been demonstrated that β_2-adrenergic agonists inhibit the activation of macrophages and T cells (Sekut *et al.* 1995; Panina-Bordignon *et al.* 1997; Malfait *et al.* 1999) which have been shown to exacerbate muscle pathology in *mdx* mice (Spencer *et al.* 2001; Wehling, Spencer and Tidball 2001) (Table 1-6).

1.16 Increasing proliferation and regeneration of muscle cells

1.16.1 Myostatin inhibition

Myostatin (growth determining factor-8) is a member of the transforming growth factor (TGF) β superfamily of proteins that regulate the proliferation and differentiation of specific cell types (McPherron, Lawler and Lee 1997). The myostatin gene is genetically well conserved throughout

evolution and is identical between human and mouse species (Lee and McPherron 2001). Myostatin is expressed from developing somites through adulthood and is a highly conserved negative regulator of muscle mass (Bogdanovich *et al.* 2004). A double-muscle phenotype-Is observed in mice expressing dominant negative transgenes (Zhu *et al.* 2000; Lee and McPherron 2001; Yang *et al.* 2001; Nishi *et al.* 2002) or carrying a targeted myostatin deletion (McPherron, Lawler and Lee 1997).

Studies have shown that endogenous myostatin blockade using anti-myostatin antibodies results in biochemical, anatomical, and physiological improvement in *mdx* mice (cited in Ruegg *et al.* 2002). Wagner *et al.* (2002) showed improved phenotype in dystrophin and myostatin mutated mice. Myostatin inhibition also has been shown to induce muscle hypertrophy and/or hyperplasia with a corresponding decrease in body fat (Hamrick, McPherron and Lovejoy 2002; McPherron and Lee 2002; Ruegg *et al.* 2002; Zimmers *et al.* 2002). Researchers have shown increases in muscle mass and strength coupled with no adverse side effects in both *mdx* mice that are null mutants for myostatin (Wagner *et al.* 2002) and *mdx* mice treated with anti-myostatin antibodies (Bogdanovich *et al.* 2002). A case report of a child with a mutated myostatin gene and no detectable myostatin in his sera imply that anti-myostatin treatments in children may be without deleterious effects (Schuelke *et al.* 2004). As with other pharmacological treatments, myostatin inhibition fails to correct the underlying genetic defect and the muscles remain dystrophin deficient with any hypertrophy of the muscle increasing vulnerability to potential mechanical damage. Despite this, myostatin inhibition remains a potential therapeutic strategy for DMD treatment (Table 1-6).

1.16.2 Leukaemia inhibitory factor

Leukaemia inhibitory factor (LIF) is a potent mitogen of primordial germ cells (Resnick *et al.* 1992), haematopoietic stem cells (Leary *et al.* 1990; Verfaillie and McGlave 1991), and embryonic stem cells (Williams *et al.* 1988). Three major studies have found the following positive effects of LIF; LIF increases muscle regeneration in acutely injured muscles (Barnard *et al.*

1994), LIF prevents muscle damage after denervation (Finkelstein *et al.* 1996), and mice that are null mutants for LIF show decreased muscle repair (Kurek *et al.* 1997). Challenges in developing a safe method for systemic delivery of LIF to muscle continue to impede its use in the clinical treatment of DMD (Table 1-6).

Table 1-6 Therapeutics agents in DMD treatment and research

Therapeutic agent	Mode of action	DMD or *mdx*	Reported benefits	Adverse effects reported	Reference(s)
Norethandrolone	anabolic pathways	DMD	modest improvement in muscle strength	negative rebound effect, androgenic effects (priapism, acne, hair growth)	(Dowben and Perlstein 1961)
methandrostenolone	anabolic pathways	DMD	modest improvement in muscle strength	negative rebound effect, androgenic effects (priapism, acne, hair growth)	(Gamstorp 1964) (Charash 1965)
oxandrolone	anabolic pathways, activates gene transcription, antagonises cortisol binding to glucocorticoid receptors	DMD	lean body mass increase, improvement in quantitative muscle function	minor androgenic effects in children, no improvement in function, no significant adverse effects	(Fenichel *et al.* 1997) (Fenichel *et al.* 2001) (Wong and Christopher 2002) (Zhao *et al.* 2004) (Church 2004)
growth hormone (GH)	anabolic pathways	DMD	small improvement in cardiac function	no effect or a catabolic effect on skeletal muscle	(Rudman *et al.* 1972) (Cittadini *et al.* 2003)
mazindol	inhibits GH	DMD	increased muscle function	no improvement in muscle function, slowed growth, sleep disturbances, increased heart rate (HR)	(Zatz, Betti and Frota-Pessoa 1986) (Zatz *et al.* 1987) (Coakley *et al.* 1988) (Zatz *et al.* 1989) (Griggs *et al.* 1990)
IGF-1	anabolic pathways, satellite cell proliferation	*mdx*	increased whole body muscle mass and strength, reduced pathology, hyperplasia, improved respiratory muscle strength, decrease in the catabolic effects of prednisone, reduced fibrosis in *mdx*	none reported	(Mauras and Beaufrere 1995) (Barton *et al.* 2002) (Gregorevic *et al.* 2002) (Day *et al.* 2002) (De Luca *et al.* 2003)

Therapeutic agent	Mode of action	DMD or *mdx*	Reported benefits	Adverse effects reported	Reference(s)
calpastatin transgene	inhibits proteolysis by calpains in muscle	*mdx*	reduced necrosis	none reported	(Tidball and Spencer 2002)
albuterol	inhibits proteolysis, inhibits activation of macrophages and T cells, β_2-adrenergic agonist	DMD	increased muscle strength	none reported	(Fowler *et al.* 2004) (Spencer *et al.* 2002)
MG132	inhibits proteolysis via the ubiquitin/proteasome system	*mdx*	reduced muscle pathology	none reported	(Bonuccelli *et al.* 2003)
prednisone (see also Table 2-4)	immunosuppressant, inhibits proteolysis, enhances myogenesis, modulates Ca^{2+} handling, inhibits apoptosis	DMD and *mdx*	maintenance of strength and function, preservation of respiratory function, reduction in inflammation	weight gain, slowed longitudinal growth, behavioural disturbances, hirsutism, bone demineralisation (lowered density)	(Kissel *et al.* 1993) (Kawai *et al.* 1993) (Connolly *et al.* 2002) (Dubowitz *et al.* 2002) (Kinali *et al.* 2002) (Muntoni *et al.* 2002) (Merlini *et al.* 2003) (Wehling-Henricks, Lee and Tidball 2004) (Yilmaz, Karaduman and Topaloglu 2004)
		mdx	improved cardiac contractility		(Gosselin and McCormick 2004) (Hoey *et al.* 2002)
DFZ	immunosuppressant	DMD	similar to prednisone	similar to prednisone but generally less severe, cataracts	(Angelini *et al.* 1994) (Bonifati *et al.* 2000) (Biggar *et al.* 2001) (Silversides *et al.* 2003) (Alman, Raza and Biggar 2004) (Biggar *et al.* 2004)

Therapeutic agent	Mode of action	DMD or *mdx*	Reported benefits	Adverse effects reported	Reference(s)
					(Alman 2005)
azathioprine	inhibits DNA synthesis (not an immunosuppressant *per se*)	DMD	reduces inflammatory cells in muscle	no functional improvement	(Arizon, Anguita, Valles, Lopez-Rubio *et al.* 1993) (Griggs *et al.* 1993) (Kissel *et al.* 1993)
cyclosporine	inhibits calcineurin myoblast transfer	DMD and *mdx*	increased muscle strength	no effect or a detrimental effect on muscle function and histology, inhibition of muscle growth	(Weller *et al.* 1991) (Law *et al.* 1992) (Sharma, Mynhier and Miller 1993) (Law *et al.* 1993) (Mendell *et al.* 1995) (Miller *et al.* 1997) (Stupka *et al.* 2004)
herbal medicine	unknown	DMD	improved muscle function	weight gain, short stature, hirsutism, cushingoid appearance	(Urtizberea *et al.* 2003) (Courdier-Fruh *et al.* 2003)
green tea extract	antioxidant	*mdx*	reduced muscle necrosis (EDL)	no reduced muscle necrosis (SOL)	(Buetler *et al.* 2002)
tumour necrosis factor-alpha (TNF-α) transgene	TNF-mediated processes in muscle and inflammatory cells	*mdx*	reduced muscle pathology at some stages, improved respiratory function	worsened muscle pathology at some stages	(Spencer, Marino and Winckler 2000) (Gosselin *et al.* 2003)
anti-TNF-α therapy (infliximab/remicade®)	blocks TNF function	*mdx*	delayed muscle pathology	none reported	(Grounds and Torrisi 2004)
nNOS transgene	NO-mediated processes in muscle	*mdx*	reduced muscle pathology and inflammation	none reported	(Wehling, Spencer and Tidball 2001)
L-arginine	increased NO production by NOS	*mdx*	reduced muscle pathology, increased utrophin expression	none reported	(Chaubourt *et al.* 1999) (Chaubourt *et al.* 2000) (Voisin *et al.* 2005)

Therapeutic agent	Mode of action	DMD or *mdx*	Reported benefits	Adverse effects reported	Reference(s)
NO	vasodilatation and mediation of the inflammatory response			excess level of NO are cytotoxic	(Zhuang *et al.* 2001)
anti-myostatin	myostatin function blockade	*mdx*	muscle hypertrophy, hyperplasia, reduced pathology, improved muscle function	none reported	(Bogdanovich *et al.* 2002) (Wagner *et al.* 2002)
LIF	increased muscle cell growth and proliferation	*mdx*	muscle hypertrophy, hyperplasia, reduced fibrosis, improved muscle strength	none reported	(Austin *et al.* 2000) (White *et al.* 2001) (White *et al.* 2002)
perindopril	angiotensin converting enzyme (ACE) inhibitor	DMD	delayed onset and progression of prominent left ventricle dysfunction	consistent with ACE inhibitors	(Duboc *et al.* 2005)

1.17 Gene based therapies

Gene therapy can be broadly divided into two categories; somatic gene therapy and germ-line gene transfer. Somatic gene therapy aims to correct a gene defect in somatic cells and involves either the introduction of a normal gene directly to the affected tissue or *ex vivo* gene transfer to cells in culture followed by reimplantation into the patient. Germ-line gene transfer involves the introduction of genetic material to fertilised ova (thus integrating the DNA permanently into somatic and germ cell lines) (Turner, Dunckley and Dickson 1997).

Gene therapy in the treatment of DMD has met a number of obstacles, including immune reaction to viral vectors, difficulty in penetrating through the basal lamina, and the limited spread of action to a relatively small number of fibres (Dubrovsky *et al.* 1998). DMD affects muscles body-wide and an effective treatment ideally requires dystrophin induction in all muscles. For example, an adeno-associated virus (AAV) vector-mediated gene transfer has a limitation in insertion size up to 4.9 kb. Therefore, a full-length dystrophin cDNA (14 kb) cannot be incorporated into an AAV vector (Takeda 2004).

Another genetic approach involves regeneration of muscle using stem cells. There are many barriers to overcome in attempts to treat patients with bone marrow isolated stem cells. The most difficult challenge is in culturing the stem cells to increase their numbers (proliferate) for application and how to then introduce the normal dystrophin gene into these cells (Nonaka 2004). A 2005 study by Payne *et al.* demonstrated that murine skeletal muscle-derived stem cell (MDSC) transplantation into the hearts of *mdx* mice resulted in the expression of dystrophin-positive myocytes and concluded that MDSC transplantation warranted further investigation as a potential therapy for cardiac dysfunction in DMD.

The current vector based research and treatment strategies in DMD are illustrated in Figure 1-16.

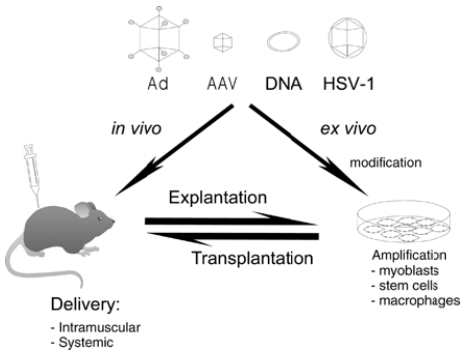


Figure 1-15 Vector based therapeutic strategies in DMD

Vector--based therapeutic approaches in DMD. Viral or non-viral vectors may be used for (i) *in vivo* gene therapy and may be delivered either directly into skeletal muscle or systemically; and (ii) *in vitro* gene therapy where they are used for infecting cultured cells that are then transplanted into the recipient animal. Ad, (adenovirus), AAV (adeno-associated virus), DNA (naked plasmid DNA), and HSV-1 (herpes simplex virus) (Allamand and Campbell 2000).

1.17.1 Aminoglycoside antibiotic therapy

Aminoglycoside antibiotic therapy aims to bypass the primary genetic defect in DMD and represents a novel treatment regimen for DMD cases specifically caused by premature stop codon mutations (approximately 15% of cases) (Bogdanovich *et al.* 2004). *In vitro*, gentamicin (an aminoglycoside antibiotic) has been shown to increase the frequency of erroneous insertion of nonsense codons hence permitting the translation of alleles carrying nonsense mutations to continue reading to the end of the gene thus allowing translation to suppress a gene mutation (Wilschanski *et al.* 2000; Hamilton 2001). Barton-Davis *et al.* (1999) further identified a gentamicin *mdx* mouse treatment regime that resulted in a 10% to 20% increase in dystrophin expression and functional improvement (demonstrating that aminoglycosides potentially suppress stop codons *in vivo*). However, Dunant *et al.* (2003) (Dunant *et al.* 2003) failed to replicate the results of Barton-Davis (1999). To date, no human gentamicin trials have demonstrated improved dystrophin expression (Dunant *et al.* 2003). It is also known that the specific isomeric structure of gentamicin has profound effects on the drug's propensity for inducing stop codon misreading (Loveless,

Kohlhepp and Gilbert 1984; Yoshizawa, Fourmy and Puglisi 1998). This may further limit the current therapeutic application of gentamicin treatment and it is clear that additional research is required to fully evaluate the potential of aminoglycoside therapy of DMD (Dunant *et al.* 2003).

1.17.2 Antisense oligonucleotides

Antisensense oligonucleotides (AOs) are targeting vectors designed to repair genetic mutations, to modify genomic sequences in order to compensate for gene deletions, or to modify ribonucleic acid (RNA) processing in order to ameliorate the effects of the underlying gene mutation (Rando 2002). AOs induce the specific skipping of a single exon, permitting the restoration of the reading frame, and allowing the synthesis of a largely functional dystrophin protein (Aartsma-Rus *et al.* 2004).

Increased levels of the cyclin-dependent kinase inhibitor p21 associated with decreased myoblast proliferation may be involved in the dystrophic process in DMD. Improved proliferation of primary myoblasts in DMD patients may result by a reduction in p21 using AOs.

Endesfelder *et al.* (2005) found that 80% of dystrophin-deficient cell culture samples revealed an 8% and 36% increase in muscle cell proliferation in untransfected control cells compared to cells transfected with scrambled AOs. Fletcher *et al.* (2005) further demonstrated dystrophin expression in *mdx* mouse tissues after localised and systemic delivery of an AO designed to target the dystrophin exon 23 donor splice site thus concluding that AOs are eminently suitable for consideration for therapeutic exon skipping in dystrophin mutations (Fletcher *et al.* 2005).

1.18 Sex hormones and DMD

Steroid and peptide hormones, together with nutrition and exercise are the primary regulators of muscle growth and maintenance in humans yet there is limited knowledge as to the effects of steroid hormones on individual muscle types (Sciote *et al.* 2001).

Glenmark *et al.* (1992) showed that whilst fibre areas remain constant there is a different development of fibre-type composition with

increased age in women and men: the type-I percentage tends to increase in the women and decrease significantly in men (suggesting a sex related fibre adaptation to increased age). Zatz *et al.* (1988) evaluated the relationship between height and rate of clinical progression in boys with DMD. Ninety two DMD patients with age ranging from two to 23 years (mean 8.2 ± 3.4 years) were assessed. Clinical course was determined through Vignos scale of functional disability, motor ability, and timed functional tests. All patients had grossly elevated serum CK and PK levels. When height was adjusted for patients' age, a statistically significant correlation was found between height and clinical course (positive with Vignos scale and negative with motor ability), suggesting that smaller boys have a better clinical course than taller DMD patients of comparable age. These results support the suggestion that growth inhibition may be effective in diminishing the clinical progression of DMD (Zatz *et al.* 1988).

In 2003, a growth hormone (GH) deficient DMD patient was reported by Ghafoor *et al.* The patient was clinically asymptomatic in terms of DMD. GH replacement therapy was initiated with the resultant emergence of clinical DMD symptoms including proximal muscle weakness and increased fatigability. Serum CK levels subsequently confirmed a diagnosis of DMD.

1.18.1 Testosterone

Testosterone is the primary androgen in the human body and its biosynthesis in males takes place primarily in the testis with lower level synthesis occurring in the adrenal cortex. The testosterone levels resulting from adrenal cortex production alone are insufficient in maintaining masculine characteristics. Testosterone is present in very low levels in prepubertal boys. At puberty, pulsatile secretion of gonadotrophin releasing hormone (GnRH) causes the anterior pituitary to produce leutinising hormone (LH) and follicle stimulating hormone (FSH). Circulating LH induces the Leydig cells of the testis to produce testosterone. As circulating testosterone levels rise, there is a negative feedback on the production of GnRH at the hypothalamic level, and LH and FSH at the pituitary level.

Circulating testosterone is present in several forms. The highest testosterone concentration is bound to sex hormone binding globulin (SHBC) and is not bioavailable. Testosterone may also circulate unbound (or bound weakly to albumin), and this unbound testosterone is bioavailable and responsible for exerting androgenic effects.

Testosterone is converted to other clinically important compounds in the peripheral circulation and/or tissues. Oestradiol is produced by esterification of testosterone. Elevated levels of oestradiol can downregulate (via a negative feedback mechanism) the hypothalamic pituitary gonadal axis, resulting in decreased GnRH secretion and decreased circulating testosterone levels. Subsequently, increased testosterone levels can stimulate the conversion of testosterone to oestradiol. In males, serum testosterone levels vary hourly (ultradian rhythm) together with a diurnal rhythm in which highest levels occur in early morning.

Testosterone is known to act differentially on skeletal muscle from different regions of the body. Two genes likely to mediate the testosterone effect are IGF-I (growth regulator acting in an autocrine and paracrine way) and the androgen receptor (AR) (receptor density may determine differential muscle growth).

Androgens stimulate myogenesis, but it is not known what specific cell types within human skeletal muscle express the AR protein or which specific cells are the target of androgen action (Sinha-Hikim *et al.* 2004). In 2002, the first study demonstrating that androgens induce skeletal muscle fibre hypertrophy in healthy young human males showed that the cross-sectional areas (CSAs) of type-I and type-II fibres increased with increased testosterone concentrations (whilst the proportion of type-I and type-II fibres remained constant). These results were consistent with findings from rodent studies that reported an increase in fibre diameter in levator ani muscle in response to androgen treatment (Sinha-Hikim *et al.* 2002). However, Souccar *et al.* (2005) showed that testosterone does not prevent the progress of muscle disease in the *mdx* levator ani muscle, but androgen withdrawal accelerates muscle wasting suggesting a nominal beneficial effect. Gonadectomy of young adult *mdx* mice caused atrophy of the levator ani muscle, accelerated muscle wasting, and reduced tetanic force by 31%.

The mechanisms by which testosterone causes muscle hypertrophy are unknown, as are the effects of testosterone on muscle fibre size and composition in humans. Data from animal studies are limited and contradictory (Sinha-Hikim *et al.* 2002).

In human males, the linear growth and increase in muscle and bone mass during puberty are mediated predominantly through the action of testosterone (Mauras 1995a). In DMD, the exacerbation of symptoms clearly coincides with the onset of puberty and arguably the most prominent clinical event is the loss of ambulation which occurs between seven and 13 years of age (Dubowitz 1978). Eagle *et al.* (2002), in a study of 197 DMD patients reported no significant differences in the mean age that walking independently ceased, and concluded that loss of ambulation occurred at age 9.3 years.

In 1981 Zatz *et al.* reported a unique case of a 13 year old Japanese boy who possessed both an idiopathic GH deficiency and DMD. Clinically, the child presented with hypertrophied calves, no visible muscle wasting, waddling gait, Gowers' manoeuvre when rising from the floor, short stature (104 cm), weight 21 kg, normal IQ, a bone age of four and a half years, and normal thyroid hormone levels. GH deficiency was confirmed through insulin tolerance testing and un-responsiveness to L-dihydroxyphenylalanine (L-dopa) (Liberman, Cesar and Wajchenberg 1979). Serum CK and PK levels were grossly elevated, electromyography (EMG) and muscle biopsy results were consistent with progressive muscle disease, and pedigree analysis confirmed DMD. The child had two younger male siblings and seven other male relatives with DMD, all of which showed a typical clinical course of the disease and were non-ambulant by 12 years old. The fascinating thing about this unique case is that the child showed an uncharacteristically benign clinical course of DMD, thus suggesting that the GH deficiency was, in some way, contributing to the slowed progression of the neuromuscular disease. This hypothesis was further supported by the work of Chyatte *et al.* (1973, 1974) who found that GH produced a catabolic response in DMD patients and not the expected anabolic response as occurred in controls (Chyatte *et al.* 1973; Chyatte *et al.* 1974). Titsuka *et al.* (1981) further reported that muscular dystrophy was not expressed in

genotypically dystrophic mice which were growth compromised by a dwarfism (dw) gene and that dystrophic symptoms could be alleviated in mice if bone lengthening was arrested (Totsuka, Watanabe and Kiyono 1981). In 1986 Zatz reported a five year follow-up study of the original abovementioned case. The patient, at 18 years of age, had a height of 120 cm, weighed 41 kg, a bone age of seven years, genitalia of pre-pubertal appearance, raised serum CK and PK levels, normal thyroid and adrenal function, and hypogonadism due to gonadotrophin deficiency. Extraordinarily the patient, despite being grossly obese, was still ambulant and able to walk short distances unaided. Zatz *et al.* (1986) reaffirmed the original hypothesis which suggested that the overtly benign course of DMD in the original case was in some way related to GH deficiency.

Based on the conclusions of Zatz *et al.* (1981), Collipp *et al.* (1984) evaluated the effect of the GH inhibitor mazindol in nine DMD boys. These researchers had previously observed that GH affected both zinc levels (Cheruvanky *et al.* 1982; Collipp *et al.* 1982) and, in turn, cell membrane lipid composition and function (Clejan, Collipp and Maddaiah 1980; Clejan *et al.* 1981; Maddaiah *et al.* 1981; Clejan *et al.* 1982). Mazindol treatment resulted in no significant change in baseline GH levels, no improvement in muscle strength, and no improvement in peak expiratory flow. However, mazindol did reduce weight gain and significantly increased serum selenium levels but the researchers concluded that the effect of GH suppression through mazindol treatment in DMD was inconclusive and required further study. In 1986 Zatz *et al.* conducted a therapeutic trial of mazindol in a pair of seven and a half year old monozygotic twins with DMD. After one year the study was aborted due to the overwhelming evidence that the disease course in the control child was significantly worse than the mazindol-treated twin and it was strongly suggested that GH inhibition may potentially have a role in DMD treatment (Zatz, Betti and Frota-Pessoa 1986). To date the specific mechanism of action of both GH and testosterone on normal human muscle remains unknown and even less information is available on the role of GH and testosterone in the *mdx* mouse model and DMD affected muscles.

1.18.2 Oestrogens

The biosynthesis of oestrogens from C19 steroids is catalysed by the aromatase cytochrome P450, a product of a single gene, *cyp*19. This enzyme is widely expressed in many tissues, including Sertoli and Leydig cells of the testes in males (Kelch *et al.* 1972), the brain (Ryan *et al.* 1972), adipocytes, liver, muscle (Longcope *et al.* 1978), hair follicles (Schweikert, Milewich and Wilson 1975), and bone (Sasano *et al.* 1997). Oestrogens play a major role in many metabolic processes in both men (De Ronde *et al.* 2003) and women.

Androgen and oestrogen hormones influence skeletal muscle size and the characteristics of skeletal myocyte types. Specific androgen levels are necessary for sex-specific patterns of growth in male muscles. Hypogonadism in this murine model generally retards muscle development in males, but has no apparent influence or enhances muscle development in females. Type-IIB fibres are most dependent upon sex hormones for appropriate development, but this relationship is muscle-specific (Sciote *et al.* 2001).

Oestrogens appear to have significant effects in the male biological system. Oestrogens produce significant beneficial effects on skeletal growth and bone maturation. In old age oestrogens are better predictors of bone fractures than androgens. Oestrogens exert positive effects on the brain: on cognitive function, co-ordination of movement, pain and affective state. The positive effects of oestrogen on the cardiovascular (CV) system include those on lipid profiles, fat distribution, endocrine/paracrine factors produced by the vascular wall (such as endothelins, NO), blood platelets, inflammatory factors and coagulation (Gooren and Toorians 2003). A number of the known physiological effects of oestrogens in non-dystrophic animals and humans are described in Table 1-7. Oestrogens also play a role in eNOS expression as summarised in Figure 1-17.

Table 1-7 Known physiological effects of oestrogen(s) in non-dystrophic animals and humans

Reported physiological effects of oestrogen(s)	Reference
▪ antioxidant and membrane stabiliser, protects against peroxidative damage of membrane lipids and low density lipoproteins (LDLs)	(Tiidus 1995)
▪ at high doses exert an immunosuppressant effect (rats)	(Baral, Kwok and Berczi 1991)
▪ enhances immunological responses (anti-inflammatory) in humans at lower doses	(Sorachi *et al.* 1993)
▪ the stimulation of GH secretions in males during puberty is via the oestrogen receptor (ER) after aromatisation	(Metzger and Kerrigan 1994)
▪ stimulates muscle protein deposition and skeletal muscle growth in cattle	(Hayden, Bergen and Merkel 1992)
▪ produces an increase in GH production in prepubertal and postmenopausal females	(Mauras, Rogol and Veldhuis 1990) (Veldhuis *et al.* 2004)
▪ a facilitatory role in the neuroendocrine control of the somatotrophic axis during male puberty	(Metzger and Kerrigan 1994)
▪ no anabolic effect on skeletal muscles in prepubertal females	(Mauras 1995a)
▪ beneficial effects on neurones, bone, the CV system, and body composition	(Persky *et al.* 2000).
▪ no effect on fatigability in EDL muscles	(Tiidus, Bestic and Tupling 1999)

In order to elucidate the relationship between DMD and gonadal hormones, Usuki *et al.* (1989) measured the levels of serum oestrogens and other sex-related hormones in humans diagnosed with DMD. The values were compared with those for age-matched controls. The baseline levels of serum oestradiol were significantly higher in all DMD patients compared to age-matched unaffected subjects. Serum baseline testosterone, LH and FSH levels were all essentially normal, whereas FSH levels were low. The patients had no weight, liver or glandular abnormalities, thus it was concluded that the elevated serum oestradiol levels resulted from increased peripheral androgen-to-oestrogen conversion in DMD patients.

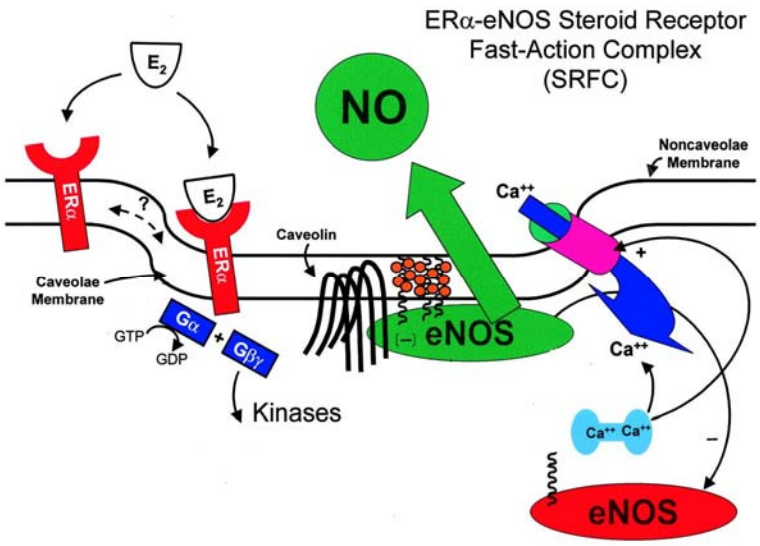


Figure 1-16 Oestrogen activation of eNOS.

Oestrogen activation of eNOS involves oestrogen receptor (ER) coupling to the enzyme in a steroid receptor fast-action complex (SRFC) in endothelial cell caveolae. Oestrogen binding to ER leads to G protein activation, which mediates downstream events. After oestrogen exposure, eNOS translocates from the membrane to intracellular sites, resulting in diminished NOS activity (change from *green* to *red*) (Nuedling *et al.* 1999).

In addition to the physiological roles and functions of testosterone and oestrogen discussed above, steroid hormones have a major influence on vascular cells. In various cells of the vascular wall, testosterone can exert direct effects either by activation of the AR or by nongenomic effects on plasma membrane receptors and channels. However, the expression of aromatase and 17ß-hydroxysteroid dehydrogenase in smooth muscle cells, endothelial cells, and macrophages opens the possibility of local conversion of testosterone (and DHEA) to oestradiol. Both the classic oestrogen receptor α (ERα) and the alternative oestrogen receptor β (ERß) are expressed by various vascular cells, so that testosterone can also modulate vascular physiology indirectly via local oestradiol production (Wu and Von Eckardstein 2003).

1.19 Aims of study

This dissertation aimed to examine the role of sex hormone status in cardiac and skeletal muscle function in the *mdx* mouse model of DMD through the co-quantification of muscle-, sex-, and age-specific differences in the *mdx* mouse. Initially, the effects of testosterone-treatment were examined, followed by an investigation of sex (gender) differences, determination of the effects of surgical castration, in addition to the effects of oestrogen treatment in both the *mdx* and C57 control strains. Furthermore, cardiac and skeletal muscle function was characterised across a range of ages in order to improve the applicability of the *mdx* mouse model for research. Representative muscles included the left atrium (cardiac muscle), extensor digitorum longus (fast-twitch skeletal muscle, type-I), and soleus (slow-twitch skeletal muscle, type-II).

2 METHODOLOGY

2.1 Ethical considerations

Ethical approval for all procedures was obtained from the Animal Ethics Committee at the University of Southern Queensland (USQ). All experiments complied with the ethical guidelines of the USQ Animal Ethics Committee and the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the care and use of animals for scientific purposes 7th Edition 2004 (available from http://www.nhmrc.gov.au).

2.2 Experimental animals

Both male and female C57BL/10ScSn (C57) mice and mutant C57BL/10ScSn (*mdx*) mice were used. Mice were obtained from the Animal Resource Centre (ARC), Nedlands, Western Australia and the USQ breeding colony. The *mdx* mouse strain was obtained by mating homozygous (*mdx/mdx*) females to hemizygous (*mdx*/Y) males. Genetic variation was considered insignificant throughout as both mouse colonies were initiated from the same genetic pool. Animals were fed standard mouse chow with water *ad libitum* and housed at 21-24 °C under a 12 hour light-dark cycle (6 am to 6 pm).

2.3 Treatment of animals - allometric scaling

In preliminary trials, to establish dosage regimes and experimental protocols, human equivalent clinical doses were administered to animals without apparent functional effect. The concept of allometric scaling was then investigated (Kim, Kim and Lee 1998; Mahmood 1999a; Mahmood 1999b; Mahmood and Balian 1999; Mahmood and Yuan 1999; Christiansen 2002) suggesting that responsiveness to drugs may optimally be transferred between species on the basis of relative surface areas and not simply on the basis of weight. It was concluded, therefore, that clinical and physiological responses potentially occur in higher doses in mice than in humans. The

fundamental principles of allometric scaling were then applied in order to determine future dosage regimes for experimental animals.

Subcutaneous (SC) injections were consistently limited to volumes not exceeding 0.1 mL in order to minimise potential irritability.

2.4 Individual trial methodologies

2.4.1 Testosterone trial

In the testosterone trial male mice were weaned at 21 days of age and treatment commenced at 25 days of age. Mice were administered an SC injection of testosterone (Sigma Chemical Company, St Louis USA) dissolved in sesame oil, at an equivalent dose rate of 0.2, 1.0, 1.5, and 2.0 mg/kg/day. Animals were treated over a ten day period with the total dose divided into two treatments which were administered on Day 1 and Day 5. Control *mdx* and C57 mice were administered an equivalent volume of sesame seed oil. Animals were then euthanased at 35 days of age (the conclusion of 10 days treatment).

2.4.2 Sex (gender) trial

Male and female C57 and *mdx* mice were weaned at 21 days of age. Adult mice at the following ages were utilised for the trial: 90, 180, and 330 days. C57 female mice aged 180 days were not available for the trial. No drug treatments were administered prior to euthanasia.

2.4.3 Castration trial

The castration trial utilised male mice weaned at 21 days of age. Surgical castration or sham operation (as detailed below) occurred at 42 days of age. Prior to the administration of drugs, mice were weighed and the anaesthetic and post operative drug doses calculated. For castration and sham operations mice were anaesthetised with an intraperitoneal (IP)

injection of ketamine 10 μg/g (Ketamav, Mavlab, Slacks Creek, Australia) and xylazine 50 μg/g (Xylazil, Troy Laboratories, Smithfield, Australia). Once surgical anaesthesia was established a single drop of sterile saline was placed on each eye of the mouse and the eyes covered to prevent them from drying. Throughout the anaesthesia the mouse was carefully monitored with attention to paw pinch reflex (to maintain sufficient depth of anaesthesia) and body temperature (the animal was kept warm under a lamp).

The scrotal area was close clipped using electric clippers and the testes area and groin swabbed with chlorhexidine gluconate (Hibiclens®, Regent Medical, USA). A longitudinal (3–5 mm) incision was made in the right scrotum exposing the right testicle. The testicular cord was then clamped with mosquito forceps prior to ligation on the distal side, then excised. The forceps remained clamped in position for 2 minutes before removal. The remaining tissues were re-inserted into the right scrotal sac and the wound closed with a single suture of 5/0 silk surgical suture thread (Dysilk™, Dynec Pty Ltd, South Australia). The identical surgical procedure was then repeated on the left side. Anaesthesia was reversed by administration of a 1.0 μg/g SC injection of atipamazole (Antisedan®, Ciba Geigy, Basel, Switzerland). Postoperative analgesia was provided by a 0.05 μg/g SC injection of buprenorphine (Temgesic®, Reckitt and Coleman, West Ryde, Australia). For recovery, the animal was placed on its side, head extended, in clean, dry cage under a lamp and monitored continuously until full recovery from anaesthesia was apparent. All procedures were conducted under standard veterinary operating theatre conditions. Control (sham operated) animals were exposed to the same surgical procedures albeit without the ligation of the testicular cord and subsequent removal of the testes. Mice were postoperatively monitored twice daily for 10 days and euthanased at 6 months of age.

2.4.4 Oestrogen trial

In the oestrogen trial male mice were weaned at 21 days of age with drug treatment commencing at 25 days of age. During the 10 day treatment period, mice were administered a daily SC injection of 17β-oestradiol (Sigma

Chemical Company, St Louis, USA) dissolved in sesame oil, at a concentration sufficient to deliver 0.08, 0.16, and 0.32 mg/kg/day. Control *mdx* and control C57 mice were administered an equivalent SC volume of sesame seed oil. Animals were euthanased at 35 days of age.

2.4.5 Influence of age trial

In the influence of age trial, mice 14 to 21 days of age were not weaned prior to experimentation. Mice from 23 to 330 days of age were weaned at 21 days of age. Male mice were utilised and no drug treatments were administered prior to euthanasia. In order to minimise the use of mice, the *mdx* mice at 90, 180, and 330 days of age were the same as those used in the gender trial.

2.5 Euthanasia

In all trials euthanasia was achieved by exsanguination (decapitation) under general anaesthesia. Only the method of general anaesthesia varied. In the testosterone trial mice were anaesthetised with an IP injection of ketamine 75 μg/g (Ketamav®, Mavlab, Slacks Creek, Australia) and xylazine 15 μg/g (Xylazil®, Troy Laboratories, Smithfield, Australia). In all other trials general anaesthesia was achieved using IP zolazepam 50 μg/g (Zoletil, Virbac, Peakhurst, Australia).

2.6 Dissection of tissues

Post euthanasia, the whole heart was dissected and placed in carbogenated (95% O_2, 5% CO_2) ice-cold Tyrodes physiological salt solution (TPSS) (see Appendix I). The left atrium (LA) was dissected and retained for functional experiments. The right atrium (RA), left ventricle with septum (LV+S), right ventricle (RV), left soleus (SOL), left (EDL) muscles were dissected out and stored in a volume of 10% neutral buffered formalin (Amber Scientific, Belmont, Australia) - with a minimum ratio of 10 times

formalin volume to tissue volume - and stored at room temperature (22º C-23º C). The testes were dissected free and stored at -70º C.

The right SOL and right EDL limb muscles were dissected and placed into carbogenated (95% O_2, 5% CO_2) ice-cold Krebs physiological salt solution (KPSS) (see Appendix I). Particular care was taken to ensure that both the proximal and distal tendons were sufficiently intact and of appropriate length to enable the tendons to be tied with a silk suture thread (Dysilk™, Dynec Pty Ltd, South Australia) without contacting or damaging any functional skeletal myocytes.

2.7 Organ bath (functional) experiments

Force transducers and pipettes used in organ bath experiments were calibrated in order to maintain experimental accuracy. Prior to the commencement of new protocols, organ baths were cleaned with concentrated nitric acid and manually scrubbed and rinsed repeatedly with reverse osmosis water at the completion of each experiment.

2.8 Skeletal muscle experiments

EDL and SOL skeletal muscles were maintained briefly in ice-cold carbogenated KPSS until they were mounted in individual 25 mL glass water-jacketed organ baths. A 5/0 silk surgical (Dysilk™, Dynec Pty Ltd, South Australia) thread was tied to the tendon at each end of the muscle. The skeletal muscle was then mounted with the proximal end connected to a force transducer (FT-102, CB Sciences, Milford, MA, USA) and the distal end anchored to a fixed pin securing the muscle in a vertical position. Secured muscles were then immersed in organ baths containing 25 mL carbogenated KPSS and maintained at 23.0±0.5º C. Skeletal muscle preparations were positioned between two platinum electrode pins along the length of the muscle (care was taken to ensure that muscles did not touch the electrodes directly).

Muscles were field stimulated via a Grass S48 stimulator (W. Warwick, RI, USA) and current intensity was amplified using a pre-amplifier

(EP500B. Audio Assemblies, Campbellfield, Victoria, Australia). Data were collected via a PowerLab system and analysed using Chart 4.1.1 software (AD Instruments, Castle Hill, New South Wales, Australia).

An initial preload of 10 mN was placed on each skeletal muscle during the equilibration period. Preliminary equilibration of preparations was achieved by washing muscles with 25 mL fresh Krebs physiological solution at 10 minute intervals (total 30 minutes). Skeletal muscles continued to be washed at 10 minute intervals throughout the duration of the experiment.

The optimum preload was established by eliciting twitch contractions (20 V) at a range of preloads (10, 7, 5, 3, and 1 mN) and, once determined, was then maintained for subsequent procedures. The optimum voltage, to induce maximum absolute twitch force (Pt) was determined by delivering a single stimulation at incremental voltages ranging from 1.5 to 30 V.

Having determined optimal preload and voltage, 3 distinct Pt readings were recorded. These readings were then averaged to establish a final value for absolute Pt (sPt).

For the tetanic protocol, optimum preload and optimum voltage was utilised as determined in the protocol described above. All tetanic stimuli occurred at 3 minute intervals throughout the experiment and, where necessary, optimal preload was re-established before further electrical stimulation of the muscle.

Optimum frequency (pulse duration 0.5 ms, frequency range 10 to 180 Hertz (Hz)) was determined for the point of maximum tetanic force (Po). At optimum preload, voltage, and frequency, maximum tetanic force was recorded 3 times. These readings were then averaged and a value for maximum tetanic force normalised for cross-sectional area (sPo) was determined.

At the completion of the twitch and tetanic recordings, muscle length (Lo) and midpoint diameter were recorded at optimum preload using a digital micrometer (Mitutoyo, Japan). Muscles were then removed, tendons dissected free, muscles blotted for approximately 3 seconds and weighed.

Muscle length (Lf) was calculated by multiplying Lo by fibre length to muscle length ratios as determined by Brooks and Faulkner (0.71 for SOL

and 0.44 for EDL) (Brooks and Faulkner 1988). The mean CSA of each skeletal muscle was then calculated by dividing muscle weight by the product of myocyte length and 1.06 mg/mm^3, the density of mammalian skeletal muscle (Mendez and Keys 1960).

Twitch force normalised for cross-sectional area (sPt) was calculated by normalising Pt for muscle CSA and specific maximum tetanus (sPo) was calculated by normalising Po for CSA. The respective mathematical formulae used are defined in Table 2-1 below;

Table 2-1 Definitions and mathematical formulae used in skeletal muscle results calculations

Symbol	Definition	Mathematical formulae	
		SOL muscle	EDL muscle
Pt (mN)	absolute twitch force		
Lo (mm)	muscle length at optimum preload		
Po (mN)	maximum tetanic force		
Lf (mm)	standardized muscle length	Lo x 0.71	Lo x 0.44
CSA (mm^2)	total fibre CSA	weight (mg)/Lf x 1.06	weight (mg)/Lf x 1.06
sPt (mN/mm^2)	absolute twitch force (Pt) normalised for CSA	Pt/CSA	Pt/CSA
sPo (mN/mm^2)	maximum tetanic force (Po) normalised for CSA	Po/CSA	Po/CSA

(Adapted from Brooks and Faulkner 1988).

2.9 Cardiac muscle experiments

A 5/0 silk surgical suture thread (Dysilk, Dynec Pty Ltd, South Australia) was tied to the proximal end of the atria and attached to a force transducer (FT-102, CB Sciences, Milford, MA, USA). A stainless steel hook attached to the distal end of the muscle secured the LA in a vertical position. Atria were positioned between two platinum electrode pins with care taken to ensure that they did not directly contact the electrodes. Each LA was mounted in an individual water-jacketed glass organ bath containing 25 mL TPSS at 35.0±0.5º C, under optimal preload of approximately 5 mN. During a 30 minute equilibration period the atria were washed with fresh TPSS at 10 minute intervals.

The initial calcium chloride ($CaCl_2$) concentration in the organ baths was 1.8 mM. Throughout the duration of the experiment, the LA were field stimulated (at 20% above threshold) via an AMPI Master 8 stimulator at a frequency of 1 Hz and pulse width 5 ms. A concentration response curve (CRC) to calcium (Ca^{2+}) was generated in order to determine the maximum force of contraction (FOC) of the LA. At 3 minute intervals $CaCl_2$ was added cumulatively to the organ bath resulting in final $CaCl_2$ concentrations of 1.8, 2.4, 3.2, 4.0, 5.6, 7.2, 9.0, and 12.0 mM. Prior to the addition of each subsequent volume of $CaCl_2$ the mean maximum FOC was recorded.

Data were collected via a PowerLab system and analysed using Chart 4.1.1 software (AD Instruments, Castle Hill, New South Wales, Australia). At the conclusion of each experiment the LA was removed, blotted for 3 seconds and weighed.

2.10 Histology protocol

The histological techniques involved fixing, pre-blocking, blocking, cutting, de-waxing, rehydrating, staining, mounting, and analysis procedures. Tissues were stored in formalin and prepared by being placed sequentially in 70% ethanol (EtOH) for 24 hours, 90% EtOH (12-16 hours), 100% EtOH (120 minutes), 100% EtOH (60 minutes), and twice in toluene (20 minutes) (Asia Pacific Specialty Chemicals Limited, Seven Hills, New South Wales, Australia).

Tissues were then embedded in paraffin wax blocks and 10 µm sections cut with a Reichert-Jung 2035 Biocut microtome (Cambridge Instruments, West Germany) using a stainless steel Feather S35 microtome blade (Feather Safety Razor Company Limited, Medical Division, Japan). Ribbons were floated via a water bath (Electrothermal, London) at 50º C on to a Polysine™ microscope slide (Menzel-Glaser® GmbH, Braunschweig, Germany) and dried overnight on a warming plate (Thermoline Scientific Equipment Pty Ltd, Wetherill Parke, Australia) at 40º C.

To achieve de-waxing, rehydration, and haematoxylin and eosin staining of slides, the following method was adopted;

Slides were placed in a Labmaster oven at 56º C (60 minutes), then, in a fume hood at room temperature (22º C-23º C), before the following washes were undertaken; three xylene (Asia Pacific Specialty Chemicals Limited, Seven Hills, Australia) washes (five minute intervals), two 100% EtOH washes (three minute intervals), 90% EtOH (three minutes), 70% EtOH (three minutes), water bath (two minutes), double distilled water (ddH_2O) (one minute), haematoxylin stain (see Appendix I) (seven minutes), water bath (one minute), Scott's solution (see Appendix I) (0.5 minute), water bath (four minutes), 70% EtOH (one minute), eosin stain (see Appendix I) (11 minutes), 95% EtOH (0.5 minute), and three 100% EtOH treatments (0.5 minute intervals) were completed.

For mounting, slides initially underwent three xylene washes (5 minute intervals). A small amount of DePeX mountant (BDH Chemicals Australia Pty Limited, Kilsyth, Australia) – thinned with xylene - was then applied to the tissue surface of the slide and a Deckglaser 100 glass cover slip (Menzel-Glaser® GmbH, Braunschweig, Germany) was placed on top of the slide and stored at room temperature (22º C to 23º C). All slides were coded randomly to minimise experimenter bias during histological analysis.

2.11 Histological analysis

Histological slides were viewed using a Nikon Eclipse E600 microscope. Muscle fibre sizes were determined using the minimal Ferets diameter method as described by Briguet *et al.* 2004 (Briguet *et al.* 2004). Approximately 20-100 fibres (limited by the quality of the histological slide) of SOL and EDL muscles were analysed using AnalySIS (Soft Imaging System, GmbH, Münster, Germany) software determining mean cell diameter and percentage of cells with centralised nuclei. Upon completion of the analysis the code was broken to permit statistical analysis.

2.12 Data analysis

Data was compiled initially in Microsoft Excel 2000. Results were then converted to Statistical Product for Service Solutions (SPSS) 11.0 for

Windows Student version format for further analyses. Means (expressed as the mean ± the standard error of the mean (SEM)) were calculated. One-way Analysis of Variance (ANOVA) statistical analyses were used to determine differences between experimental groups. A result with a p value less than 0.05 ($p<0.05$) was deemed statistically significant.

3 Testosterone trial

3.1 Influence of testosterone on body weight

All groups of C57 mice were significantly heavier than *mdx* mice ($p<0.01$). C57s were 50%, 51%, 29%, 25%, and 33% heavier than corresponding *mdx* mice across increasing doses of testosterone, respectively. Testosterone treatment did not, however, cause any significant change in body weight (BW) in either mouse strain over the 10 day treatment period (Figure 3-1).

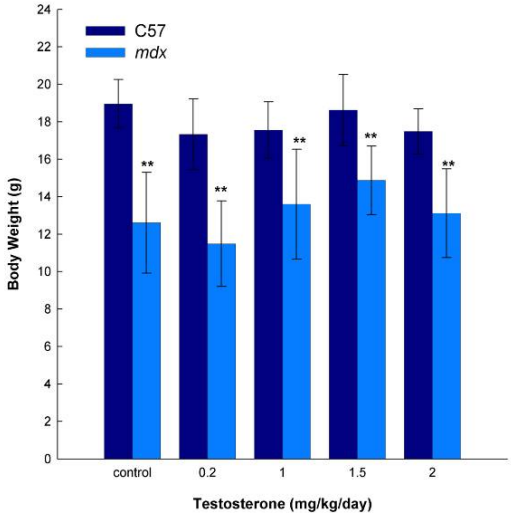


Figure 3-1 Body weights of male C57 and *mdx* mice at varying testosterone doses.

Comparison of male mouse body weights between strains and within mouse strains at varying testosterone doses. * p<0.05, ** p<0.01 between strain comparison. age = 35 days old. n = 9-16 mice.

3.2 Influence of testosterone on testes weight

Testosterone treatment had no significant effect on absolute testes weight within C57 mice. In contrast, absolute testes weights in *mdx* decreased with 0.2 mg/kg/day testosterone and increased at 1.0 and 1.5 mg/kg/day treatment, compared to controls. The testes of C57 mice were consistently heavier than *mdx* equivalents. This was not significant at doses of 1.0 and 1.5 mg/kg/day despite C57s having significantly higher BW than *mdx* across all treatments. Control, 0.2 mg/kg/day, and 2.0 mg/kg/day C57

mouse testes were 3.63-, 7.42-, and 2.25-fold heavier ($p<0.01$) than *mdx* mice testes, respectively (Figure 3-2).

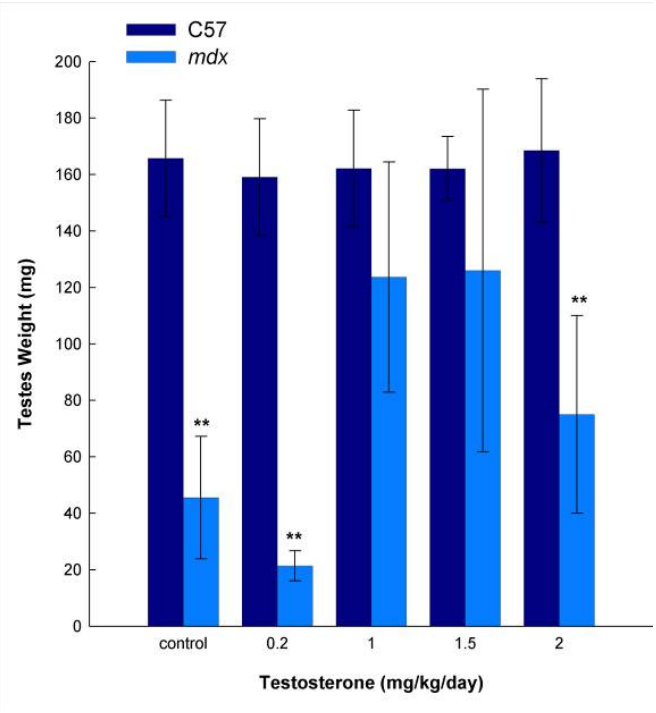


Figure 3-2 Testes weights of male C57 and *mdx* mice at varying testosterone doses.

*** p<0.05, ** p<0.01 between strain comparison; † p<0.05, †† p<0.01 treatment comparison with same strain. age = 35 days old. n = 9-16 mice.**

3.3 Influence of testosterone on cardiac characteristics

3.3.1 Cardiac morphometry

Despite a significant difference in the BWs of C57s compared to *mdx* mice across all treatments ($p<0.01$), there was no significant difference in the weight of the LA of control, 1.0, 1.5, or 2.0 mg/kg/day testosterone-treated mice between strains. Testosterone treatment at 1.0, 1.5, and 2.0 mg/kg/day did cause an increase in LA weight in *mdx* mice ($p<0.05$) but not in C57s. An aberration occurred at a dose of 0.2 mg/kg/day testosterone in which the *mdx* LA weight was 58% lower than C57s ($p<0.05$) and 38% lower than control *mdx* ($p<0.05$) (Figure 3-3).

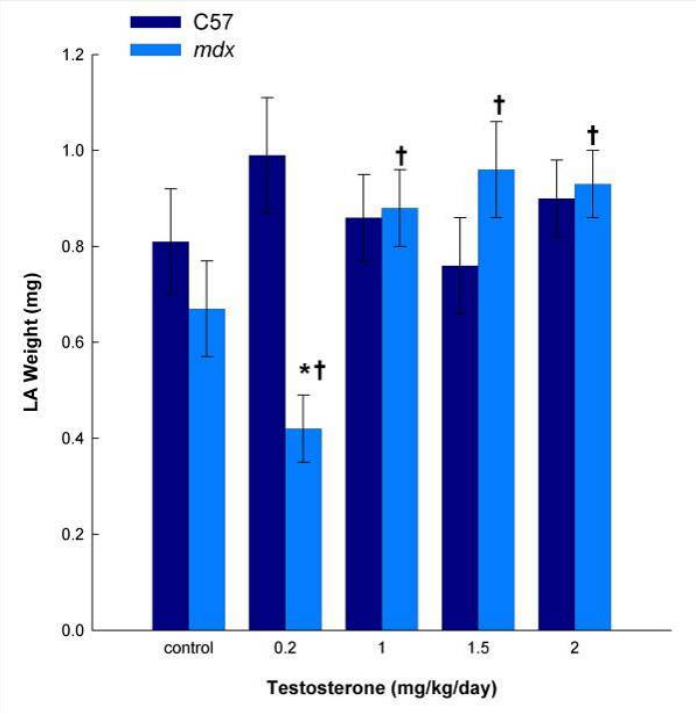


Figure 3-3 Left atrial weights of male C57 and *mdx* mice at varying testosterone doses.

*** p<0.05, ** p<0.01 between strain comparison; † p<0.05, †† p<0.01 treatment comparison with same strain. age = 35 days old. n = 9-16 mice.**

3.3.2 Cardiac function

Controls, and doses of 0.2 and 1.0 mg/kg/day testosterone, showed no significant difference in basal force of contraction (FOC) between *mdx* and C57 LA. Due to the variability between LA contractility of different mice there was also no significant difference in force when comparing LA from testosterone-treated mice to controls within each strain. Testosterone treatment did, however, produce a non-significant trend of decreased forces (for doses over 1.0 mg/kg/day) in the *mdx* with increasing doses that was not evident in C57s.

At testosterone doses of 1.5 mg/kg/day ($p<0.05$) and 2.0 mg/kg/day ($p<0.01$) C57s had a significantly higher basal FOC (normalised for LA weight) than *mdx* mice (Figure 3-4).

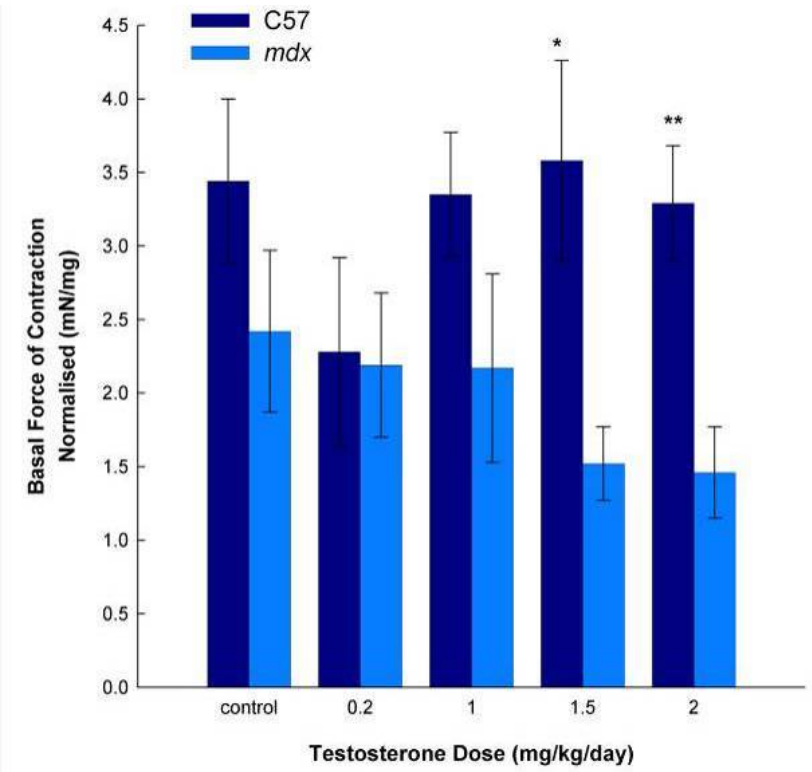


Figure 3-4 Basal force of contraction of left atria in male mice at varying testosterone doses.

Comparison between strains depicting basal FOC of LA (normalised for LA weight) versus testosterone dose (mg/kg/day). * p<0.05, ** p<0.01 between strain comparison; † p<0.05, †† p<0.01 treatment comparison with same strain. age = 35 days old. n = 9-16 mice. Force values normalised for LA weight.

The analysis of Ca^{2+} CRCs revealed no significant difference between strains at a dose of 0.2 mg/kg/day (Figure 3-5B). In controls, and at all other testosterone doses, C57s had a significantly greater atrial FOC at Ca^{2+} concentrations of 4.0, 5.6, and 7.2 mM, respectively compared to *mdx* (Figure 3-5).

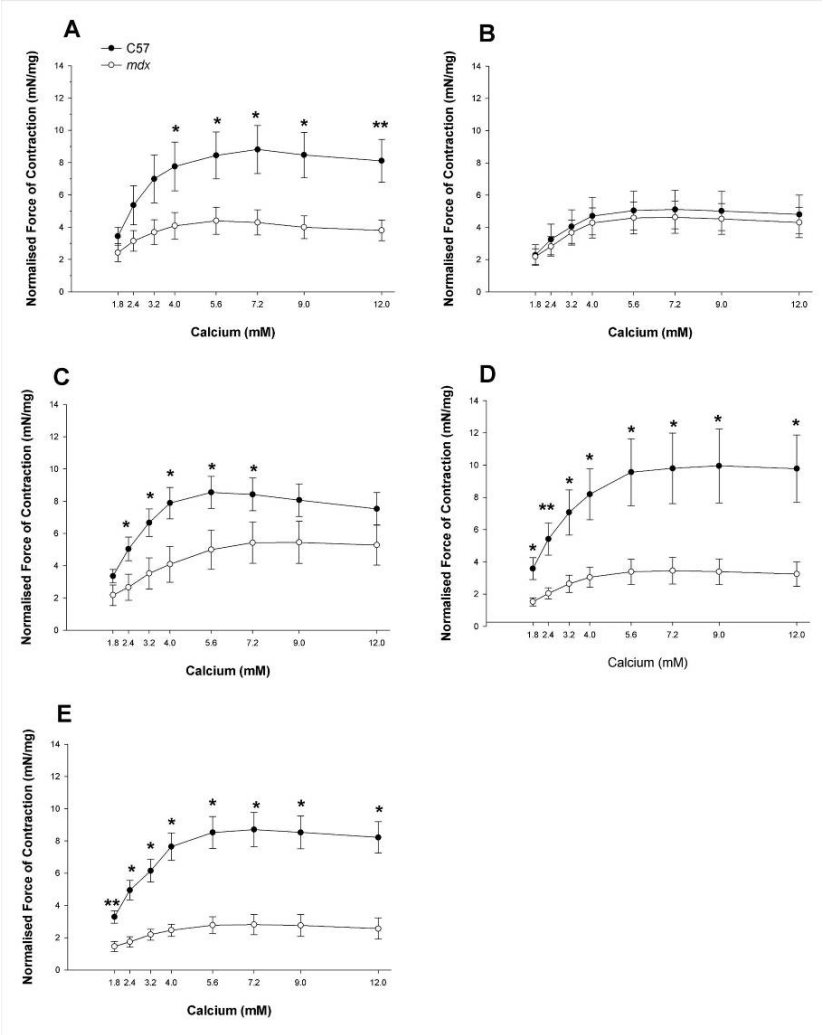


Figure 3-5 Concentration response curves to calcium at varying testosterone doses in male mice.

(A) controls (B) 0.2 mg/kg/day testosterone (C) 1.0 mg/kg/day testosterone (D) 1.5 mg/kg/day testosterone and (E) 2.0 mg/kg/day testosterone. * p<0.05, ** p<0.01. age = 35 days old. n = 9-16 mice. Force values normalised for LA weight.

The maximum LA FOC (normalised for LA weight) was significantly higher ($p<0.05$) in C57s for controls and all doses of testosterone except 0.2 mg/kg/day. In the *mdx*, testosterone treatment caused a trend of decreased maximum FOC (non-significant) with increasing doses similar to that seen in basal forces, but again this was not evident in C57s (Figure 3-6).

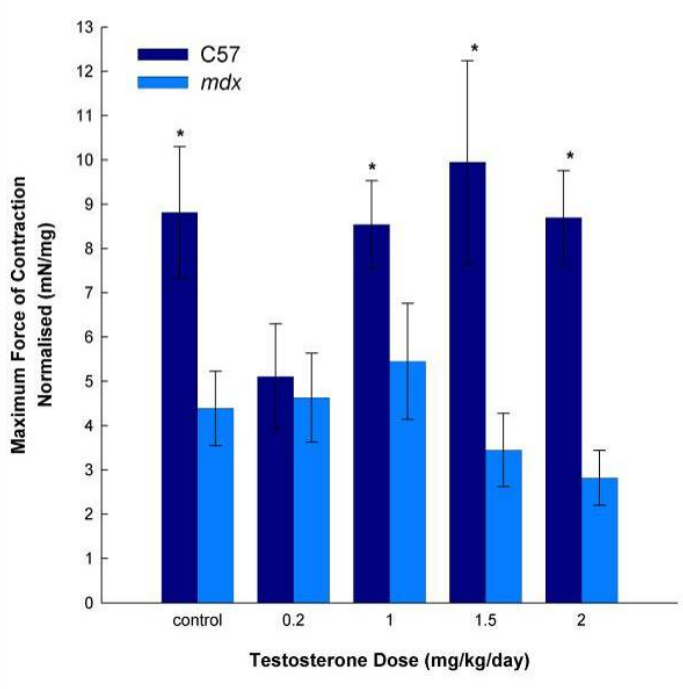


Figure 3-6 Maximum force of contraction of the LA at varying testosterone doses in male mice.

Comparison between strains of maximum FOC of the LA (normalised for LA weight) across testosterone doses (mg/kg/day). * p<0.05, ** p<0.01. age = 35 days old. n = 9-16 mice. Force values normalised for LA weight.

3.4 Influence of testosterone on skeletal muscle morphometry

There was no significant difference in SOL weights between *mdx* and C57 mouse strains or between the groups of C57 mice. All treated *mdx* mice had heavier SOL muscles than control *mdx* with this reaching significance for doses of 1.0 and 1.5 mg/kg/day testosterone where SOL weights were 43% and 62% heavier than control *mdx* ($p<0.05$).

In C57s, EDL weights in treated mice were not significantly different to control mice. However, in *mdx* mice, all testosterone-treated mice had heavier EDL muscles than controls but this was only significant ($p<0.01$) for 1.5 mg/kg/day treated mice which had 46% heavier EDL muscles than control *mdx*. *Mdx* EDL muscle weights in controls, 0.2 mg/kg/day, and 2.0 mg/kg/day treated mice were significantly lower (35%, 24%, and 24%) than C57 equivalents ($p<0.01$) (Table 3-1).

Table 3-1 **Weight of SOL and EDL muscles in male C57 and *mdx* mice at varying testosterone doses.**

testosterone (mg/kg/day)	C57 SOL weight (mg)	*mdx* SOL weight (mg)	C57 EDL weight (mg)	*mdx* EDL weight (mg)
Control	4.9±0.9	3.7±1.2	5.4±1.1	3.5±1.1**
0.2	4.9±0.8	4.1±1.1	4.9±0.5	3.7±0.8**
1.0	4.9±0.9	5.3±1.1†	5.0±0.9	4.5±1.2
1.5	5.8±1.2	6.0±1.8†	5.3±0.9	5.1±0.8††
2.0	5.3±1.0	4.8±0.8	5.4±0.7	4.1±0.8**

*** p<0.05, ** p<0.01 between strain comparison; † p<0.05, †† p<0.01 treatment comparison within same strain. age = 35 days old. n = 9-16 mice.**

3.5 Discussion

In human males, the linear growth and increase in muscle and bone mass during puberty are mediated predominantly through the action of the sex steroid hormone testosterone (Mauras 1995b). In DMD, the exacerbation of clinical symptoms clearly coincides with the onset of puberty with arguably the most prominent clinical event being the loss of ambulation which typically occurs between seven and 13 years of age (Dubowitz 1978).

In 1981 Zatz, Betti and Levy reported a unique case of a 13 year old Japanese boy who possessed both idiopathic GH deficiency and DMD. Clinically, the child presented with hypertrophied calves, no visible muscle wasting, a waddling gait, positive Gowers' manoeuvre when rising from the floor, short stature (104 cm), weight 21 kg, normal IQ, a bone age of four and a half years, and normal thyroid hormone levels (Zatz, Betti and Levy 1981). GH deficiency was confirmed through insulin tolerance testing and unresponsiveness to L-dihydroxyphenylalanine (L-dopa) (Liberman, Cesar and Wajchenberg 1979). Serum CK and PK levels were grossly elevated, electromyography and muscle biopsy results were consistent with progressive muscle disease, and pedigree analysis confirmed DMD. The child had two younger male siblings and seven other male relatives with DMD, all of which showed a typical clinical course of the disease and were non-ambulant by 12 years of age. The fascinating thing about this unique case is the fact that the child showed an uncharacteristically benign clinical course of DMD. In 1986 Zatz reported a five year follow-up study of the

original abovementioned case. The patient, at 18 years of age, short stature (120 cm), weighed 41 kg, had a bone age of seven years, genitalia of pre-pubertal appearance, raised serum CK and PK levels, normal thyroid and adrenal function, together with hypogonadism resulting from gonadotrophin deficiency. Extraordinarily, the patient, despite being grossly obese, was still ambulant and able to walk short distances unaided. Zatz *et al.* (1986) reaffirmed their original hypothesis which suggested that the overtly benign course of DMD in the case was in some way related to GH deficiency in this boy. Furthermore, the clinical observation in the case study by Zatz, Betti, and Levy (1981) that a GH deficient DMD patient had "genitalia of pre-pubertal appearance" was further thought-provoking in that it implied that the boy potentially had compromised androgen (testosterone) levels. This raises the question as to whether it is possible that lowered testosterone levels had a role in the benign nature of this case and, conversely whether increased levels of testosterone potentially exacerbate the clinical symptoms of DMD. This study investigated the effects of testosterone treatment in *mdx* and C57 control strain mice on cardiac muscle function and response to exogenous Ca^{2+}.

In this study all groups of 35 day old C57 mice were heavier than *mdx* equivalents and testosterone treatment failed to produce any significant BW change within either strain over the 10 day treatment period. Despite C57s being heavier in BW, testes weights were consistently greater than in *mdx*. This may be indicative of an inherently raised peripheral aromatisation of testosterone to oestradiol in *mdx*. Spearow *et al.* (1999) found that oestrogen treatment of C57 mice during juvenile development resulted in the lowered testes weights therefore an increase in serum oestrogens would predictably lower testes weight. In human studies, Usuki *et al.* (1989) reported that DMD patients had significantly higher baseline levels of serum oestrone than age-matched unaffected boys. Serum baseline testosterone, LH and FSH levels were all essentially normal in DMD, but FSH levels were significantly lowered. This led to the conclusion that the elevated serum oestrone levels resulted from an increased peripheral conversion of testosterone to oestrogen in DMD boys. A possible increase in the conversion of testosterone to oestradiol in *mdx* (as in DMD boys) could

explain the lowered testes weights in control *mdx* compared to C57 equivalents.

In terms of cardiac morphometry, LA weights were not different between control mice (despite differences in body weight). Testosterone treatment caused no change in C57 LA weight whereas *mdx* mice responded differently with increases in LA weights at 1.0, 1.5, and 2.0 mg/kg/day treatments ($p<0.05$), further suggesting a unique responsiveness to testosterone in the *mdx* strain. Testosterone produced an overall hypertrophic response in *mdx* LA that was not evident in C57s. Testosterone is a risk factor for cardiac hypertrophy as abundant binding to the AR (Marsh *et al.* 1998) and its translocation into the nucleus may lead to exaggerated cardiac-specific gene expression (Morano *et al.* 1990). Lin, McGill, and Shain (1981) showed that tissue-specific metabolism of testosterone can also lead to the production of high-affinity ligands of the AR such as DHT (Lin, McGill and Shain 1981). Increased DHT synthesis may accelerate cardiac hypertrophy via the AR signalling pathway.

With respect to cardiac contractility studies, some researchers have argued that the LA is poorly representative of ventricular function in the heart. Despite this, Sanyal (1980) demonstrated gross histological and ultrastructural changes in all heart chambers of DMD patients compared to unaffected age and sex-matched children. Minajeva *et al.* (1997) showed atrial and ventricular cardiac tissues exhibit the same sensitivity to Ca^{2+} and Alloatti *et al.* (1995) and Lu and Hoey (2000a) have shown cardiac abnormalities in the *mdx* mouse model using LA preparations. On the basis of this it was deemed appropriate to use LA preparations as a model of cardiac function in the *mdx* mouse. Results showed that *mdx* absolute basal forces were 61% lower than C57s and 29% lower than C57s when corrected for LA weight (non-significant). It must be noted that there was large variability in basal FOC of LA generated by *mdx* compared to C57s and this was reflected in the large SEMs in the results. The lack of significance in these findings is inconsistent with a number of previous studies that have reported significant differences in basal LA contractility between strains however this may also be reflective of age-specific differences. For example, Woolf *et al.* (2006) reported a significant decrease in basal LA contractility in

male 12 week and 12 month old *mdx* mice compared to C57 equivalents. The mice in this study were 35 days old so age-matched comparisons are not possible. Most importantly, these results (albeit non-significant) indicate that LA basal contractility is compromised in the *mdx* mouse and that despite the fact that testosterone produced significant LA hypertrophy this did not translate to an increase in muscle contractility.

Testosterone also had an impact on LA response to Ca^{2+} with the form of Ca^{2+} CRCs showing a varied pattern between strains. Compared to the C57 control strain, *mdx* showed a more flattened Ca^{2+} CRC (rather than the traditional sigmoid curve shown in the control strain). This implies a dampened responsiveness to Ca^{2+} and is consistent with the findings of Lu and Hoey (2000). Furthermore, maximum LA forces generated by C57s were significantly greater than *mdx* forces for controls, 1.0, 1.5, and 2.0 mg/kg/day testosterone doses ($p<0.05$) although testosterone treatment did not significantly alter maximum LA contractility in either strain.

It is known that normal cardiac function is dependent on efficient Ca^{2+} homeostasis. During a cardiac action potential, the influx of Ca^{2+} through L-type Ca^{2+} channels activates the release of Ca^{2+} from the SR. Relaxation occurs when the influx of Ca^{2+} ceases and Ca^{2+} is transported from the cytoplasm by the Na^{+}/Ca^{2+} exchanger and sequestered in the SR by the sarcoplasmic endoplasmic calcium ATP-ase (SERCA) (Bers and Perez-Reyes 1999; Bers 2000; Bers 2002). However, Alloatti *et al.* (1995) showed that isolated *mdx* cardiac myocytes have elevated $[Ca^{2+}]_i$. This elevated Ca^{2+} results in cardiac myocyte necrosis and diminished cardiac function (Bia *et al.* 1999) as a direct consequence of Ca^{2+} dependent (calpain mediated) proteolysis (Tidball and Spencer 2000). In addition to increased $[Ca^{2+}]_I$, *mdx* cardiac myocytes exhibit altered Ca^{2+} handling (Lucas-Heron *et al.* 1987). It has been hypothesised that the Ca^{2+} overload in the *mdx* heart could be due to a 'leaky membrane' similar to that observed in *mdx* skeletal muscle in which increased leak channel activity has been reported (Turner *et al.* 1991). Alternatively, *mdx* cardiac Ca^{2+} overload may encompass physiological processes involved in Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} handling, and functions of the SR. Golden (2004) provided the first evidence that testosterone regulates gene expression of the major Ca^{2+} regulatory proteins in isolated rat

ventricular myocytes. Testosterone treatment (six to 24 hours) induced an increase in β_1-adrenergic receptor density, L-type calcium channel mRNA levels, Na^+/Ca^{2+} exchanger mRNA levels, and stimulated a 300-fold increase in androgen receptor (AR) message abundance. In 2005 Golden (2005) showed that acute testosterone treatment resulted in an increase in peak shortening and a decrease in time to peak shortening in isolated rat cardiac myocytes. Based on the results of Golden in 2004 and 2005 one would expect testosterone treatment to produce an increase in cardiac contractility. However, this study showed that in the *mdx* mouse testosterone treatment produced no significant change in basal LA contractility, no effect on maximum LA contractility, and a dampened response to Ca^{2+} as evidenced by the shape of Ca^{2+} CRCs.

Skeletal muscle morphometry showed no difference in SOL weights between strains or between treatment groups of C57s. Conversely, *mdx* mice showed a differential response to testosterone treatment with all testosterone-treated *mdx* SOLs being heavier than control *mdx* (significant for 1.0 and 1.5 mg/kg/day doses).

Importantly, this study showed that testosterone failed to produce an increase in cardiac contractility in the *mdx* mouse and, in fact, appeared to have a deleterious effect on LA cardiac contractility and responsiveness to Ca^{2+}. These results warrant extensive further investigation as to the specific physiological effects of testosterone in the *mdx* mouse in both cardiac and skeletal muscles.

4 Sex (gender) trial

4.1 Influence of sex on body weight and heart weight

In 90 day old mice, *mdx* were significantly heavier in BW than C57s of the same sex and age. *Mdx* males were 9% heavier than C57 males ($p<0.05$) but not different in BW to *mdx* females. C57 males were 29% heavier than C57 females ($p<0.01$) and *mdx* females were 30% heavier than C57 females ($p<0.01$). In 180 day old mice there was no difference in BWs between mice. In 330 day old mice, *mdx* males and females were 19% and 23% respectively heavier than their C57 age and sex-matched controls ($p<0.01$). C57 males were 6% heavier than C57 females ($p<0.05$) which was in contrast to the *mdx* mice (where there was no significant difference in BWs between sexes) (Figure 4-1A).

For all groups there was a trend of increasing HW with age (non-significant) (Figure 4-1B). In 90 day old mice males had no difference in HW. Female *mdx* hearts were 16% heavier than C57 female hearts ($p<0.01$). Male and female *mdx* had no difference in HW whereas C57 male hearts were 20% heavier than C57 female hearts ($p<0.01$). In 180 day old mice C57 males and *mdx* females had a 15% ($p<0.05$) and 24% ($p<0.01$) respectively heavier HW than *mdx* males. Male 330 day old mice had no difference in HW. Female *mdx* hearts were 39% heavier than C57 female hearts ($p<0.01$). Male and female *mdx* had no difference in HW whilst C57 male hearts were 19% heavier than C57 female hearts ($p<0.01$).

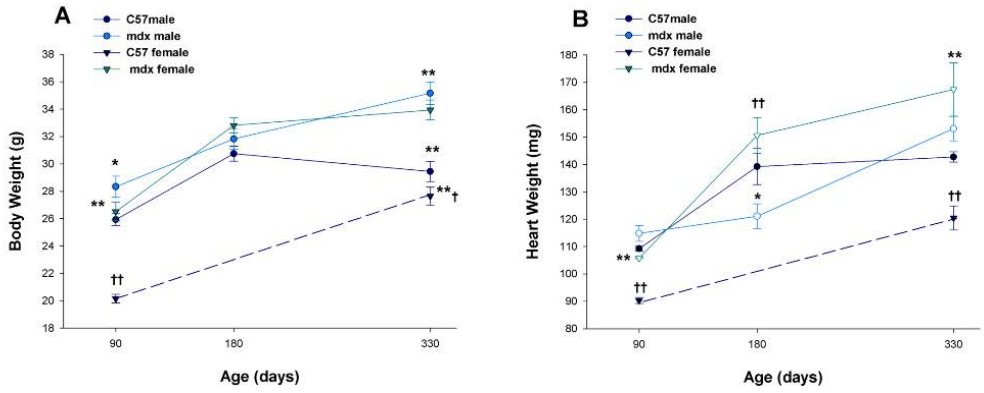


Figure 4-1 Influence of sex on body weight and heart weight between strains and sexes.

Comparison of mouse body weights (A) and heart weights (B) at varying ages between strains of the same sex and within mouse strains of different sexes.
*** p<0.05, ** p<0.01 between strain comparison at equivalent sex and age;**
† p<0.05, †† p<0.01 sex comparison within same strain and age.
n = 6-10 mice.

4.2 Influence of sex on cardiac characteristics

4.2.1 Cardiac morphometry

In 90 day old mice there was no significant difference in heart weight as a percentage of body weight (HW%) between males. Conversely, C57 females had a 13% ($p<0.01$) and 7% ($p<0.05$) higher HW% than *mdx* females and C57 males, respectively. There was no difference in RA weight between mice (Figure 4-2A) and the only difference in LA weight between mice was the fact that *mdx* males had 48% heavier LA than C57 males ($p<0.01$). LA weights of C57 females and males did not differ as did the LA weights of C57 females and *mdx* females (Figure 4-2B).

With respect to right ventricular free wall (RV) weight, there was no difference between males or between *mdx* males and females. Both C57 males and *mdx* females had 24% and 43% heavier RVs than C57 females ($p<0.01$) (Figure 4-2C). *Mdx* males had a 6% heavier left ventricle plus septum (LV+S) than C57 males ($p<0.05$). *Mdx* females were 12% heavier in LV+S weight than C57 females ($p<0.01$). C57 males and *mdx* males had a 12% ($p<0.01$) and 11% ($p<0.05$) higher LV+S weight than their same strain females (Figure 4-2C).

In 180 day old mice there were no differences between males in any of the measured cardiac parameters despite differences in BW (Figure 4-2). C57 males and *mdx* females had 18% and 21% respectively higher HW%s than *mdx* males ($p<0.01$). There was no difference in RA weight between mice (Figure 5-2A) or in LA weight between males. The LA of *mdx* females were 60% heavier than *mdx* males ($p<0.01$) (Figure 4-2B). There was no difference in RV weight between mice (Figure 4-2C). C57 males and *mdx* females had a 20% ($p<0.05$) and 27% ($p<0.01$) respectively heavier LV+S than *mdx* males (Figure 4-2C).

In 330 day old mice; *mdx* males had an 11% lower HW% than C57 males ($p<0.01$). There was no difference in HW% between females. The *mdx* mice did not differ in terms of HW% whereas C57 males were 11% higher in HW% than C57 females ($p<0.05$). The LA of *mdx* females were 40% ($p<0.05$) and 48% ($p<0.01$) heavier than *mdx* males and C57 females, respectively. All other cardiac parameters measured showed no differences between strains or sexes of the same mouse strains (Figure 4-2).

There was no difference in RA weight between males. *Mdx* females had an 84% heavier RA weight than C57 females ($p<0.05$). Neither mouse strain differed between sexes in terms of RA weight (Figure 4-2A). There was no difference in LA weight between males. *Mdx* females were 148% heavier in LA weight than C57 females ($p<0.01$). *Mdx* males had a 29% lower LA weight than females ($p<0.05$). There was no sex difference in LA weight with C57s (Figure 4-2B). *Mdx* males and females had a 22% ($p<0.05$) and 57% ($p<0.01$) higher RV weight than C57 males and females, respectively. Neither mouse strain differed between sexes in terms of RV weight (Figure 4-2C). There was no difference between males in terms of LV+S weight. *Mdx* females were 33% heavier in LV+S weight than C57 females ($p<0.01$). There was no difference in LV+S weight in *mdx*. C57 males had an 18% higher LV+S weight than C57 females ($p<0.01$) (Figure 4-2C).

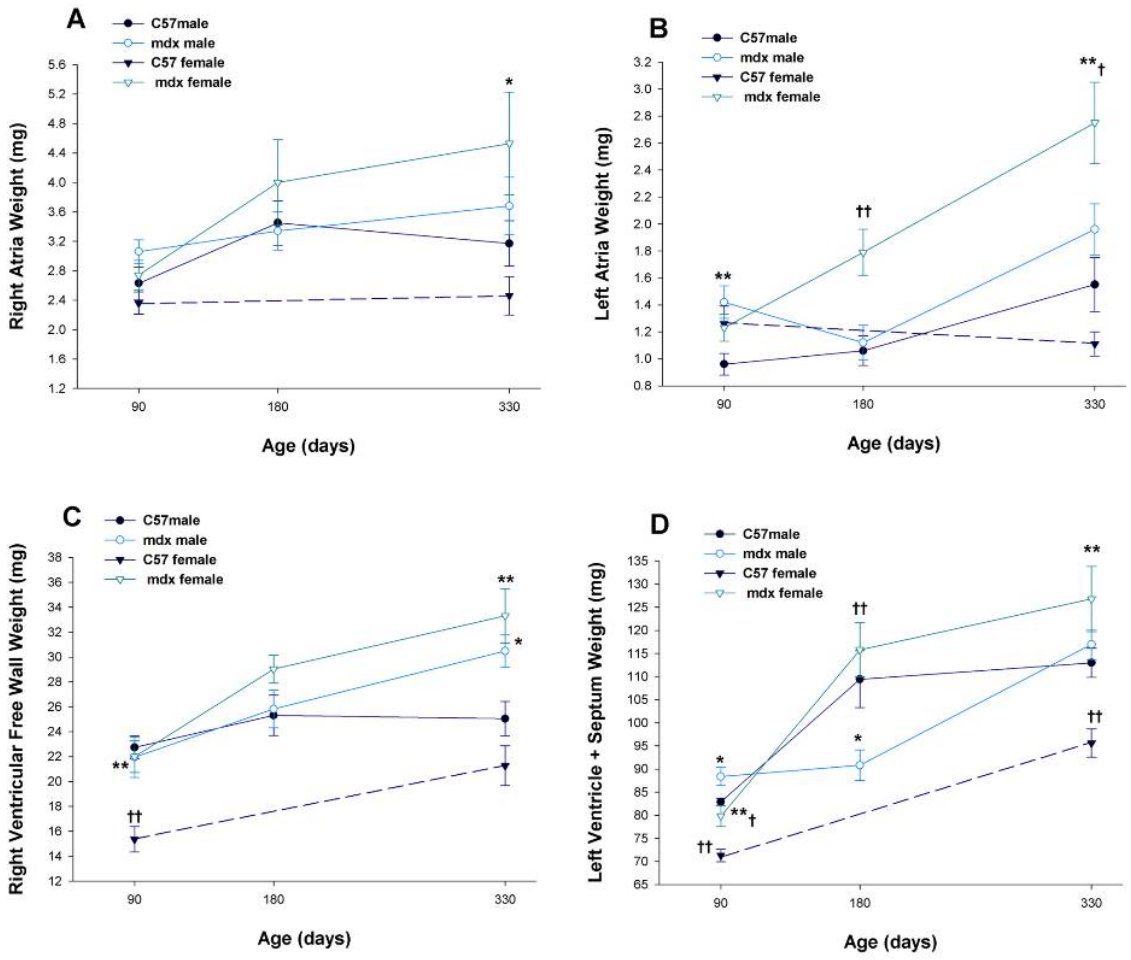


Figure 4-2 Effects of age on cardiac tissue weights between strains and sexes.

Comparison of mouse RA weights (A), LA weights (B), RV weights (C), and (D) LV+S weights at varying ages; between strains of the same sex and within mouse strains of different sexes. * p<0.05, ** p<0.01 between strain comparison at equivalent sex and age; † p<0.05, †† p<0.01 sex comparison within same strain and age. n = 6-10 mice.

4.2.2 Cardiac function

At 90 days of age basal cardiac FOC in C57 males was 48% ($p<0.05$) and 112% ($p<0.01$) higher than *mdx* males and C57 females, respectively. The differences were even greater for normalised FOC with C57 males being 129% ($p<0.01$) and 184% ($p<0.01$) higher than *mdx* males and C57 females, respectively. There were no differences in basal FOCs in *mdx* between sexes in any age groups, with a trend toward convergence of values at 330 days of age (Figure 4-3).

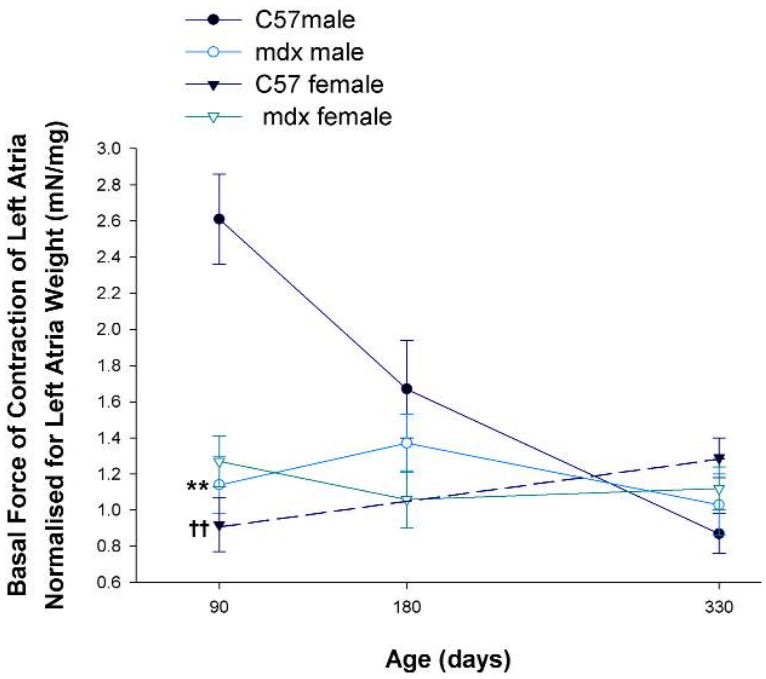


Figure 4-3 Basal FOC of LA (normalised for LA weight) in *mdx* and C57 males and females across ages.

Comparison between strains (same sex) and within mouse strains (different sexes) at varying ages. * p<0.05, ** p<0.01 between strain comparison at equivalent sex and age; † p<0.05, †† p<0.01 sex comparison within same strain and age. n = 6-10 mice.

Figure 4-4 illustrates that, at 90 days of age there was no difference in Ca^{2+} CRCs between females or between *mdx* mice. Interestingly, C57 males showed significantly higher ($p<0.01$) forces than both *mdx* males and C57 females across all Ca^{2+} concentrations.

Figure 4-5A shows 180 day old *mdx* males generating some lower forces than age-matched C57 males. However, there was no difference between *mdx* males and *mdx* females in terms of Ca^{2+} CRCs (Figure 4-5B).

Results showed no difference in Ca^{2+} CRCs between males of different mouse strains (Figure 4-6A) or between *mdx* sexes at 330 days of age (Figure 4-6D). At Ca^{2+} concentrations of 9.0 mM and 12.0 mM C57 males have a higher normalised FOC of LA than C57 females ($p<0.05$, Figure 4-6B) and C57 females had a higher FOC than *mdx* females ($p<0.05$, Figure 4-6C).

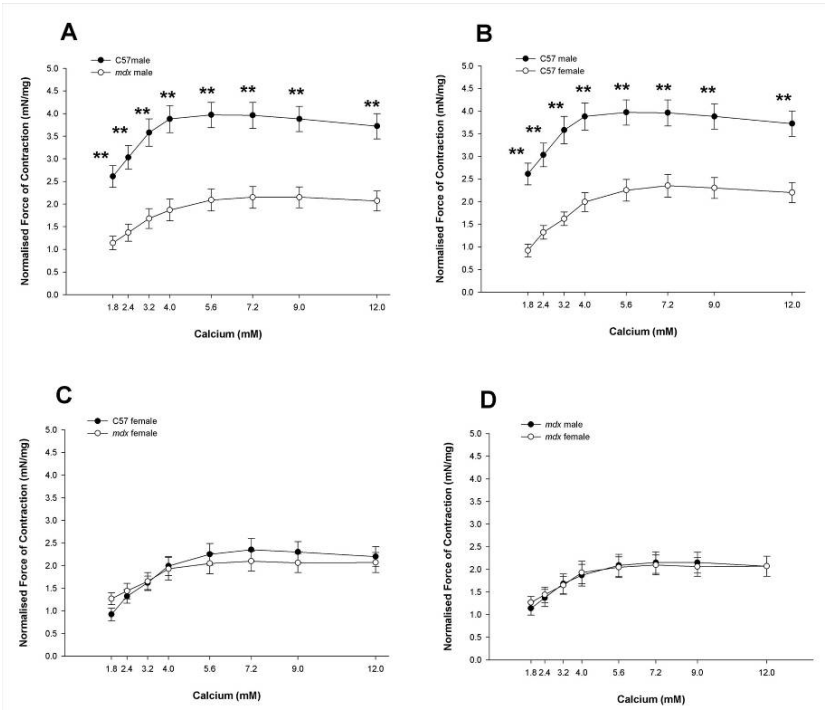


Figure 4-4 Concentration response curves to calcium comparing male and female 90 day old mice.

Comparison between strains and sexes for 90 day old mice. Comparisons between (A) C57 males and *mdx* males (B) C57 males and C57 females (C) C57 females and *mdx* females and (D) *mdx* males and *mdx* females. * p<0.05, ** p<0.01.
n = 8-9 mice.

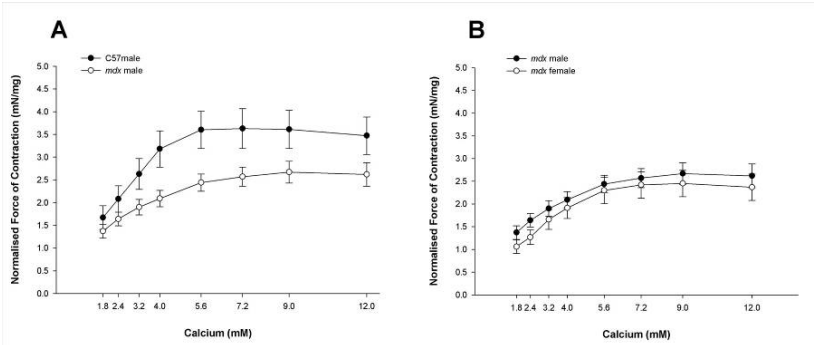


Figure 4-5 Concentration response curves to calcium comparing male and female 180 day old mice.

Comparison between strains and sexes for 180 day old mice. Comparisons between (A) C57 males and *mdx* males and (B) *mdx* males and *mdx* females. * p<0.05, ** p<0.01.
n = 8-10 mice.

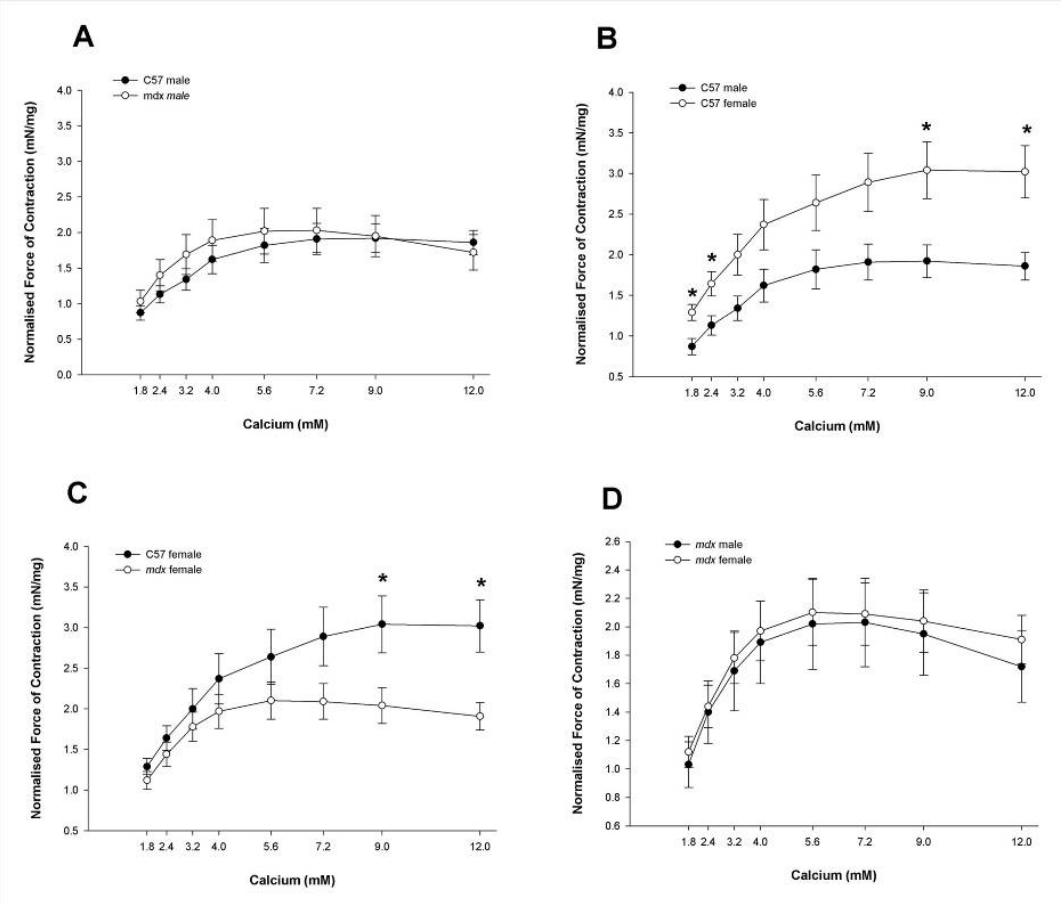


Figure 4-6 Concentration response curves to calcium comparing male and female mice 330 day old.

Comparison between strains and sexes for 330 day old mice. Comparisons between (A) C57 males and *mdx* males (B) C57 males and C57 females (C) C57 females and *mdx* females and (D) *mdx* males and *mdx* females. * p<0.05, ** p<0.01. n = 7-10 mice.

Figure 4-7 shows that, in 90 day old mice, there was no difference between mice in maximum FOC of LA. However, when adjusted for LA weight, maximum FOC (normalised for LA weight) results show C57 males with 87% higher forces than *mdx* males ($p<0.01$) and 71% higher forces than C57 females ($p<0.01$) with still no difference between *mdx* males and *mdx* females. There were no significant force differences between groups at other ages.

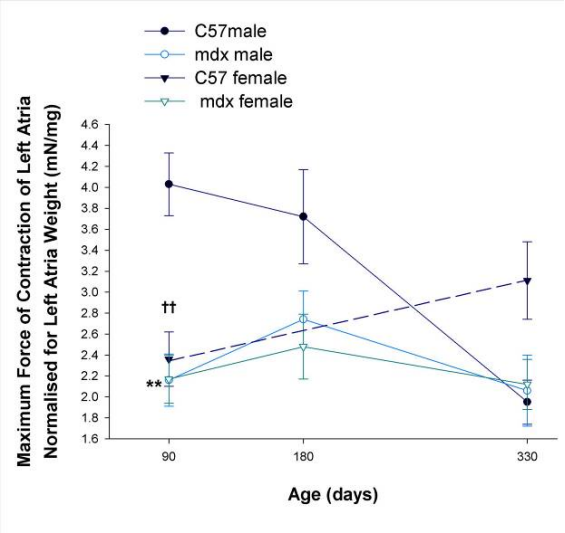


Figure 4-7 Maximum FOC of LA (normalised for LA weight) in male and female mice across ages.

Comparison between strains of the same sex and within mouse strains of different sexes across varying ages. * p<0.05, ** p<0.01 between strain comparison at equivalent sex and age; † p<0.05, †† p<0.01 sex comparison within same strain and age. n = 6-10 mice.

4.3 Influence of sex on EDL characteristics

4.3.1 EDL morphometry

In 90 day old mice; *mdx* males had 31% heavier EDL than C57 males (p<0.01) but were not significantly different to *mdx* females. C57 males and *mdx* females had 59% and 72% heavier EDL than C57 females (p<0.01) (Figure 4-8A). At the same age, *mdx* males had a 22% larger EDL CSA (CSA) than C57 males (p<0.01) but were not different to *mdx* females. C57 males and *mdx* females had a 34% and 49% larger EDL CSA than C57 females (p<0.01) (Figure 4-8C).

In 180 day old mice; *mdx* males had 30% heavier EDL than C57 males (p<0.01) but were not significantly different to *mdx* females (Figure 4-8A). *Mdx* males had a 27% larger EDL CSA than C57 males (p<0.01) but were not different to *mdx* females (Figure 4-8C).

330 day old *mdx* males had 73% and 52% heavier EDL weight than C57 males and *mdx* females respectively (p<0.01). C57 males had a 26% heavier EDL weight than C57 females (p<0.01). *Mdx* female EDL weight was almost double (+94%) that of C57 females (p<0.01) (Figure 4-8A). *Mdx*

males had a significantly larger (+18%) EDL CSA than C57 males ($p<0.01$), with no significant difference to *mdx* females. C57 males and *mdx* females had a 24% and 68% larger EDL CSA than C57 females ($p<0.01$) (Figure 4-8C).

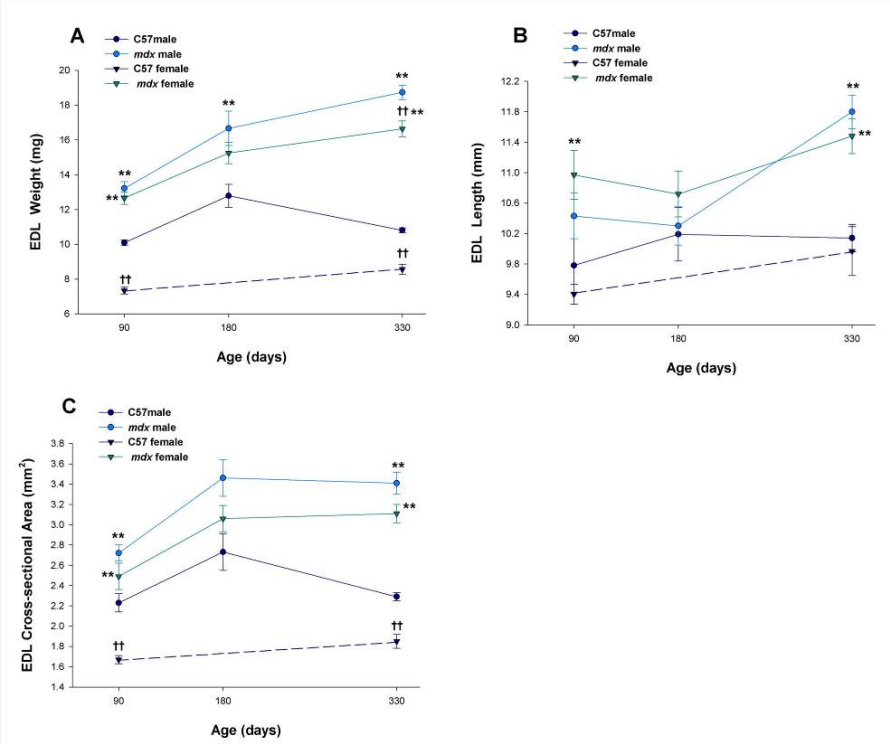


Figure 4-8 EDL skeletal muscle morphometry comparing mouse strains and sexes.

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. A = EDL weight, B = EDL Lo and C = EDL CSA. n = 8-9 mice.**

4.3.2 EDL function

In 90 day old mice; *mdx* males had a 24% and 27% higher Pt than C57 males ($p<0.05$) and *mdx* females ($p<0.01$). C57 males and *mdx* females had a 51% ($p<0.01$) and 50% ($p<0.05$) higher Pt than C57 females. There was no difference in Po between males or between *mdx* males and females. C57 males and *mdx* females had a 45% and 49% higher Po than C57 females ($p<0.05$). There were no significant differences in sPt, sPo, or

tetanus to twitch ratio (Po:Pt) between strains or between sexes of the same strain (Table 4-1).

Table 4-1 EDL muscle function in 90 day old mice.

mouse strain sex	C57 male	*mdx* male	C57 female	*mdx* female
BW (g)[1]	25.93±0.43	28.33±0.77*	20.14±0.32††	26.52±0.67**
Pt (mN)	38.99±2.73	48.17±2.39*	25.76±3.64††	38.26±2.87*††
Po (mN)	158.94±15.03	205.71±17.74	109.54±14.84†	163.13±20.16*
sPt (mN/mm^2)	17.95±1.71	17.70±0.75	15.55±2.33	15.71±1.56
sPo (mN/mm^2)	73.55±8.60	75.79±6.76	65.68±8.79	67.15±9.55
Po:Pt	4.05±0.21	4.26±0.31	4.30±0.20	4.20±0.35

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. n = 8-9 mice. [1]this information is described in Figure 4-1A.**

Table 4-2 confirms no significant changes in any of the measured EDL parameters between strains or between sexes of the same strain in 180 day old mice.

Table 4-2 EDL muscle function in 180 day old mice.

mouse strain sex	C57 male	*mdx* male	*mdx* female
BW (g)[1]	30.74±0.54	31.82±0.76	32.82±0.57
Pt (mN)	39.54±2.64	43.64±3.90	48.21±3.00
Po (mN)	155.74±12.85	179.25±20.76	191.69±15.19
sPt (mN/mm^2)	15.32±1.71	12.87±1.24	15.93±1.10
sPo (mN/mm^2)	60.58±7.19	53.55±6.98	63.57±5.74
Po:Pt	3.97±0.23	4.09±0.30	3.97±0.19

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age.**
n = 8-10 mice. [1]this information is described in Figure 4-1A.

In 330 day old mice, C57 males had a 43% higher Pt than C57 females (p<0.01). C57 males had a 45% higher Po than C57 females (p<0.01). *Mdx* females had a 32% higher Po than C57 females (p<0.05). *Mdx* males had a 30% lower sPt than C57 males (p<0.01) but no difference to *mdx* females. *Mdx* males had a 33% lower sPo than C57 males (p<0.01) but no difference to *mdx* females. Other comparisons between strains or within strains and between sexes revealed no significant differences in EDL parameters (Table 4-3).

Table 4-3 EDL muscle function in 330 day old mice.

mouse strain sex	C57 male	*mdx* male	C57 female	*mdx* female
BW (g)[1]	29.44±0.73	35.16±0.82**	27.65±0.65†	33.94±0.73**
Pt (mN)	43.02±1.66	44.83±3.00	30.01±3.88††	39.22±2.50
Po (mN)	200.87±12.73	202.64±15.71	138.37±12.35††	182.80±9.45*
sPt (mN/mm^2)	18.99±3.50	13.20±2.56**	16.48±2.27	12.69±9.45
sPo (mN/mm^2)	89.02±23.59	59.46±12.10**	76.09±8.16	58.96±3.27
Po:Pt	4.62±0.51	4.64±1.44	4.78±0.27	4.71±0.20

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age;**
† p<0.05, †† p<0.01 sex comparison with same strain and age.
n = 7-12 mice. [1]this information is described in Figure 4-1A.

4.3.3 EDL histology

Figure 4-9 shows that, at all ages, *mdx* male and female muscle tissues had a significantly higher percentage of centrally nucleated fibres (CNFs) than their C57 equivalents ($p<0.01$). Age-matched males and females of same strain were not significantly different with respect to percentage of CNFs with the exception of 180 days of age where *mdx* females had a significantly higher percentage of CNFs than *mdx* males ($p<0.01$).

Figure 4-10 shows that there was no difference in EDL cell diameter between strains or between sexes within the same mouse strain at either 90 or 180 days of age. However, at 330 days of age *mdx* males and females had significantly larger ($p<0.01$, $p<0.05$) EDL cell diameters than C57 equivalents.

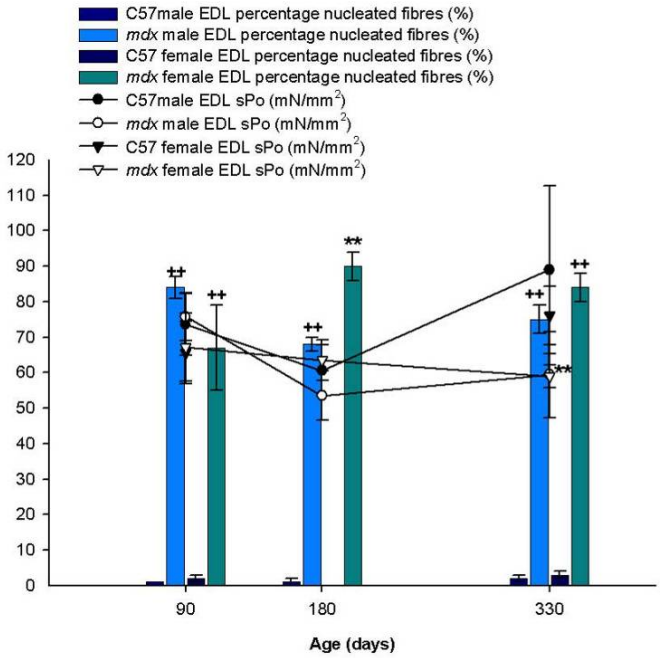


Figure 4-9 **Percentage of CNFs and maximum tetanic forces in EDL muscles at varying ages and between sexes.**

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age.**
n = 7-12 mice.

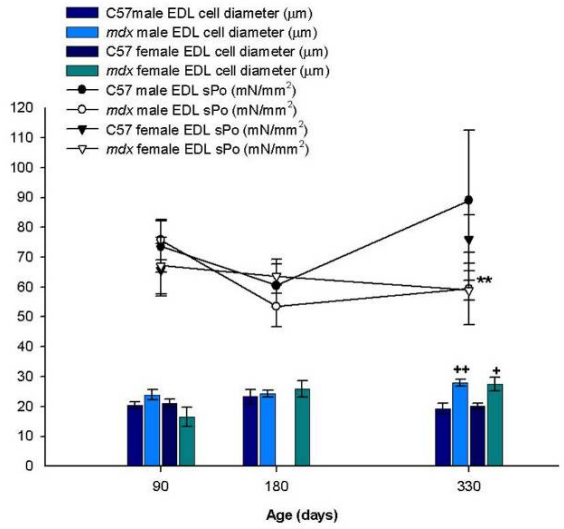


Figure 4-10 **EDL cell diameters and maximum tetanic forces at varying ages and between sexes.**

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age.**
n = 7-12 mice.

4.4 Influence of sex on SOL characteristics

4.4.1 SOL morphometry

Figure 4-11A clearly depicts that, at 90 days of age, *mdx* males had 50% ($p<0.01$) and 22% ($p<0.05$) heavier SOL than C57 males and *mdx* females, respectively. C57 males and *mdx* females had 26% and 66% heavier SOL than C57 females ($p<0.01$). 180 day old *mdx* males had a 23% larger SOL weight than C57 males ($p<0.05$) but were not different to *mdx* females. *Mdx* males had a 16% larger SOL CSA than C57 males ($p<0.05$) but were not different to *mdx* females. *Mdx* males, at 330 days old, had a SOL weight of more than double (2.13-fold) that of C57 males ($p<0.01$). Similarly, *mdx* females had a 70% higher SOL weight than C57 females ($p<0.01$). There was no difference between male and female C57 with respect to SOL weight whereas *mdx* males had a 37% higher SOL weight than *mdx* females ($p<0.01$).

Mdx males had a 75% and 28% larger SOL CSA than C57 males and *mdx* females respectively ($p<0.01$). There was no difference in SOL CSA between C57s. *Mdx* females had a 6% larger SOL CSA than C57 females ($p<0.01$) (Figure 4-11C).

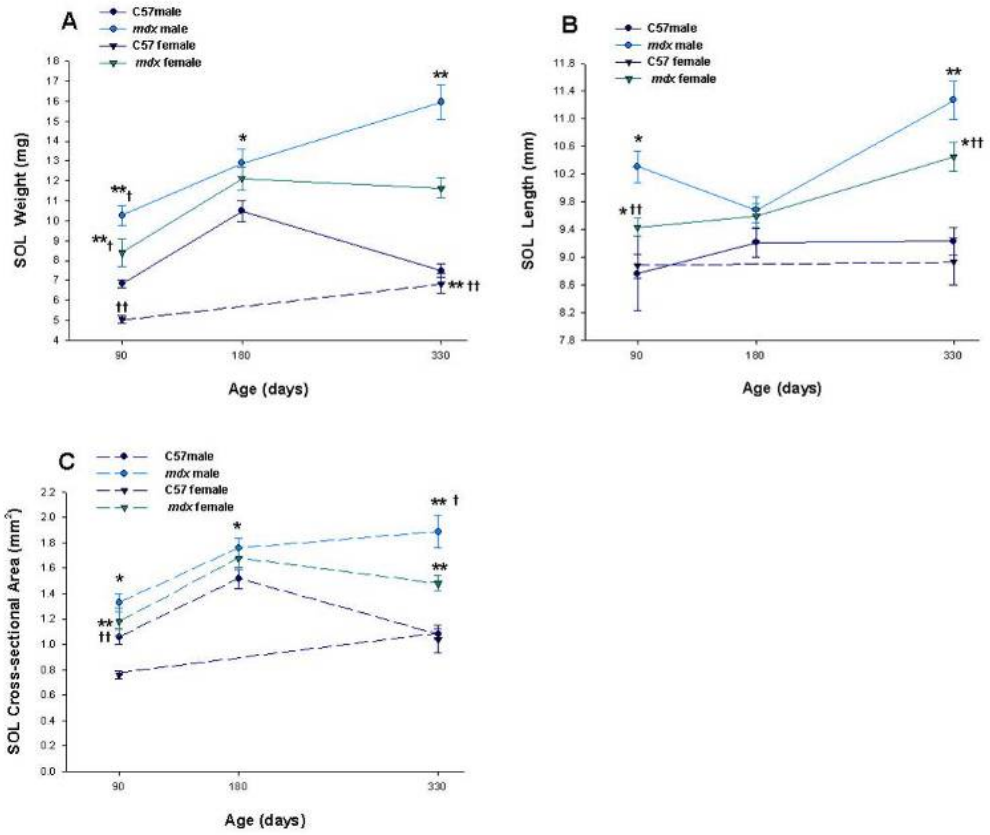


Figure 4-11 SOL skeletal muscle morphometry comparing mouse strains and sexes.

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age.**
A = SOL weight, B = SOL Lo, and C = SOL CSA. n = 8-9 mice.

4.4.2 SOL function

Table 4-4 shows that in 90 day old mice, *mdx* males and females have significantly higher SOL Pt and Po values than C57s of equivalent sex ($p<0.01$). However, when normalised for CSA (namely sPt and sPo values), these differences lose statistical significance.

Table 4-4 SOL muscle function in 90 day old male and female mice.

mouse strain sex	C57 male	*mdx* male	C57 female	*mdx* female
BW (g)[1]	25.93±0.43	28.33±0.77*	20.14±0.32††	26.52±0.67**
Pt (mN)	18.26±1.80	27.35±1.60**	15.65±1.65	25.10±1.05**
Po (mN)	109.24±14.05	173.58±8.60**	102.61±11.70	166.55±5.23**
sPt (mN/mm^2)	17.50±1.88	20.75±0.98	21.29±2.83	22.08±1.77
sPo (mN/mm^2)	104.06±12.91	132.00±6.79	139.21±18.79	146.52±11.05
Po:Pt	5.90±0.38	6.42±0.35	6.60±0.35	6.68±0.22

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. n = 8-9 mice. [1]this information is described in Figure 4-1A.**

In 180 day old mice; *mdx* males had a 25% (p<0.01) and 21% (p<0.05) higher SOL Pt than C57 males and *mdx* females, respectively. *Mdx* males had 43% higher Po than C57 males (p<0.01) but no difference to *mdx* females. There were no differences in sPt . *Mdx* males had a 22% higher sPo than C57 males (p<0.05) but no difference to *mdx* females. *Mdx* males had a 15% higher Po:Pt than C57 males (p<0.05) but no difference to *mdx* females (Table 4-5).

Table 4-5 SOL muscle function in 180 day old male and female mice.

mouse strain sex	C57 male	*mdx* male	*mdx* female
BW (g) [1]	30.74±0.54	31.82±0.76	32.82±0.57
Pt (mN)	21.09±1.95	26.35±0.87**	21.85±1.54†
Po (mN)	126.75±11.12	181.52±5.07**	160.97±10.89
sPt (mN/mm^2)	14.34±1.54	15.39±0.74	13.39±1.32
sPo (mN/mm^2)	85.73±8.44	104.84±3.07*	98.64±9.30
Po:Pt	6.09±0.31	6.99±0.27*	7.42±0.28

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. n = 8-10 mice. [1]this information is described in Figure 4-1A.**

In 330 day old mice; there was no difference in SOL Pt between males or C57s. *Mdx* males had an 18% higher Pt than *mdx* females (p<0.05). *Mdx* females had a 36% higher Pt than C57 females (p<0.01). *Mdx* males had a 42% higher Po than C57 males (p<0.01) but no difference to *mdx* females. There was no difference between C57s in Po. *Mdx* females had an 88% higher Po than C57 females (p<0.01). *Mdx* males had a 31% lower sPt than C57 males (p<0.05). There was no difference in sPt between males and

females in respective mouse strains or between females. There was no difference in sPo between males, females, or between the sexes in the respective mouse strains. *Mdx* males and *mdx* females had an 11% ($p<0.05$) and 34% ($p<0.01$) higher Po:Pt compared to C57 males and C57 females respectively. There was no difference in Po:Pt in C57s whereas *mdx* males had a 10% lower ratio to *mdx* females ($p<0.05$) (Table 4-6).

Table 4-6 SOL muscle function in 330 day old male and female mice.

mouse strain sex	C57 male	*mdx* male	C57 female	*mdx* female
BW (g)[1]	29.44±0.73	35.16±0.82**	27.65±0.65†	33.94±0.73**
Pt (mN)	20.93±1.77	24.77±1.43	15.52±1.81	21.06±0.64**†
Po (mN)	125.58±12.14	178.16±11.58**	89.72±10.38	168.48±5.59**
sPt (mN/mm^2)	19.60±1.72	13.60±1.35*	16.42±2.79	14.32±0.50
sPo (mN/mm^2)	116.61±10.97	98.42±11.15	94.23±16.08	115.02±5.80
Po:Pt	6.05±0.39	7.19±0.22*	5.97±0.51	8.02±0.20**†

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. n = 7-12 mice. [1]this information is described in Figure 4-1A.**

4.4.3 SOL histology

At all ages, *mdx* male and female muscles had a significantly higher percentage of CNFs than their C57 equivalents. Across the ages, males and females of same strain were not significantly different with respect to percentage of CNFs (Figure 4-12).

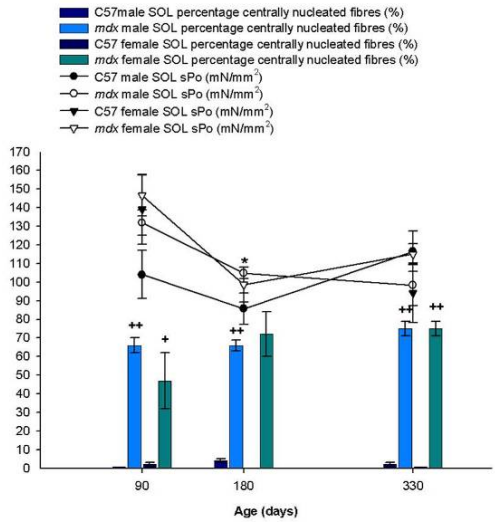


Figure 4-12 Percentage of CNFs and sPo in SOL muscles at varying ages and sexes.

*** p<0.05, ** p<0.01 between strain comparison with equivalent sex and age;**
† p<0.05, †† p<0.01 sex comparison with same strain and age.
n = 7-12 mice. No 180 day old C57 females were available.

There was no difference in SOL cell diameters between males and females within the same mouse strain at any age. Cell diameters were not different between strains at 90 or 180 days of age, however, at 330 days of age *mdx* males and females had significantly larger (p<0.05, p<0.01) cell diameters than C57 equivalents (Figure 4-13).

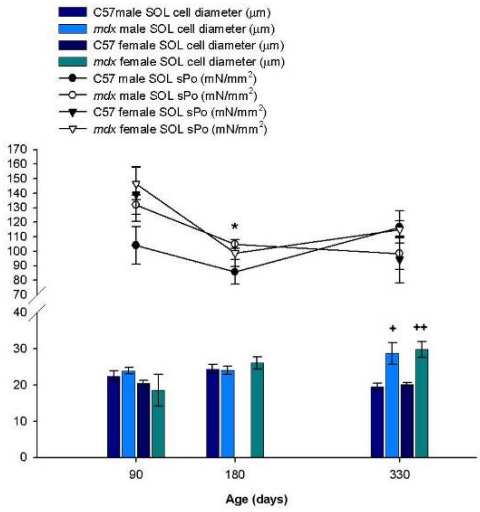


Figure 4-13 Cell diameters and sPo in SOL muscles at varying ages and between sexes.

p<0.05, ** p<0.01 between strain comparison with equivalent sex and age; † p<0.05, †† p<0.01 sex comparison with same strain and age. n = 7-12 mice. No 180 day old C57 females were available.

4.5 Discussion

This trial examined the body weight, cardiac, and skeletal muscle differences between sexes and mouse strains across specific ages. Despite no significant differences in birth weight between sexes in humans, males are significantly heavier than age-matched females throughout human adulthood (Hamill *et al.* 1979; Brainard and Burmaster 1992; Murray and Burmaster 1992; Burmaster and Crouch 1997). This study showed that BWs in the *mdx* mouse do not correlate with human growth patterns, particularly with respect to sex differences. All mice in the study had reached adulthood and at 90 days of age *mdx* males were heavier than C57 males and females. Lu and Hoey (2004) found no difference in BW between *mdx* and C57 males at 12 to 14 weeks old. There were no differences in BWs between mice at 180 days of age but at 330 days *mdx* mice were actually heavier than age- and sex-matched controls. This was in contrast to Lu and Hoey (2000) who found 12 month old C57 males heavier than *mdx* age-matched males and Laws (2005) who reported no difference in BWs between *mdx* and C57s from six months to 12 months of age but that C57s were heavier than *mdx* from 15 to 24 months of age .

At all age groups C57 males were heavier than females but there were no sex differences in *mdx* BWs. The maintenance of 'male-equivalent' weight in 330 day old female *mdx* may be due to the increased deposition of adipose tissue in ageing *mdx* females as has been reported by Salimena *et al* (2004).

The incidence of cardiac involvement in human female DMD carriers is extremely high and manifests predominantly as cardiomyopathy and progresses with age (Cox and Kunkel 1997). Sex-related differences in cardiac function have been well documented in normal humans (Harrison and Smith 1980; Liao *et al.* 1995; Yamasaki *et al.* 1996) and rats (Watanabe 1988; Vizgirda *et al.* 2002) and yet extremely limited data is available on sex differences in cardiac function in the *mdx* mouse. CV disease in humans shows a male to female ratio of 2.2 across different populations (Kalin and Zumoff 1990) and the sex disparity in cardiac disease has been interpreted primarily as reflecting oestrogen-mediated protection against the deposition

of intra-arterial plaque (atherogenesis) (Liu, Death and Handelsman 2003). It is important to study the sex differences in the *mdx* mouse model of DMD with a view to providing valuable research information directly applicable to female DMD carriers and to further elucidate the role of sex hormones (both male and female) in the disease.

At 90 days old *mdx* males had lower LV+S and LA weight compared to C57s, whereas, at this age *mdx* males had a higher LV+S and HW compared to *mdx* females. These results are in contrast to findings by Lu and Hoey (2000) who found that 12 to 14 week old male *mdx* actually had lower LV+S weights than C57s. At 180 days old, *mdx* males had lower LV+S, whole heart, and HW as a percentage of BW compared to C57 males and *mdx* females, in addition to a lower LA weight than *mdx* females. In 330 day old mice *mdx* males had heavier RVs but lower HW as a percentage of BW compared to C57s males and lower LA weight and heart rate (HR) as a percentage of BW compared to *mdx* females. Lu and Hoey (2000) also reported lower RV weights in 12 month old *mdx* males despite lower BWs.

When comparing females aged 90 days, *mdx* RV, LV+S, HW, and HW as a percentage of BW values were higher than C57s. Then, in the oldest groups (330 days of age), all cardiac tissues measured in females were heavier in *mdx* compared to C57s. In 12 month old females, Lu and Hoey (2000) found that despite C57s being heavier in BW than *mdx*, the RV of *mdx* females was heavier than age-matched C57 females.

Cardiac abnormalities are well documented in DMD patients and it is thought that the left ventricular posterobasal and lateral walls are most extensively affected, sparing the right ventricle and the atrium (Finsterer and Stollberger 2003). Therefore, one would expect to see lowered LV+S weights in *mdx* compared to C57s, particularly in groups where BWs are not different. This occurred in two groups; 180 day old *mdx* males when compared to both C57 males and *mdx* females. In other groups where there was a significant difference in LV+S values there was a corresponding significant difference in BW which confounds interpretation. It must be noted that the LV+S differences were not mirrored by functional results or response to exogenous Ca^{2+} as reflected by Ca^{2+} CRCs.

The comprehensive 2000 study by Lu and Hoey examined atrial function in 12 to 14 week old *mdx* and C57 males and 12 month old *mdx* and C57 females. This study was the first research to show both age-related deterioration of β_1-adrenoceptors in the male *mdx* heart and differences in β_1-adrenoceptor function between male and female *mdx* (Lu and Hoey 2000b).

This study found significant differences in basal LA FOC between strains was evident at 90 days of age at which C57 males generated higher forces than both C57 females and *mdx* males. This concurs with the work of Woolf (2003) who found basal contractility in *mdx* was lower than C57s in 12 to 14 week old male LAs . LA forces in *mdx* were not different between males and females at any age tested. The work of Lu and Hoey (2000) also illustrated reduced responsiveness to Ca^{2+} in both male and female *mdx*. This study supported their work in that it showed that C57 males (across all ages) generated higher forces than both *mdx* males and C57 females, and that both sexes in *mdx* had a dampened responsiveness to Ca^{2+} as shown by Ca^{2+} CRCs generated. No differences in Ca^{2+} CRCs between *mdx* males and females were seen at any age. These results suggest that both male and female *mdx* mice from 90 to 330 days of age have cardiac abnormalities not evidenced by C57 control mice.

Despite the fact that this study did not measure serum CK levels in experimental animals, the results of studies which have found sex differences in serum CK activity in *mdx* mice are notable. In 2004 Salimena *et al.* found that at six weeks of age *mdx* males showed non-significant increased serum CK levels compared to age-matched females, by 12 weeks of age *mdx* males had an over 100% increase in CK levels compared to *mdx* females, whereas, by the age of 48 weeks, *mdx* males actually had an approximately 4.5-fold decrease in CK activity compared to age-matched females. Valentine (1988) found that in the *cxmd* dog, females had lower serum CK levels than hemizygous males but that muscle damage was greater in elderly (24 week old) *mdx* female dogs (Valentine *et al.* 1988).

Some researchers (Amelink, Kamp and Bar 1988; Tiidus *et al.* 2001; Tiidus 2003) suggest that human sex differences are such that female skeletal muscle has a greater regenerative capacity after exercise induced damage, compared to males. Tiidus (2001) concluded that female hormones

had an anti-inflammatory role in muscle protection, Paroo *et al.* (2002) showed that men have higher levels of stress protein HSP70 and CK levels in response to exercise, than women (Paroo, Dipchand and Noble 2002) and Komulainen (1999) reported no changes to the architecture of muscle fibres in females compared with males immediately after exercise and up to six hours later, thus indicating female muscles are protected against membrane damage (Komulainen *et al.* 1999).

Bar (1988) showed that oestrogen (in rats) had a protective role in skeletal muscles of both males and females (Bar *et al.* 1988) and two studies, specifically using the *mdx* mouse model, showed that oestrogen could attenuate the inflammatory-related leucocyte infiltration into muscles (Wise and Dubal 2000; Zhai *et al.* 2000).

EDL morphometry at 90 days of age revealed that *mdx* males and females had higher BWs, EDL weights, and EDL CSAs than sex-matched C57s, this results was mirrored in a comparison between C57 sexes, whilst there was no difference in EDL muscle weight or CSAs between *mdx* sexes despite males having higher BW. At 180 days old *mdx* males were not different in EDL morphometry to females but had heavier EDLs with greater CSAs than C57 males. At 330 days of age both *mdx* males and females had greater BWs, EDL weights and CSAs than sex-matched C57s, with the same differences occurring between C57 males and females. *Mdx* males at this age had heavier EDLs but not different BWs or EDL CSAs than *mdx* females. Clearly, both male and female *mdx* showed hypertrophy of the EDL muscles across the ages studied.

Functional studies were then conducted to determine whether the EDL muscle hypertrophy seen in *mdx* males and females actually translated to increased forces in these fast-twitch muscles. Protocols were developed and incorporated the optimisation of muscle preload, stimulation voltage, and stimulation frequency and these were consistently used for the remainder of the project. Kaminski (1992) argued that fast-twitch, extremity muscles (such as EDL), are affected preferentially in DMD (Kaminski *et al.* 1992). Clearly, *mdx* EDL muscles were hypertrophied but the results showed no differences in EDL forces generated (when normalised for CSA) at 90 or 180 days of age when compared to females or male controls. At 330 days of age EDL muscle

weakness became apparent in *mdx* males. These males did not generate different EDL forces to *mdx* females but had a 33% lower sPo than C57 males, despite being heavier in BW and EDL weight. Conclusively, hypertrophied EDL muscles did not generate greater forces than controls in either male or female *mdx.*

In 330 day old mice, SOL skeletal muscle morphometry mirrored the results for EDL muscles except that C57 males and females differed only in BW and *mdx* males had larger SOL CSAs than *mdx* females. Both fast-twitch (EDL) and slow-twitch (SOL) skeletal muscles were hypertrophied in *mdx* males and females. As in EDL studies, there were no functional differences (normalised for CSA) for 90 or 180 day old mice. Again, at 330 days of age there were no SOL functional differences (normalised for CSA) between *mdx* males and C57 males, *mdx* males and *mdx* females, or *mdx* females and C57 females. C57 males at this age generated higher sPo values than C57 females. As with EDL muscles, hypertrophied SOL muscles did not translate to muscles capable of generating larger mechanical forces *in vitro*. As with EDL muscles, hypertrophied SOL muscles seen in male and female *mdx* did not generate greater forces than control C57 males and females.

This is the first study to show sex-specific differences in skeletal muscle function between *mdx* and C57 mice.

At the 76th European Neuromuscular Centre (ENMC) International Workshop in The Netherlands 2000 it was agreed that, in order to compare emerging treatment strategies, standardisation of protocols for the assessment of functional and histological parameters in *mdx* mouse research be a priority (Rudel and Brinkmeier 2002). It can be argued that this is yet to be achieved (Harper *et al.* 2002; Nguyen *et al.* 2002), however, Briguet *et al.* (2004) has developed a method of reliably determining muscle fibre diameter in the *mdx* mouse and this method was utilised in this project (Briguet *et al.* 2004).

Since 1987, the histopathological changes in *mdx* skeletal muscle over the lifespan of the mouse have been determined by a number of researchers (Carnwath and Shotton 1987; Torres and Duchen 1987; Coulton *et al.* 1988; Pastoret and Sebille 1995a). Limited data was available that specifically addressed the issue of sex differences in *mdx* and C57s.

However, Salimena *et al.* (2004) noted that six week old *mdx* males showed more extensive sarcolemmal damage in EDL muscle than age-matched females whereas the situation was reversed at 24 weeks of age. Histological analysis showed consistent differences between age-matched *mdx* sexes. Twelve week old *mdx* females showed less muscle inflammation and necrosis than males and at all ages studied (six, 12, and 24 weeks of age) female *mdx* showed a higher percentage of regenerating fibres compared to age-matched males. These researchers also found that 48 week old *mdx* females presented an 80% increase in fibrosis and deposition of fatty tissue compared to age-matched males. Salimena *et al.* (2004) thus concluded that the characteristic histological features of *mdx* skeletal muscles may be influenced by sex dimorphism.

Organ bath functional studies showed hypertrophy of both fast-twitch (EDL) and slow-twitch (SOL) *mdx* muscles failed to translate to increased forces *in vitro* when compared to age- and sex-matched controls. Histological analysis showed that across the ages studied, both *mdx* males and females had significantly higher percentages of CNFs compared to sex-matched C57s. This is clearly consistent with the current literature.

Throughout the lifespan of the *mdx* there is a vicious cycle (Figure 2-14) of degeneration and regeneration which has been documented by a number of researchers (Zacharias and Anderson 1991; Beilharz *et al.* 1992; Grounds and McGeachie 1992; Pacioretty, Cooper and Gilmour 1992; Lefaucheur, Pastoret and Sebille 1995; Pastoret and Sebille 1995a). The presence of mononuclear cells located in epimysial space of *mdx* muscle cells indicates that an inflammatory processes is occurring in response to continual vicious cycles as described above (Anderson, Bressler and Ovalle 1988; Coulton *et al.* 1988; Pastoret and Sebille 1993; Lefaucheur, Pastoret and Sebille 1995). Elevated numbers of CNFs are commonly observed in the histopathology of both DMD patients and animal models of the disease including the *mdx* mouse (Narita and Yorifuji 1999), however in DMD patients the population of CNFs is considered to be lower than in milder forms of muscular dystrophy (Tohgi *et al.* 1994).

This project examined the histological differences between strains and sexes with respect to CNFs and cell diameters with a view to

determining whether correlations existed between functional and histological results. Percentages of CNFs were determined for both EDL (fast-twitch) and SOL (slow-twitch) skeletal muscles. Consistent with the literature (Narita and Yorifuji 1999) both muscle types in *mdx* showed exponentially higher percentages of CNFs compared to C57 sex-matched controls. In EDL muscles, percentages of CNFs did not differ between age-matched strains, the exception being 180 day old female *mdx* which had higher percentages than *mdx* males. At all ages, *mdx* SOL muscles had a significantly higher percentages of CNFs than sex-matched C57s and, across all ages, males and females of same strain did not differ with respect to percentage of CNFs.

In DMD and *mdx* mice, the fundamental deleterious consequence of dystrophic gene expression is segmental necrosis of skeletal muscle fibres. The nature of the gene defects and the pathogenesis of muscle fibre damage are not clear. However, clinical and experimental evidence indicates that muscle fibres, whose girth is below a certain level (estimated at approximately 20 to 25 μm in diameter in *mdx* mice) are not susceptible to necrosis. This apparent protection against functional damage has been observed in muscle fibres that are naturally of small diameter (such as the EOMs), and in mouse muscle fibres that have been prevented from growing normally by experimental procedures or by pathological processes in DMD patients (Karpati, Carpenter and Prescott 1988). Briguet (2004) also noted that both small diameter and large diameter muscle fibres were more abundant in *mdx* dystrophic muscle than in age- and sex-matched controls (Briguet *et al.* 2004). This suggests that even in situations where there may be no significant difference in muscle cell diameter between strains there may, in fact, be a greater variance in *mdx* values. This was supported by this trial and reflected in larger standard error values (SEMs) seen in histological results of *mdx* compared to C57s. This trial offered the first opportunity to test that hypothesis that small diameter fibres were in some way immune to disease progression in *mdx* mice.

Results for both fast-twitch (EDL) and slow-twitch (SOL) muscles showed no difference in cell diameters between strains or sexes at 90 or 180 days of age. A difference between strains emerged at 330 days where both *mdx* males and females had significantly larger cell diameters than sex-

matched C57s. Groups that had EDL mean cell diameters below 25 μm included 90 day old C57 males, C57 females, and *mdx* females, together with C57 males and females at 330 days of age. None of the 90 day old groups (with cell diameters below 25 μm) showed statistically significant difference in function when compared to sex-matched mice. However, C57 males and females at 330 days old showed higher EDL sPo values than *mdx* sex-matched mice. Groups with SOL muscles of diameters less than 25 μm included all mice at 90 days of age together with both male and female 330 day old C57s. None of these groups showed and statistically significant difference in function (sPo values) when compared to sex-matched mice. Neither fast-twitch (EDL) or slow-twitch (SOL) skeletal muscle function correlated with histological findings within or between strains and the study did not find a direct link between muscle cells of diameters less than 25 μm and skeletal muscle function.

5 Castration trial

5.1 Influence of castration on body weight

Castration caused a lower BW at age 6 months as evident in Figure 5-1. However, there was no difference in BW between *mdx* and C57 entire males or castrated males. *Mdx* and C57 entire mice were 29% heavier than castrated mice of the same strain ($p<0.01$).

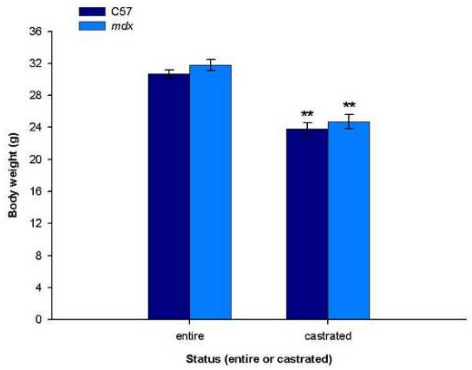


Figure 5-1Influence of castration on body weight in C57 and *mdx* male mice.

Comparison within mouse strains as to the influence of castration on BW . * p<0.05, ** p<0.01 treatment comparison in same strain. Age = 6 months.
n = 6-7 mice.

5.2 Influence of castration on cardiac characteristics

5.2.1 Cardiac morphometry

C57 entires had a 15% higher HW than *mdx* entires ($p<0.05$). *Mdx* and C57 entires had a 20% and 47% heavier HW than castrated mice of the same strain respectively ($p<0.01$), however, these mice also had a higher overall BW. Normalisation of their HW to BW revealed that castration caused this to be reduced to 11% in C57 and no difference at all in *mdx*. This suggests that the difference in HW is largely due to the difference in BW. As shown in Table 5-1, *mdx* have a lower HW% than C57s, however, castration negated this effect completely. This was because castration reduced the HW% in C57s whereas castration had no effect on HW% in *mdx*.

C57 entires had an 18% higher HW% than *mdx* entires ($p<0.01$). Castration in the C57 mouse strain caused an 11% decrease in HW% ($p<0.05$) in contrast to *mdx* where castration produced no difference in HW%.

In addition, there was no difference in HW% between castrated mouse strains.

RA weights between strains of entires or between entire and castrated *mdx* were not significantly different. C57 entires had a 64% heavier RA than C57 castrates ($p<0.01$). Castrated *mdx* had an 80% heavier RA than C57 castrates ($p<0.05$). There was no difference in LA or RV weights between strains or as a result of castration within mouse strains. Castration caused no difference in RV in *mdx* whereas C57 castrates had a 29% lower RV than C57 entires ($p<0.01$). *Mdx* entire had a 20% heavier LV+S than C57 entires ($p<0.05$) whereas there was no difference in LV+S between strains in castrates. C57 and *mdx* entires had a 23% and 55% heavier LV+S than their respective castrates ($p<0.01$).

Table 5-1 Body and cardiac weights in entire and castrated male mice.

mouse strain status	C57 entire	C57 castrated	*mdx* entire	*mdx* castrated
BW (g)	30.74±0.54	23.78±0.83††	31.82±0.76	24.67±1.01††
HW (mg)	139.24±6.58	94.87±6.61††	121.10±4.49*	100.57±3.24††
HW% (%)	0.45±0.01	0.40±0.02†	0.38±0.01**	0.41±0.01
RA weight (mg)	3.45±0.30	2.10±0.19††	3.34±0.26	3.77±0.59*
LA weight (mg)	1.06±0.11	0.81±0.11	1.12±0.13	1.02±0.10
RV (mg)	25.31±1.62	17.90±1.15††	25.82±1.51	25.07±4.99
LV+S (mg)	90.81±3.28	74.06±5.45††	109.42±6.12*	70.72±3.79††

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment; † p<0.05, †† p<0.01 treatment comparison with same strain. n = 6-7 mice.**

5.2.2 Cardiac function

There was no difference in both basal FOC of LA between strains for entires or castrates and normalised FOC between strains of entires or between entires and castrates of the same strain (Figure 5-2). *Mdx* castrates had a 38% lower normalised FOC than C57 castrates ($p<0.05$). There was no difference in maximum basal FOC between strains or between entires or castrates.

Castration caused no significant functional cardiac effect within mouse strains (Figures 5-2B and 5-2D). Castrated C57s did have

significantly higher forces than *mdx* castrates across all Ca^{2+} concentrations ($p<0.05$) (Figure 5-2C).

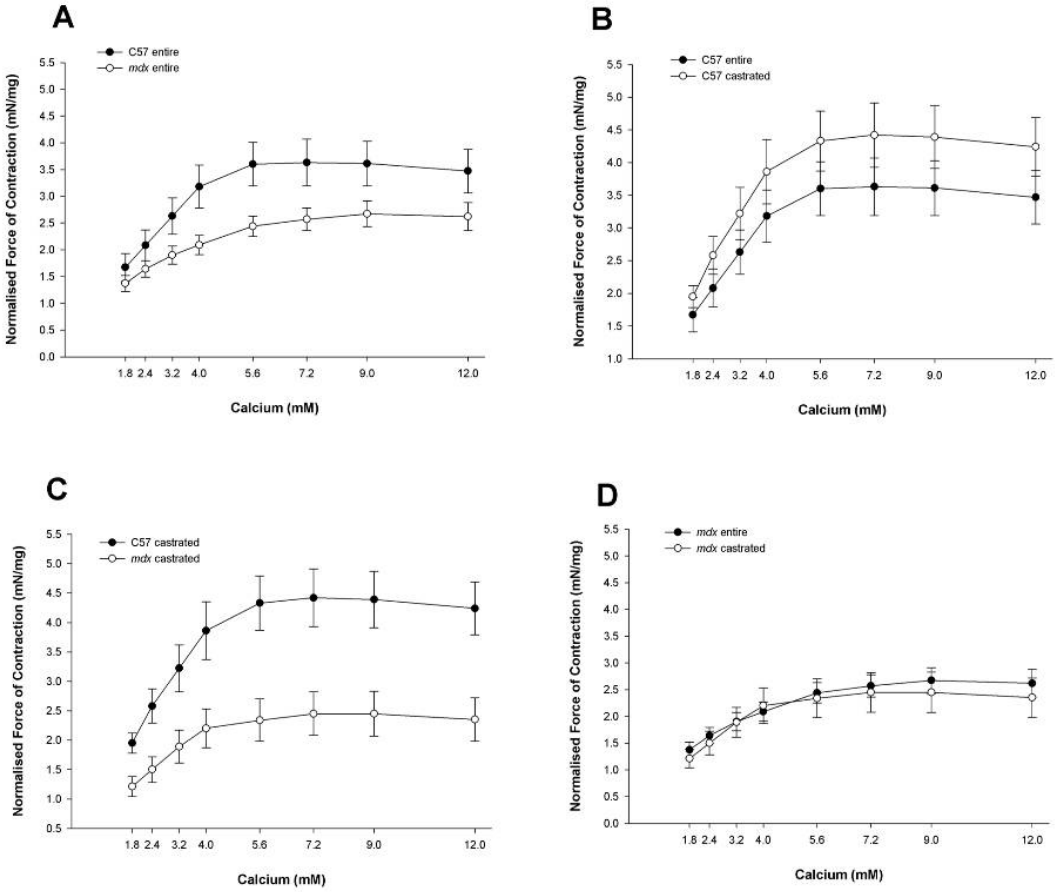


Figure 5-2 Concentration response curves to calcium in entire and castrated male mice.

(A) C57 entire versus *mdx* entire (B) C57 entire versus C57 castrated (C) C57 castrated versus *mdx* castrated and (D) *mdx* entire versus *mdx* castrated. * p<0.05, ** p<0.01. age = 6 months. n = 6-7 mice.

There was no difference in maximum FOC (normalised for CSA) between strains of entires or between entires and castrates of the same strain. The *mdx* castrates had a 45% lower maximum FOC (normalised) than C57 castrates ($p<0.05$) (Table 5-2).

Table 5-2 Cardiac function of entire and castrated male mice.

mouse strain status	C57 entire	C57 castrated	*mdx* entire	*mdx* castrated
BW (g) [1]	30.74±0.54	23.78±0.83 ††	31.82±0.76	24.67±1.01 ††
LA weight (mg)	1.06±0.11	0.81±0.11	1.12±0.13	1.02±0.10
FOC (mN)	1.62±0.16	1.48±0.11	1.54±0.22	1.20±0.17
FOC (normalised for LA weight) (mN/mg)	1.67±0.27	1.95±0.18	1.37±0.16	1.21±0.18*
maximum FOC (mN)	3.72±0.36	3.44±0.46	3.08±0.41	2.47±0.42
maximum FOC (normalised for LA weight) (mN/mg)	3.72±0.45	4.46±0.51	2.74±0.27	2.47±0.41*

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice. [1]this information is described in Figure 5-1.

5.3 Influence of castration on skeletal muscle morphometry

Mdx entires and castrates had a 30% and 29% higher EDL weight than C57 entires and castrates (p<0.01). *Mdx* and C57 entires had a 54% and 52% higher EDL weight than their respective castrates (p<0.01). *Mdx* entires and castrates had a 27% and 26% higher EDL CSA than C57 entires and castrates (p<0.01). *Mdx* and C57 entires had a 51% and 50% higher EDL CSA than their respective castrates (p<0.05). *Mdx* entires and castrates had a 23% and 32% higher SOL weight than C57 entires (p<0.05) and castrates (p<0.01). *Mdx* and C57 entires had a 32% (p<0.05) and 41% (p<0.01) higher SOL weight than their respective castrates. There was no difference in SOL CSA within mouse strains of entires or castrates. *Mdx* and C57 entires had a 28% (p<0.05) and 36% (p<0.01) higher SOL CSA than their respective castrates (Table 5-3).

Table 5-3 Skeletal muscle morphometry of entire and castrated male mice.

mouse strain status	C57 entire	C57 castrated	*mdx* entire	*mdx* castrated
BW (g) [1]	30.74±0.54	23.78±0.83††	31.82±0.76	24.67±1.01††
EDL weight (mg)	12.79±0.66	8.43±0.32††	16.66±0.99**	10.85±0.39**††
EDL Lo (mm)	10.19±0.35	10.00±0.25	10.30±0.25	10.24±0.41
EDL CSA (mm^2)	2.73±0.18	1.82±0.10††	3.46±0.18*	2.29±0.12*††
SOL weight (mg)	10.47±0.52	7.40±0.39††	12.89±1.03*	9.77±0.30**†
SOL Lo (mm)	9.21±0.21	8.89±7.96	9.68±0.28	9.60±0.38
SOL CSA (mm^2)	1.52±0.08	1.12±0.07††	1.76±0.11	1.37±0.08†

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice. [1]this information is described in Figure 5-1.

5.4 Influence of castration on EDL characteristics

5.4.1 EDL function

Castration resulted in a lower final BW in both *mdx* and C57 mice ($p<0.01$). This was reflected by a proportional decrease in the weights of their EDL muscles. Despite the lower muscle weights, there were no differences in absolute twitch or tetanic forces between any of the four groups tested. However, when the forces are normalised to CSA, castrated mice had a significantly larger tetanic force in both C57 (+52%) and *mdx (*+25%) (Table 5-4).

Table 5-4 EDL skeletal muscle function of entire and castrated male mice.

mouse strain status	C57 entire	C57 castrated	*mdx* entire	*mdx* castrated
BW (g) [1]	30.74±0.54	23.78±0.83††	31.82±0.76	24.67±1.01††
Pt (mN)	39.54±2.64	34.24±2.21	43.64±3.90	36.51±1.31
Po (mN)	155.74±12.85	165.43±7.07	179.25±20.76	166.00±9.77
sPt (mN/mm^2)	15.32±1.71	18.83±0.67	12.87±1.24	16.20±1.14
sPo (mN/mm^2)	60.58±7.19	92.01±4.58††	53.55±6.98	73.53±7.26*
Po:Pt	3.97±0.23	4.93±0.32†	4.09±0.30	4.50±0.21

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice. [1]this information is described in Figure 5-1.

5.4.2 EDL histology

For both entire and castrated mice, *mdx* had a significantly ($p<0.01$) higher percentage of EDL CNFs than C57 equivalents. However, castration did not produce a significant difference in CNFs within either mouse strain (Figure 5-3).

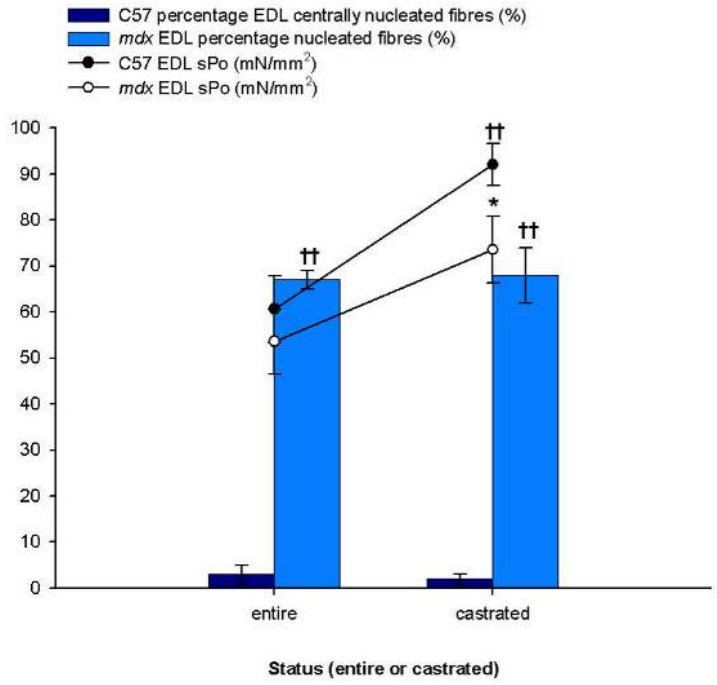


Figure 5-3 EDL percentage CNFs and sPo for entire and castrated male mice.

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice.

There was no difference in EDL cell diameters between strains of entire or castrated mice. Interestingly, castration produced a significant decrease in cell diameter in *mdx* mice ($p<0.05$) that was not evident in C57s (Figure 5-4).

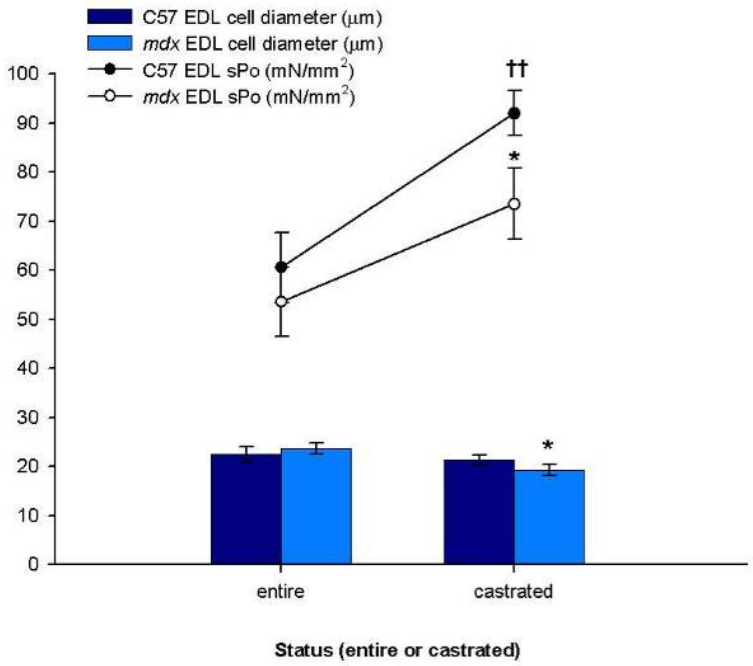


Figure 5-4 EDL cell diameter and sPo between strains and for entire and castrated male mice.

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice.

5.5 Influence of castration on SOL characteristics

5.5.1 SOL function

Mdx entires had a 25% higher Pt than C57 entires ($p<0.05$) whilst there was no difference in Pt between C57s, *mdx*, or castrates. *Mdx* entires had a 43% higher Po than C57 entires ($p<0.01$). There was no difference in Po in castrates between strains. *Mdx* entires had a 33% higher Po than *mdx* castrates ($p<0.05$) whereas there was no difference in Po as a result of castration in C57s. There was no difference in sPt between strains of entires or between entires and castrates of the same strain.

Table 5-6 shows no difference in sPo or in Po:Pt between strains of entires or castrates or between *mdx* entires and *mdx* castrates. C57 castrates had a 45% higher sPo than C57 entires ($p<0.05$) (Table 5-5).

Table 5-5 SOL muscle function in entire and castrated male mice.

mouse strain status	C57 entire	C57 castrated	*mdx* entire	*mdx* castrated
BW (g) [1]	30.74±0.54	23.78±0.83††	31.82±0.76	24.67±1.01††
Pt (mN)	21.09±1.95	20.36±1.80	26.35±1.27*	20.81±3.29
Po (mN)	126.75±11.12	133.14±14.15	181.52±7.40**	136.73±21.97†
sPt (mN/mm^2)	14.34±1.54	18.59±1.83	15.39±1.08	15.35±2.54
sPo (mN/mm^2)	85.73±8.44	124.54±17.11†	104.84±4.47	100.88±17.18
Po:Pt	6.09±0.31	6.59±0.54	6.99±0.39	6.48±0.39

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice. [1]this information is described in Figure 5-1.

5.5.2 SOL histology

For both entire and castrated mice, *mdx* mice had a significantly higher percentage of CNFs (p<0.01). Castration produced no difference in SOL percentage of CNFs in either mouse strain (Figure 5-5).

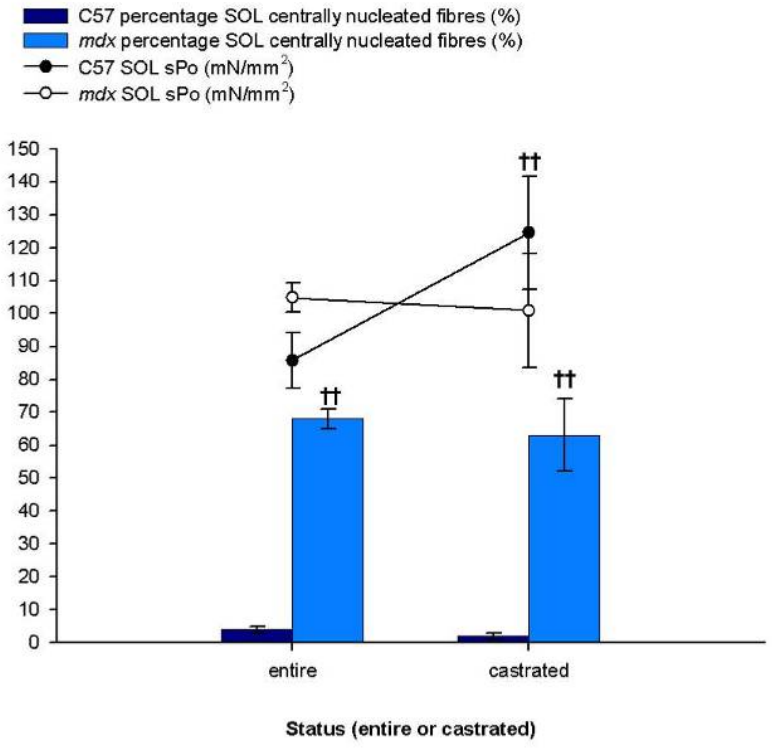


Figure 5-5 SOL percentage CNFs and sPo between strains and for entire and castrated male mice.

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice.

There was no difference in SOL cell diameters between castrated and entire mice of the same mouse strain or between strains of the same status (Figure 5-6).

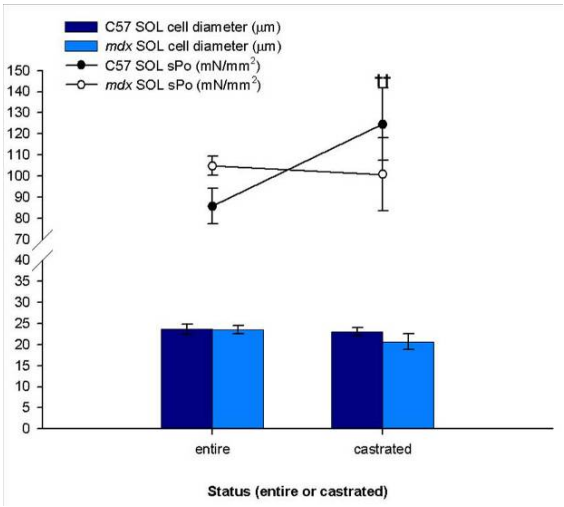


Figure 5-6 SOL cell diameters and sPo between strains and for entire and castrated male mice.

*** p<0.05, ** p<0.01 between strain comparison with equivalent treatment;**
† p<0.05, †† p<0.01 treatment comparison with same strain.
age = 6 months. n = 6-7 mice.

5.6 Discussion

Extremely limited data is available on the effects of castration on cardiac muscle function in the *mdx* mouse model of DMD. Studies on the cardiac effects of castration in male rats have shown various results. Scheuer *et al.* (1987) evaluated cardiac performance in isolated perfused rat hearts. Stroke work, ejection fraction, fractional shortening, and mean velocity of circumferential fibre shortening were all lowered in castrates. Papillary muscle studies showed increases in time to peak tension and one-half relaxation time and isotonic shortening studies showed decreased velocity of shortening in castrates (Scheuer *et al.* 1987). Golden *et al.* (2003) showed alterations in cardiac myocytes four months post-castration and provided the first evidence that androgens regulate functional expression of L-type Ca^{2+} channels and the sodium-Ca^{2+} exchanger in isolated rat ventricular myocytes (Golden *et al.* 2003). Testosterone treatment of castrated animals completely reversed the effects of castration allowing Golden *et al* (2003) to conclude that testosterone may play a role in modulating cardiac performance in males

and thereby contribute to previously observed differences in cardiac function between males and females.

In the spontaneously hypertensive rat (SHR) model males have been shown to have higher blood pressure than females (Masubuchi *et al.* 1982). Sainz *et al.* (2004) investigated the effect of castration on N_{ω}-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats and found that castration lowered blood pressure. Masubuchi (1982) demonstrated the same physiological effect of castration in SHRs. Finally, Schaible (1984) demonstrated that both left ventricular filling and left ventricular function were impaired in hearts of castrated male rats (Schaible *et al.* 1984).

Studies by Cattanach *et al.* (1977) and Sciote *et al.* (2001) showed that hypogonadal male mice were of significantly lower BW than unaffected males (Cattanach *et al.* 1977; Sciote *et al.* 2001). In this trial, castration caused a significantly lowered BW at six months within both *mdx* and C57 mouse strains whilst there was no difference in BWs between entires or castrates in both mouse strains.

The hearts of C57 entires were heavier than *mdx* entires and, although the entires of both strains were heavier than strain-equivalent castrates, normalisation of HW to BW negated the effect in *mdx*. Castration appeared to have a greater impact on cardiac tissue weights in C57s than *mdx*. For instance; BW, HW, HW%, RA weight, RV weight, and LV+S weights were all significantly lowered in C57 castrates compared to entires. Castration only produced significantly lowered BW, HW, and LV+S weights in *mdx*.

Castration caused no significant functional cardiac effect between mouse strains and yet *mdx* castrates showed consistently lower forces than entires whereas C57 castrates showed higher LA forces than entires. Despite showing no functional difference in basal or maximum LA FOC between strains, the cardiac results demonstrated that the cardiac response to Ca^{2+} was altered in *mdx* castrates compared to C57 castrates. Therefore, this is the first study to demonstrate that castration produces a differential cardiac response to exogenous Ca^{2+} between *mdx* and C57 mouse strains.

The specific role of hormones in skeletal muscle growth and function remains unclear. It is known that skeletal muscle size is influenced

by sex hormones and skeletal muscle growth is maintained by the balance of nutrition, exercise, peptide hormones, and sex hormones. Androgens (predominantly testosterone and dihydrotestosterone (DHT)) induce skeletal muscle hypertrophy in individual muscle fibres and this has been clearly demonstrated by the endogenous depletion of testosterone through surgical castration of animal models (Sciote *et al.* 2001). The physiological role of testosterone in skeletal muscle is believed to be secondary to that of GH whose effects are mediated by IGFs (Florini, Ewton and Coolican 1996). However, testosterone can act directly on skeletal muscle to increase IGF-1 expression and mediate muscle hypertrophy (Urban 1999). Furthermore, both oestradiol and testosterone (after conversion to oestradiol by aromatase) can promote GH release via non-genomic pathways (Sciote *et al.* 2001).

It has also been shown that castration differentially affects skeletal muscle mass and androgen receptor (AR) levels in rat skeletal muscles (Antonio, Wilson and George 1999). A study of castrated versus entire bovines concluded that AR expression is muscle-specific and may be modulated by circulating testicular hormones and that divergent hormone sensitivities in skeletal muscles may account for the differential growth rates of individual muscles (Brandstetter *et al.* 2000).

Kaminski (1992) argued that fast-twitch, extremity muscles (such as EDL), are affected preferentially in DMD (Kaminski *et al.* 1992). The results of this study indicate that EDL and SOL muscles respond differently to the effects of castration in the *mdx* mouse compared to C57 controls. Entire *mdx* had heavier BWs, EDLs, and SOLs than entire C57s. Castration caused a decrease in BWs, EDL muscle weights, SOL muscle weights and subsequent muscle CSAs in both *mdx* and C57 mouse strains. Despite lowered EDL muscle weights in castrates results actually showed that castrated mice generated significantly larger EDL tetanic forces (as reflected by sPo values) than entires in both *mdx* (+37%) and C57s (+52%). SOL function studies showed that castration resulted in an increase in sPo values for C57s (+45%) but not in *mdx* mice. Castration produced a functional improvement in fast-twitch (EDL) muscles in both mouse strains but no functional improvement in slow-twitch (SOL) muscles in *mdx*.

Castration studies examining the influence of androgens on skeletal muscle fibre-types in mice have produced variable results (Sciote *et al.* 2001). Vaughan *et al.* (1974) found that castration in mice produced significant fibre-type changes in the SOL muscle. They concluded that, although muscle fibre-type is probably determined genetically and well established post-puberty, the proportion of different fibre-types can be modified by androgens (Vaughan *et al.* 1974). However, Jiang *et al.* (1989) found that castration, despite causing decreased FOC in EDL muscles in ReJ 129 mice, actually produced no difference in muscle fibre-types (Jiang and Klueber 1989).

Characteristics of skeletal muscle fibre-types are influenced by androgen (predominantly testosterone and DHT) and oestrogen hormones (Sciote *et al.* 2001). A single research paper (Salimena, Lagrota-Candido and Quirico-Santos 2004) has addressed histological parameters in *mdx* castrates. Salimena *et al.* (2004) castrated *mdx* and C57 mice at 21 days of age. The animals were then euthanased four weeks after surgery (at seven weeks of age) and results showed that castrated males did not show changes in the pattern of skeletal muscle regeneration and fibrosis as evidenced by the morphometry and histological findings in the gastrocnemius (mixed type-I and type-II fibres) muscles.

For entire and castrated mice, *mdx* had exponentially higher percentages of CNFs in both EDL and SOL skeletal muscles compared to C57s. Castration did not produce a significant change in percentage CNFs in either mouse strain. Interestingly, castration produced a 17% decrease in EDL cell diameter in *mdx*, not seen in C57s.

In SOL muscles there was no cell diameter difference between castrates and entires or within strains. All groups (both castrated and entire) had muscle cell diameters below 25 μm and no correlation was found between cell diameter and skeletal muscle function for EDL or SOL muscles in either *mdx* or C57s.

This project was unique and the first to report that castration alters the response to exogenous Ca^{2+} in *mdx* hearts, that castration results in different functional responses in fast-twitch (EDL) and slow-twitch (SOL) *mdx*

muscles, and that castration produced a significant decrease in EDL cell diameters in *mdx* mice.

6 OESTROGEN TRIAL

6.1 Influence of oestrogen on body weight

Figure 6-1 shows a significant decrease in *mdx* male mouse BW at a dose of 0.16 mg/kg/day oestrogen compared to controls ($p<0.01$). Conversely, at doses above and below this there was no change in mouse BW with oestrogen treatment.

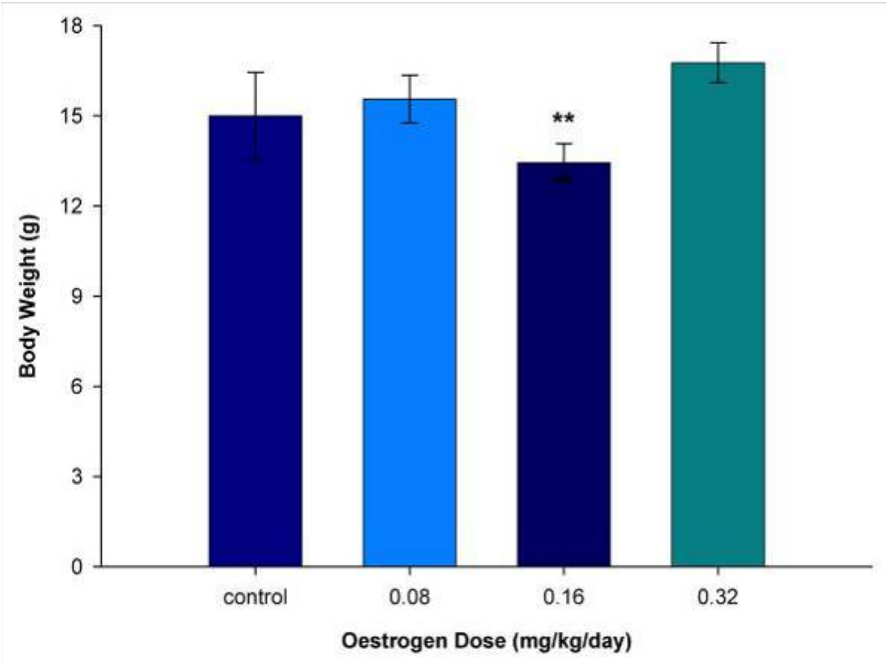


Figure 6-1 Body weights at varying oestrogen doses in male mice.

Comparison of BWs at varying oestrogen doses in *mdx* male mice.
*** p<0.05, ** p<0.01; age = 35 days old. n = 7-8 mice.**

6.2 Influence on oestrogen on testes weight

Figure 6-2 shows that oestrogen caused a trend towards decreased testes weight, significant only at 0.16 mg/kg/day oestrogen ($p<0.01$) where a 34% decrease in testes weight was evident.

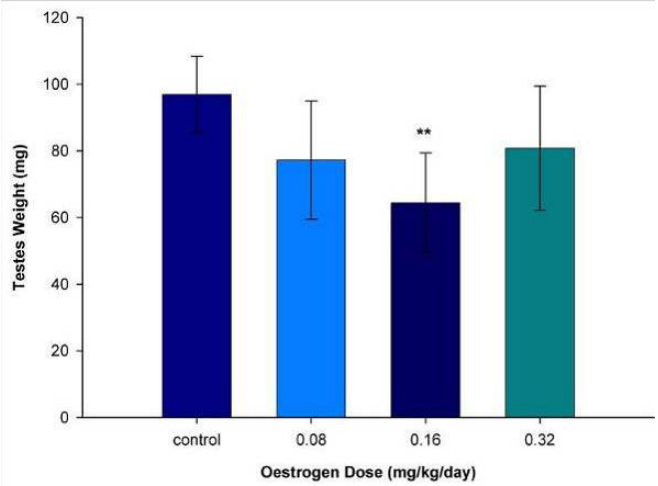


Figure 6-2 Testes weights at varying oestrogen doses in male mice.

Comparison of testes weights at varying oestrogen doses in *mdx* male mice. * p<0.05, ** p<0.01; age = 35 days old. n = 7-8 mice.

6.3 Influence of oestrogen on cardiac characteristics

6.3.1 Cardiac morphometry

A dose of 0.08 mg/kg/day oestrogen caused no significant change in body and cardiac weights. A dose of 0.16 mg/kg/day caused significantly decreased values (p<0.01) across all measured parameters other than RV weight which remained unchanged. Statistical significance was lost at a dose of 0.32 mg/kg/day (the exception being HW% which remained significantly (p<0.01) lower than that of controls (Table 6-1).

Table 6-1 Body and cardiac weights at varying oestrogen doses in male mice.

mouse strain oestrogen (mg/kg/day)	*mdx* control	*mdx* 0.08	*mdx* 0.16	*mdx* 0.32
BW (g) [1]	15.00±1.45	15.56±0.79	13.44±0.64**	16.77±0.66
HW (mg)	71.84±5.90	74.18±3.03	58.60±3.15**	74.34±4.58
HW% (%)	0.49±0.01	0.48±0.01	0.44±0.01**	0.44±0.01**
RA weight (mg)	2.16±0.15	2.14±0.47	1.80±0.17**	2.03±0.36
LA weight (mg)	0.84±0.13	0.88±0.10	0.55±0.06**	1.11±0.86
RV weight (mg)	14.49±1.32	16.83±1.47	14.71±1.10	16.90±1.96
LV+S weight (mg)	54.35±4.45	54.34±3.04	41.54±2.23**	54.30±2.75

Comparison of body and cardiac muscle weights at varying doses of oestrogen in *mdx* mice. * p<0.05, ** p<0.01; age = 35 days old (prepubertal). n = 7-8 mice.
[1]this information is described in Figure 6-1.

6.3.2 Cardiac function

Figure 6-3 depicts a trend of increased basal FOC of LA in response to oestrogen treatment. Significant force increases occurred at doses of 0.16 mg/kg/day ($p<0.01$) and 0.08 mg/kg/day ($p<0.05$) compared to controls.

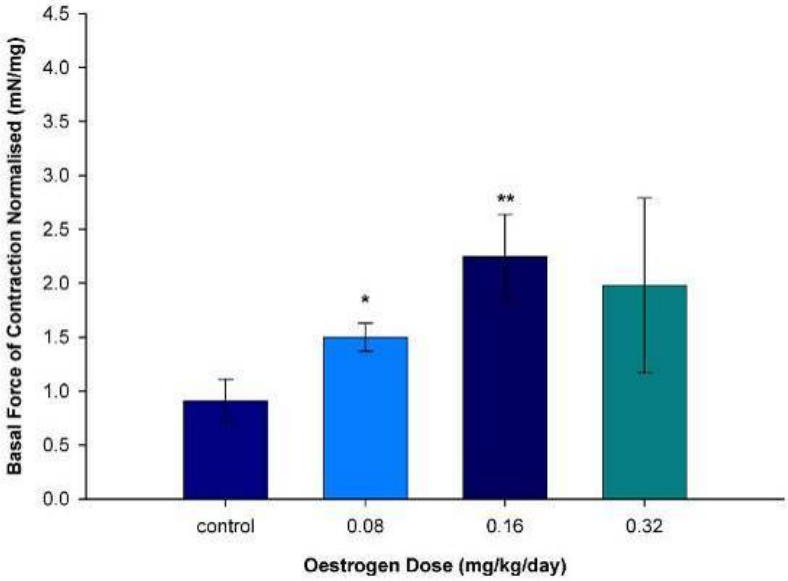


Figure 6-3 Basal FOC of the LA (normalised for LA weight) at varying oestrogen doses in male mice.

*** p<0.05, ** p<0.01; age = 35 days old. n = 7-8 mice.**

There was no difference in Ca^{2+} CRCs of control C57s compared to *mdx* controls (Figure 6-4). However, a significant increase ($p<0.01$) in FOC of LA (normalised for LA weight) occurred at an oestrogen dose of 0.16 mg/kg/day in *mdx*.

As with basal FOC, maximum FOC of LA (normalised for LA weight) increased with oestrogen treatment. Figure 6-5 shows the increase in force was only significant ($p<0.01$) at a dose of 0.16 mg/kg/day oestrogen compared to controls.

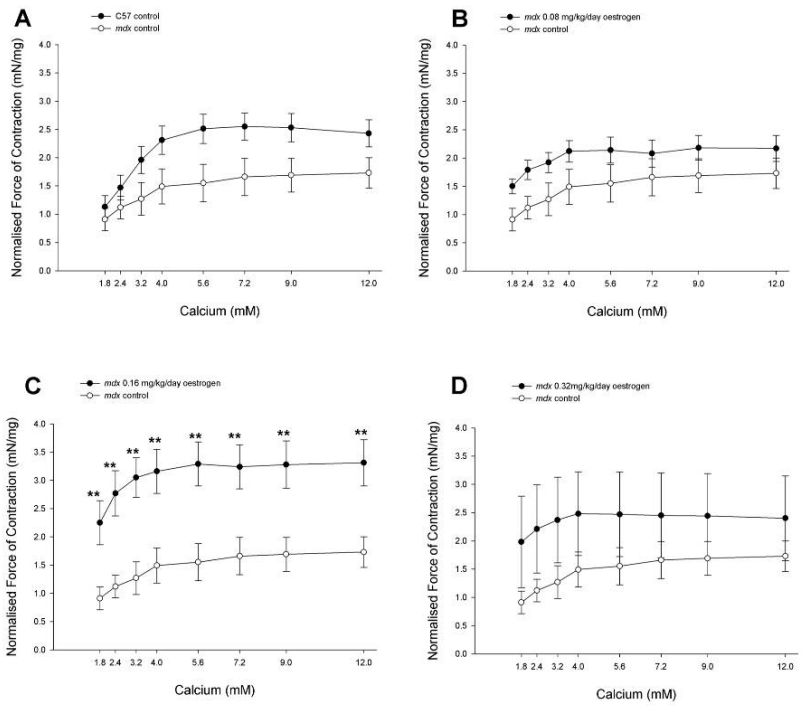


Figure 6-4 Ca^{2+} CRCs at varying doses of oestrogen in male mice.

*** p<0.05, ** p<0.01; A = controls, B = 0.08 mg/kg/day oestrogen, C = 0.16 mg/kg/day oestrogen, and D = 0.32 mg/kg/day oestrogen. age = 35 days old. n = 7-8 mice.**

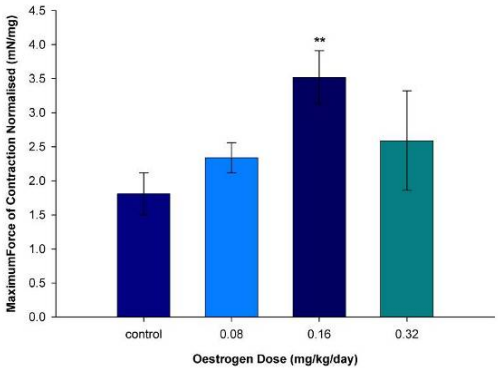


Figure 6-5 Maximum FOC of the LA (normalised for LA weight) at varying oestrogen doses in male mice.

*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice.**

6.4 Influence of oestrogen on skeletal muscle morphometry

There were no significant differences between skeletal muscle parameters depicted in Table 6-2 in mice treated at dosage rates of 0.08 or 0.32 mg/kg/day oestrogen compared to controls. The exception to this was

SOL Lo at 0.08 mg/kg/day but this did not impact on the resultant CSA. Interestingly, at a dose of 0.16 mg/kg/day, all parameters were significantly lower than that of controls but this may simply be reflective of the lowered BW at the same dosage.

Table 6-2 EDL and SOL muscle morphometry at varying oestrogen doses in male mice.

treatment (mg/kg/day oestrogen)	control	0.08	0.16	0.32
BW (g) [1]	15.00±1.45	15.56±0.79	13.44±0.64**	16.77±0.66
SOL weight (mg)	4.89±0.62	4.81±0.40	3.41±0.19**	4.09±0.66
SOL Lo (mm)	5.78±0.27	5.92±0.11*	5.45±0.16*	5.50±0.13
SOL CSA (mm^2)	0.78±0.07	0.76±0.06	0.59±0.03**	0.71±0.12
EDL weight (mg)	5.53±0.57	6.75±0.61	4.99±0.37**	6.70±0.56
EDL Lo (mm)	3.69±0.06	3.76±0.12	3.54±0.06**	3.78±0.09
EDL CSA (mm^2)	1.40±0.13	1.67±0.11	1.33±0.09*	1.68±0.15

*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice. [1]this information is described in Figure 6-1.**

6.5 Influence of oestrogen on EDL characteristics

6.5.1 EDL function

In contrast to the results found in SOL muscles as shown in Table 6-3, EDL muscle parameters measured were not significantly different to control values compared to any oestrogen doses. This applied to a dose of 0.16 mg/kg/day oestrogen despite the significantly lower BW ($p<0.01$) in these treated mice compared to controls (Figure 6-1).

Table 6-3 EDL muscle function at varying oestrogen doses in male mice.

treatment (mg/kg/day oestrogen)	control	0.08	0.16	0.32
BW (g) [1]	15.00±1.45	15.56±0.79	13.44±0.64**	16.77±0.66
Pt (mN)	18.59±2.26	26.20±3.16	18.48±1.09	24.32±3.50
Po (mN)	79.46±10.93	133.46±17.29	88.19±10.25	116.81±16.75
sPt (mN/mm^2)	13.64±1.53	15.32±1.09	14.14±0.71	14.73±2.11
sPo (mN/mm^2)	58.20±7.43	77.76±7.00	67.11±6.45	70.66±10.06
Po:Pt	4.21±0.15*	5.03±0.16	4.71±0.37	4.92±0.30

*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice. [1]this information is described in Figure 6-1.**

6.5.2 EDL histology

Oestrogen treatment resulted in a non-significant trend of decreasing percentages of CNFs in EDL muscles with oestrogen treatment compared to controls (Figure 6-6).

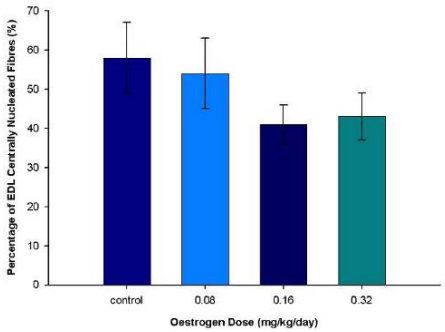


Figure 6-6 EDL percentage of CNFs at varying oestrogen doses in male mice.
*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice.**

Oestrogen treatment resulted in a trend towards decreasing EDL cell diameters. However, this was only significant (p<0.05) at an oestrogen dose of 0.16 mg/kg/day (Figure 6-7).

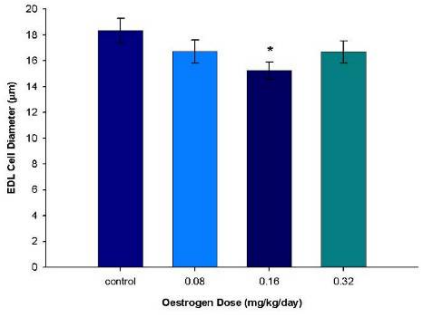


Figure 6-7 EDL cell diameter at varying oestrogen doses in male mice.
*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice.**

6.6 Influence of oestrogen on SOL characteristics

6.6.1 SOL function

A dose of 0.08 mg/kg/day oestrogen produced no functional effect in SOL muscles compared to controls. However, despite a significantly lowered ($p<0.01$) body weight, SOL weight, and SOL CSA (Table 6-4) in 0.16 mg/kg/day oestrogen-treated mice compared to controls there was a significant increase in Pt ($p<0.01$), Po ($p<0.01$), sPt ($p<0.05$), and Po:Pt ($p<0.01$), together with a non-significant increase in sPo in treated mice (Table 6-4). Interestingly, despite there being no significant differences in the SOL parameters measured in 0.32 mg/kg/day treated mice compared to controls, this oestrogen dose produced a significant increase ($p<0.05$) in sPt and sPo compared to controls.

Table 6-4 SOL muscle function at varying oestrogen doses in male mice.

treatment (mg/kg/day oestrogen)	control	0.08	0.16	0.32
BW (g) [1]	15.00±1.45	15.56±0.79	13.44±0.64**	16.77±0.66
Pt (mN)	17.22±1.06	16.43±1.61	15.11±0.72**	18.82±1.15
Po (mN)	88.68±5.35	89.06±7.71	71.88±3.10**	96.12±6.48
sPt (mN/mm^2)	24.00±3.37	21.64±2.00	26.11±1.99*	30.84±4.59*
sPo (mN/mm^2)	120.85±14.54	117.54±8.79	124.91±10.63	155.46±21.84*
Po:Pt	5.22±0.34	5.51±0.18	4.78±0.17**	5.10±0.15

*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice. [1]this information is described in Figure 6-1.**

6.6.2 SOL histology

Oestrogen treatment resulted in a 41% decrease in SOL CNFs at a dose of 0.16 mg/kg/day ($p<0.05$) compared to controls (Figure 6-8).

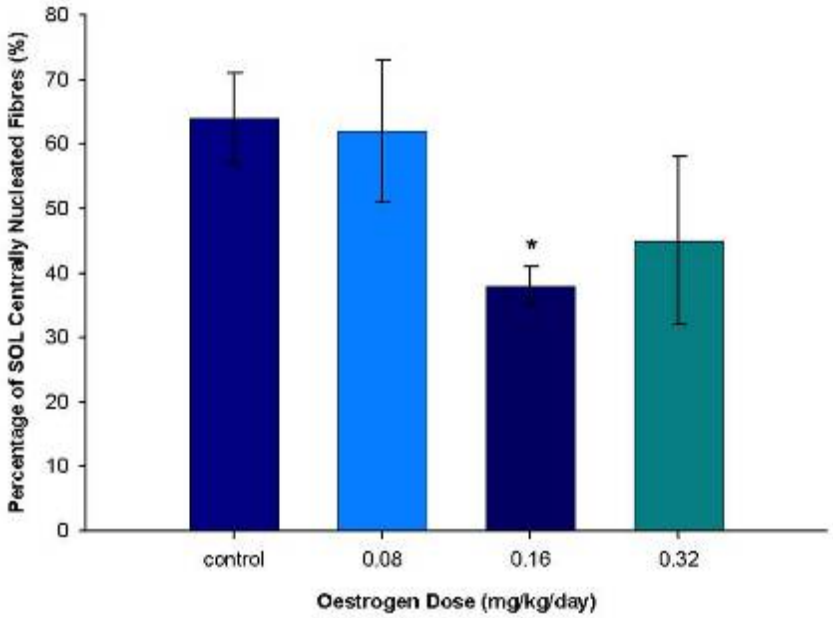


Figure 6-8 SOL percentage of CNFs at varying oestrogen doses in male mice.
*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice.**

Oestrogen treatment produced no significant difference in the SOL cell diameters compared to controls (Figure 6-9).

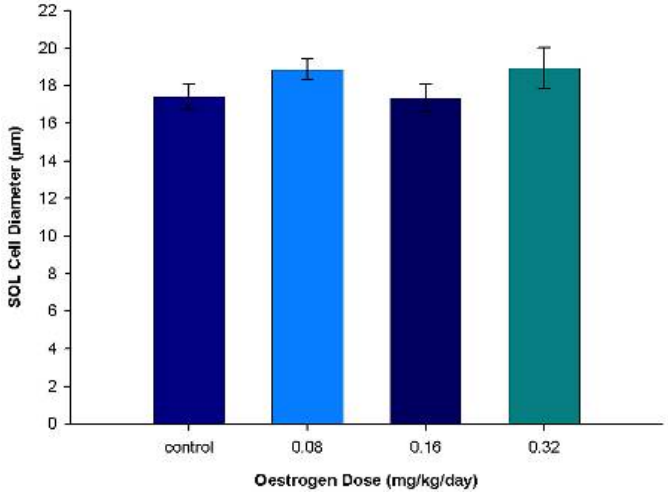


Figure 6-9 SOL cell diameter at varying oestrogen doses in male mice.
*** p<0.05, ** p<0.01; mouse strain = *mdx*. age = 35 days old. n = 7-8 mice.**

6.7 Discussion

Oestrogens have numerous significant physiological functions in humans and beneficial effects have been noted on the skeletal, nervous, and CV systems. Oestrogens produce positive effects on skeletal growth and bone maturation. Nervous system and brain oestrogenic effects include improved cognitive function, co-ordination of movement, and pain responses. Oestrogens are also known to produce cardioprotective effects which include improvements in lipid profiles, fat distribution, endocrine/paracrine factors produced by the vascular wall (i.e. endothelins and NO), blood platelets, inflammatory factors, and coagulation mechanisms (Gooren and Toorians 2003).

In males, oestrogens are predominantly the products of peripheral aromatisation of testicular and adrenal androgens (Figure 6-10). In man, testosterone synthesis declines with ageing whilst levels of total plasma oestradiol do not decline. This is thought to be due to an increase in adipose tissue (cholesterol being the substrate of peripheral aromatisation) and an associated increase in aromatase activity. However, as men age, bioavailable oestrogens may decline due to an increase in sex hormone binding globulin (Gooren and Toorians 2003).

Usuki *et al.* (1989) found that in DMD patients the baseline levels of serum oestrone were significantly higher than in age-matched unaffected boys. Serum baseline testosterone, LH and FSH levels were all essentially normal, but FSH levels were significantly lowered. This led to the presumption that the elevated serum oestrone levels resulted from increased peripheral conversion of testosterone to oestrogen in DMD (Figure 6-10) (Usuki *et al.* 1989). This is interesting in that, despite the progressive pathology of DMD, affected boys may actually be physiologically protected by increased oestrone levels, suggesting that disease progression may actually be worse in the absence of increased peripheral aromatisation of testosterone.

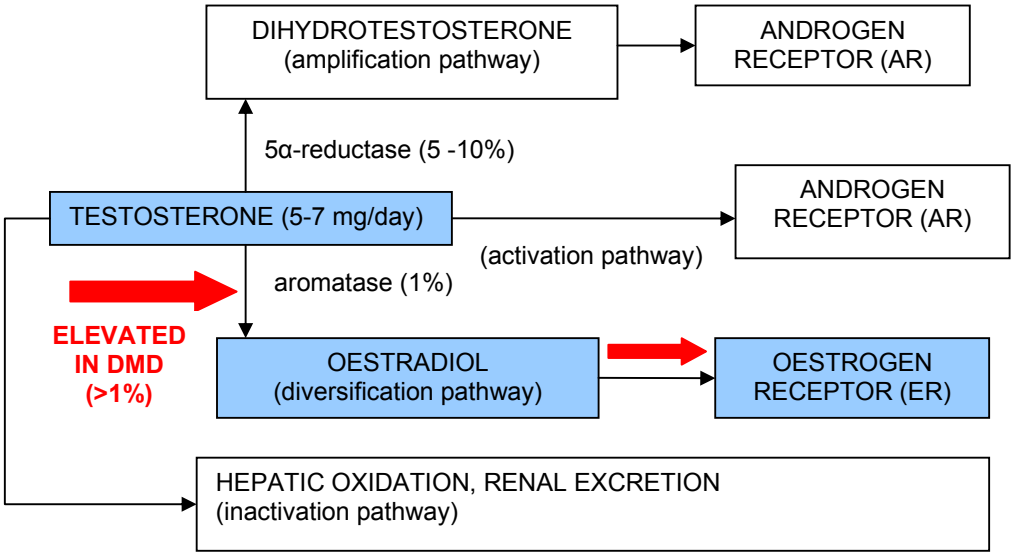


Figure 6-10 Pathways of testosterone action and the conversion to oestradiol

Rudman *et al.* (1972) reported that DMD patients were hyper-responsive to the anabolic effects of oestrogens. They speculated that this may be due to the summation of the weak intrinsic anabolic effect of oestrogen with a peripheral inhibition (by oestrogen) of the catabolic effect of endogenous GH in dystrophic muscle cells and concluded that oestrogens may have therapeutic potential in DMD (Rudman *et al.* 1972).

All DMD patients are male except for a small percentage of female patients whose disease condition is a direct consequence of chromosomal abnormalities (Nonaka 1998). Several cases of DMD affected females with X-autosome translocation have been reported in the literature (Boyd and Buckle 1986; Boyd *et al.* 1986). Zatz *et al.* (1981) documented the case of a female DMD patient that commenced menstruation at 12 years of age, underwent a normal puberty, and died at age 16 (Zatz *et al.* 1981). This suggested that the oestrogen surge associated with female puberty did not arrest the dystrophic process in the female DMD patient. However, these results failed to eliminate the potential benefits of oestrogen therapy in DMD.

There is strong support in the literature of the cardioprotective effects of oestrogen in humans (Kalin and Zumoff 1990; Grohe *et al.* 1997; Zhai *et al.* 2000; Gooren and Toorians 2003) and it is known that women with functional ovaries have a lower CV disease risk than both men and

postmenopausal women (Ren *et al.* 2003). A novel study found that in isolated perfused hearts, female rats with aortocaval fistula-induced chronic volume overload which were fed a diet of phyto-oestrogens had increased ventricular contractility compared to controls (Gardner, Brower and Janicki 2005). In male congestive heart failure (CHF) patients, oestrogen administration improves the indices of cardiac systolic performance and decreases pulmonary and systemic vascular resistance. These findings imply a beneficial effect of oestrogen in selected patients with chronic heart failure (Adamopoulos *et al.* 2002).

Cardiac myocytes contain functional oestrogen receptors (ERs) (Grohe *et al.* 1997) and oestrogen exerts CV effects via both genomic and non-genomic pathways (Curl, Wendt and Kotsanas 2001). Johnson *et al.* (1997) studied ER knockout mice and demonstrated that cardiac L-type Ca^{2+} channels are regulated by the ER. This suggests that decreased oestrogen may result in an increase in the number of cardiac L-type Ca^{2+} channels, abnormalities in cardiac excitability, and increased risk of cardiac arrhythmia and CV disease (Johnson *et al.* 1997). Curl, Wendt and Kotsanas (2001) showed that, under a variety of conditions, male rat cardiac myocytes had consistently higher $[Ca^{2+}]_i$ levels than females.

Oestrogen has a potential role in limiting Ca^{2+} entry into myocardial cells. Currently known non-genomic cardiac effects of oestrogen include protection against Ca^{2+} overload through the reduced expression of L-type Ca^{2+} channels (Curl, Wendt and Kotsanas 2001), the inhibition of L-type Ca^{2+} channel currents (Meyer *et al.* 1998), and downregulation of β-adrenergic responsiveness (Fregly and Thrasher 1977), all of which predictably lead to negative inotropic effects (Sitzler *et al.* 1996). However, in this trial, oestrogen caused a positive effect on LA contractility. Basal FOC of LA was increased at all oestrogen doses and was significant at 0.08 and 0.16 mg/kg/day although not significant at 0.32 mg/kg/day due to the large SEM values. The maximum LA FOCs mirrored basal FOC results, however only 0.16 mg/kg/day oestrogen treatment produced statistical significance. Response to Ca^{2+} was not different between controls or for 0.08 mg/kg/day or 0.32 mg/kg/day treated mice but a highly significant increase in forces occurred for all Ca^{2+} concentrations at 0.16 mg/kg/day oestrogen treatment.

Unlike the control C57 heart, the dystrophin deficient *mdx* heart is in a state of Ca^{2+} overload (Alloatti *et al.* 1995) as a result of increased Ca^{2+} influx into cardiac cells (Lu and Hoey 2000b; Woolf *et al.* 2006) and altered Ca^{2+} handling. Reducing $[Ca^{2+}]_i$ may therefore improve cardiac function in DMD and the *mdx* heart. However, two human clinical trials with compounds which lower $[Ca^{2+}]_i$ have failed to substantiate this theory. Treatment with dantrolene (which inhibits Ca^{2+} release from the SR) provided no functional improvement in DMD boys after two years treatment (Bertorini *et al.* 1991) and a double-blind clinical trial of the Ca^{2+} antagonist diltiazem proved to have no significant cardiac benefits in DMD boys after 24 to 32 months (Bertorini *et al.* 1988). Results of this study found a positive inotropic effect with oestrogen treatment which cannot be explained via the mechanisms by which oestrogen lowers $[Ca^{2+}]_i$. Therefore, other oestrogen-induced physiological mechanisms must be considered to explain the positive cardiac inotropic results. These pathways involve the increase in cardiac eNOS expression and the resulting decrease in caveolin-3 expression and/or increased NO production as discussed below.

There is extensive research interest in the role of NOS and NO in DMD and the *mdx* mouse model of the disease. Nitric oxide synthases (NOS) are the products from three distinct genes; neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelial (eNOS or NOS3). All NOS isoforms are expressed in the heart: nNOS in cardiac conduction tissue and intracardiac neurones; iNOS in virtually all cardiac cells, often in conjunction with the expression of inflammatory cytokines; and eNOS is expressed in coronary endothelium, endocardium, and cardiac myocytes. It has been shown that eNOS regulates the tone of vascular smooth muscle cells; the permeability and platelet adhesion of endothelial cells; and the receptor-effector coupling, energetics, contractility, and apoptosis of cardiomyocytes (Song *et al.* 1996).

Dystrophin anchors F-actin to the DGC, which is associated with neuronal nitric oxide synthase (nNOS). The enzyme nNOS (which produces NO) binds to the DGC in muscle and NO (a free radical) has been directly implicated in aspects of the pathogenesis of DMD. For example, NO stimulates noradrenaline (NA) and acetylcholine (ACh) release from rat

hippocampal cells (Lonart, Wang and Johnson 1992) leading to the suggestion that the conduction defects seen in dystrophic hearts may be a result of decreased nNOS activity (Bia *et al.* 1999).

Sears *et al.* (2003) concluded that the role of nNOS in cardiac myocytes may involve the following four mechanisms; (i) inhibition of the L-type Ca^{2+} channels, (ii) inhibition of Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), (iii) regulation of Ca^{2+} through increasing PLB protein levels, and (iv) regulation of Ca^{2+} dynamics through activation of the ryanodine receptor (RyR).

SERCA contributes to approximately 92% of the cytosolic Ca^{2+} removal workload in rat hearts. Phospholamban (PLB) is the main inhibitor of SERCA, acting to regulate SERCA function. An increase in PLB to SERCA ratio reduces the SERCA Ca^{2+} affinity and activity, leading to prolonged relaxation and a reduced cardiac contractility (Ren *et al.* 2003). Rohman *et al.* (2003) showed a decrease in SERCA in *mdx* myocytes (Rohman *et al.* 2003). In addition, ovariectomised rats show a reduction in the SERCA which is reversible with oestrogen therapy (Ren *et al.* 2003). Therefore, oestrogen (via SERCA modification) has the potential to reduce the elevated calcium levels characteristic of dystrophic hearts (*mdx* and DMD) and potentially restore cardiac function to that of unaffected hearts.

The nNOS isoform is associated with the SR of cardiac muscle (Xu *et al.* 1999) and potentially with the RyR (Casadei and Sears 2003). RyRs are Ca^{2+} channels that control $[Ca^{2+}]_i$ in muscle by releasing Ca^{2+} from the SR. RyRs are regulated by voltage-sensing surface membrane L-type Ca^{2+} channels known as dihydropyridine receptors (DHPRs) via a direct mechanical interaction. DHPRs are located within the cardiac T-tubule system, where dystrophin is ordinarily located (Peri *et al.* 1994). Woolf *et al.* (2006) studied 12 to 14 week old male *mdx* mice and was the first to demonstrate a reduced potency to both a DHPR agonist and antagonist in *mdx* myocytes. This was reflected by reduced receptor affinity, upregulation of DHPR mRNA and DHPR protein, together with a delayed inactivation of the L-type Ca^{2+} current. Therefore, as with the potential of nNOS to modify SERCA, there is also the potential (in dystrophic hearts) for oestrogen-mediated regulation of Ca^{2+} dynamics through activation of the RyR.

Aravamudan *et al*. (2003), in a study using caveolin-3 transgenic mice (which over-expressed caveolin-3), found that both eNOS and nNOS were equally expressed in control and caveolin-3 transgenic hearts. They concluded that because nNOS expression is reduced in dystrophin-deficient skeletal muscle tissue (DMD and *mdx*), nNOS functions may be differentially regulated in cardiac muscle (Aravamudan *et al.* 2003). However, although nNOS expression is reduced in the *mdx* mouse heart, and the physiological consequences of reduced nNOS expression are documented, current research has not shown whether oestrogen specifically increases or decreases nNOS expression in cardiac muscles. Therefore, it is not possible to deduce whether the positive effects of left atrial contractility elicited by oestrogen in this trial involved nNOS mediated pathways.

On the other hand, numerous studies have examined the effects of oestrogen on eNOS expression and function in various animal models. The development of, and subsequent experiments involving, eNOS knockout mice has led to the knowledge that the eNOS isoform is involved in modulation of cardiac contractility (Huang 1998; Huang and Lo 1998). Oestrogen is known to stimulate the expression of eNOS in neonatal and adult rat cardiomyocytes *in vivo* and *in vitro* (Nuedling *et al.* 1999) and Western blot analysis of cultured human coronary artery endothelial cells has also shown that oestrogen increases expression of eNOS whilst having no effect on $[Ca^{2+}]_i$ concentrations (Yang, Bae and Zhang 2000).

The precise molecular mechanism of oestrogen regulation of eNOS activity still remains unknown. eNOS activity appears to be under a complex and dynamic regulation and the effects of oestrogen on cardiac eNOS functional activity are not limited to simply an increase in eNOS protein expression**.** Oestrogen also reduces the association of cardiac eNOS with the inhibitory protein caveolin**-**3 and increases the association with the facilitator protein calmodulin (Wang and Abdel-Rahman 2002). Wang (2000) showed that ovariectomy in rats increased caveolin-3 expression and decreased calmodulin binding with eNOS. Subsequent oestrogen treatment of ovariectomised rats restored eNOS activity, eNOS expression, and the association of eNOS with caveolin-3 and calmodulin. Wang (2000) suggested

that oestrogen potentially reduces caveolin-3 expression via mechanisms which increase eNOS (Wang and Abdel-Rahman 2002).

It is known that caveolin-3 is over-expressed in the skeletal muscle of *mdx* mice (Vaghy *et al.* 1998). A study by Aravamudan *et al.* (2003) using caveloin-3 transgenic mice (which over-express caveolin-3 in similar proportions to the increased caveolin-3 expression in DMD and *mdx* skeletal muscle) showed that over-expression of caveolin-3 in cardiac tissues resulted in a number of histological and physiological changes including; (i) a significantly decreased expression of dystrophin, α-sarcoglycan and β-dystroglycan (down-regulation of the DGC) (ii) severe cardiac tissue degeneration, significant cardiac myocyte disorganisation, and chronic inflammation marked by increased cellular infiltrates due to muscle degeneration (evidenced by H&E staining of left ventricular and left atrial cardiac tissues), (iii) increased interstitial fibrosis (evidenced by trichrome staining of both left atrial and left ventricular areas of the heart), (iv) changes in cardiac creatine kinase levels consistent with cardiac muscle degeneration, (v) abnormal ECGs showing a prolonged QRS duration which may be indicative of either slowed conduction across the ventricles or delayed repolarisation, and (vi) reduced fractional shortening due to systolic (contraction) dysfunction (Aravamudan *et al.* 2003). This clearly suggests that an oestrogen induced decrease in caveolin-3 could potentially improve cardiac function in the *mdx* mouse heart.

Secondly, the oestrogen-mediated increased expression of eNOS and the subsequent eNOS mediated production of NO could further explain a positive inotropic effect. The actions of NO on the heart can be divided into direct effects on the cardiac myocytes and indirect effects on myocardial function (Shah *et al.* 1996). The regulation of cardiac muscle function by coronary vascular and endocardial NO has been reported in ferret papillary muscle preparations (Smith, Shah and Lewis 1991). Cardiac effects of NO include; (i) dilatation of cardiac blood vessels (Palmer, Ferrige and Moncada 1987), (ii) inhibition of platelet aggregation (Radomski, Palmer and Moncada 1987; Tanner *et al.* 2000) (iii) regulation of venous return and capacitance, and thus filling volume, with modulation of cardiac output via Frank-Starling mechanisms (Shah 1996) (iv) anti-inflammatory and anti-arteriosclerotic

properties *in vivo* (Moroi *et al.* 1998), (v) inhibition of β-adrenergic-responsiveness (Shah *et al.* 1996), and (vi) modulation of sarcolemmal Ca^{2+} influx and Ca^{2+} release from the SR (Kelly, Balligand and Smith 1996; Shah *et al.* 1996). Most importantly, research has shown that NO can induce both negative and positive inotropic effects. High levels of NO induce negative cardiac contractile responses via cGMP mediated pathways, whereas low levels of NO increase positive inotropic effects via cAMP (Vila-Petroff *et al.* 1999). The positive inotropic effects of NO donors have further been observed in isolated cat papillary muscles (Mohan *et al.* 1996) and rat ventricular myocytes (Kojda *et al.* 1996).

Furthermore, Wehling-Henricks *et al.* (2005) recently developed an *mdx* mouse model with myocardial expression of a nNOS transgene. This effectively translated to an elevated production of NO by the myocardium. Expression of the transgene prevented the characteristic progressive ventricular fibrosis seen in *mdx* mice and greatly reduced myocarditis. All of the characteristic ECG abnormalities of *mdx* mice (including deep Q-waves, diminished S:R ratios, polyphasic R-waves and frequent premature ventricular contractions) were either improved or corrected by nNOS transgene expression. In addition, defects in *mdx* cardiac autonomic function (i.e. decreased HR variability) were significantly reduced by the increased NO resulting from nNOS transgene expression (Wehling-Henricks *et al.* 2005). Therefore, the oestrogen-induced positive cardiac inotropic results in this trial can potentially be explained via mechanisms which increase cardiac eNOS expression and, in turn, decrease caveolin-3 and increase NO levels in the *mdx* heart. This is the first research to clearly demonstrate that oestrogen treatment can have a positive effect on basal and maximum cardiac contractility and improved responsiveness to Ca^{2+} in the *mdx* mouse model of DMD.

Individual skeletal muscles in the human body contain a unique composition of type-I (fast-glycolytic) and type-II (slow-oxidative) muscle fibres. Type-I fibres (predominate in EDL) are mainly responsible for movement whereas type-II fibres (predominate in SOL) are postural muscles. Physical inactivity (as progressively seen in DMD boys) places less metabolic demand on type-I muscles; however, the type-II muscles are still recruited to

maintain posture. Furthermore, an increase in BW (also seen in DMD boys) increases the metabolic load on type-II postural muscles. The higher metabolic demands on type-II muscles increase muscle free radical production and subsequent oxidative damage in DMD skeletal muscles.

With respect to sex differences, men express a higher percentage of type-I (fast-glycolytic) skeletal muscle fibres than women, whilst women have a higher expression of type-II (slow-oxidative) fibres. It is generally accepted that male skeletal muscles have a higher maximum power output than female skeletal muscles. Conversely, during eccentric contractions, female muscles are considered more fatigue resistant and recover faster, reflecting a higher proportion of type-II (slow-oxidative) fibres in females (Glenmark *et al.* 2004).

The effects of oestrogen on skeletal muscle are mediated by ligand-activated transcription factors known as oestrogen receptors (ERs) (Mangelsdorf *et al.* 1995). Human skeletal muscle cells express two functional ERs, ERα (Kahlert *et al.* 1997) and ERβ (Wiik *et al.* 2003), which are not isoforms of each other, but distinct proteins encoded by separate genes located on different chromosomes (Couse and Korach 1999).

Currently the specific effects of oestrogen on human skeletal muscle function are largely unknown. Two studies showed that oestrogen treatment in post-menopausal women increased force production in the adductor pollicis muscles (Skelton *et al.* 1999) and the isometric strength of back extensor muscles (Heikkinen *et al.* 1997). Other research (in pre-menopausal women) has shown no improved forces with increased oestrogen levels (Greeves *et al.* 1997).

Various animal studies have also reported conflicting results as to the effects of oestrogen on skeletal muscle function. Oestrogen protection against skeletal muscle damage in rats has been shown using an exercise model and yet the mechanism of this protection remains unclear (Bar *et al.* 1988). In studies using female rats, Suzuki and Yamamuro (1985) found that isomeric twitch and tetanic contractions in both EDL and SOL muscles were increased post-ovariectomy (Suzuki and Yamamuro 1985) whilst McCormick *et al.* (2004) found ovariectomy (Ovx) had no effect on maximal specific isometric force of SOL muscles (McCormick *et al.* 2004). However, in contrast to the findings of Suzuki and Yamamuro (1985) and McCormick *et*

al. (2004), a number of studies have concluded that oestrogen can have positive effects on skeletal muscle contractility. Warren *et al.* (1996) found that maximal isometric tetanic force decreased in oestrogen depleted EDL muscles of female mice (Warren *et al.* 1996). In 1999 Wattanapermpool and Reiser found a ~20% reduction in the maximum Ca^{2+} induced FOC in single, permeabilised SOL fibres from Ovx rats (Wattanapermpool and Reiser 1999). In two separate trials, researchers showed that male and Ovx female rats had higher heat stress protein expression and exercise-induced skeletal muscle damage than control female rats and subsequent oestrogen treatment reversed these effects in both trials (Bar *et al.* 1988; Paroo, Dipchand and Noble 2002). A 2005 study determining the effects of oestrogen hormone removal on force-generating capacities and contractile proteins in SOL and EDL muscles of C57 female mice showed that SOL and EDL muscles from Ovx mice generated less maximal isometric force than did those from sham operated control mice. Total and contractile protein contents of SOL and EDL muscles were not different between Ovx and sham mice, indicating that protein synthesis was not affected by oestrogen removal. These results further suggest that oestrogen depletion has detrimental effects on skeletal muscle force-generating capacities which could be explained by altered actin-myosin interactions (Moran, Warren and Lowe 2005). A recent study by Moran, Warren, and Lowe (2006) on the effects of Ovx on EDL and SOL muscles of female rats showed that the removal of ovarian hormones increased muscle mass, resulted in no change in contractile protein expression (implying that the quantity of contractile machinery was not affected), and lowered isometric force generation. Moran, Warren, and Lowe (2006) concluded that ovarian hormones directly affect contractile protein function via altering myosin structural distribution during contraction (Moran, Warren and Lowe 2006). In addition, Sitnick *et al.* (2006) found that atrophied gastrocnemius muscles in Ovx rats failed to recover under reloading and concluded that oestrogen was essential to the recovery of atrophied muscle mass (Sitnick *et al.* 2006). Therefore, the most recent research evidence appears to support the theory that oestrogen is capable of improving force generation in both type-I (fast-glycolytic) and type-II (slow-oxidative) skeletal muscles.

In this study, skeletal muscle functional results were encouraging in terms of the potential of oestrogen to improve force production in *mdx* EDL (type-I) and SOL (type-II) muscles. EDL sPo values in oestrogen-treated mice ranged from ~15% to ~34% higher than untreated controls but results were not significant. These results were reflected in SOL muscle sPo values. An oestrogen treatment of 0.32 mg/kg/day (despite producing no significant differences in muscle weight or CSA compared to controls) produced a ~29% increase in SOL specific muscle force compared to un-treated *mdx*. Oestrogen treatment clearly produced an overall increased FOC in both type-I (EDL) and type-II (SOL) skeletal muscles and these results further support studies which have shown that oestrogen can have a positive effect on skeletal muscle contractility.

The oestrogen-mediated mechanisms that potentially explain positive effects on skeletal muscle force generation include; (i) antioxidant/antiinflammatory effects, (ii) nNOS/NO mediated effects, (iii) the inhibition of GH action, (iv) muscle fibre-type switching and (v) decreases in skeletal muscle calpain activity. These mechanisms are discussed below;

The potential of oestrogen as an antioxidant (and membrane stabiliser) capable of protecting skeletal muscle is controversial. It has been shown that oestrogen has a potent antioxidant capacity that plays a protective role in cardiac muscle (Tiidus 2000); however, it is not fully understood as to whether this antioxidant capacity has the ability to protect skeletal muscle (Kendall and Eston 2002). Early studies by Amelink and Bar (1986) showed a significant *in vivo* and *in vitro* post-exercise reduction in muscle CK leakage and lowered serum CK levels in oestrogen-treated Ovx female rats (Amelink and Bar 1986). Measurements of CK released from skeletal muscles is not considered a good indicator of muscle damage but is considered a valid indicator of muscle sarcolemmal stability and Amelink and Bär (1986) concluded that oestrogen had a role in maintaining muscle membrane stability. Tiidus (2003) also concluded that a primary effect of oestrogen may be the protection of skeletal muscle membranes from exercise-induced muscle damage (Tiidus 2003). Persky (2000) showed oestrogen depletion in type-II muscles of female rats produced greater CK efflux in SOL muscles, suggesting that a lack of oestrogen increases

susceptibility to membrane and myofibril damage. Persky (2000) further demonstrated that SOL (type-II) muscles incur more damage in oestrogen deprivation than EDL (type-I) muscles and implied that oestrogen is more protective in type-II muscles than type-I muscles (Persky *et al.* 2000). Secondly, preferential protection by oestrogen may involve features of the metabolic differences in muscle fibre types. Type-II fibres have a greater supply of mitochondria, an higher oxygen dependence, and an increased ability to use fatty acids as a major energy source, causing increased free radical production. During oestrogen deprivation, there is a decline in the transport of glucose into muscle cells (Bishop and Simpkins 1995), an associated decreased in insulin sensitivity, increased fasting glucose levels (Senoz *et al.* 1996), and increased fasting insulin levels (further exacerbating free radical production and subsequent skeletal damage) (Poehlman, Toth and Gardner 1995), suggesting oestrogen may protect skeletal muscle via antioxidant pathways. However, a study examining the effects of oestrogen supplementation on markers of neutrophil infiltration and damage in the skeletal muscles of rats post-ischemia demonstrated that oestrogen did not attenuate neutrophil infiltration, thus questioning oestrogens antioxidant potential (Tiidus *et al.* 2005). Interestingly, it has been further suggested that if oestrogen does inhibit the inflammatory response process associated with the muscle damage and repair cycle, then it may actually have a negative role in restoring normal muscle function after muscle damage has occurred (Kendall and Eston 2002). Therefore, there remains questionable evidence as to the potential of oestrogens to reduce muscle damage via antioxidant pathways and, although anti-oxidative pathways may play a role in limiting skeletal muscle damage, it appears likely that other mechanisms play a more significant role in processes involved in improving skeletal muscle contractility.

Oestrogen can improve skeletal muscle function via nNOS/NO mediated vasodilatation and the subsequent improved blood flow to skeletal muscles. In exercising skeletal muscle, vasoconstrictor responses to α-adrenoceptor activation are attenuated in part by NO produced by nNOS, which is expressed constitutively in skeletal muscle cells. In skeletal muscle of pregnant animals, nNOS mRNA is upregulated, suggesting that muscle

nNOS expression is modulated by oestrogen (Fadel, Zhao and Thomas 2003). It is known that human skeletal muscle has the potential to synthesise oestrogen to the same extent as adipose tissue (Larionov *et al.* 2003). Fadel *et al.* (2003) revealed that skeletal muscle nNOS correlated directly with plasma 17β-oestradiol and inversely with the magnitude of sympathetic vasoconstrictor responses in contracting hind limbs of female rats. This implied that NO-dependent attenuation of sympathetic vasoconstriction in contracting skeletal muscle is impaired in oestrogen-deficient female rats, and that this impairment may be mediated by reduced skeletal muscle nNOS expression (Fadel, Zhao and Thomas 2003). The absence of dystrophin has been shown to correlate with a decrease in nNOS in skeletal muscle in both DMD patients (Brenman *et al.* 1995) and the *mdx* mouse (Lau *et al.* 1998). In the skeletal muscles of DMD patients, nNOS is absent from the sarcolemma and diffusely distributed at reduced levels in the cytoplasm (Brenman *et al.* 1995; Chang *et al.* 1996; Grozdanovic, Gosztonyi and Gossrau 1996). Brenman *et al.* (1995) found that, in *mdx*, nNOS was absent from the sarcolemma and the total nNOS activity was lowered. NOS activity was higher in a soluble fraction of *mdx* than C57s, suggesting that nNOS dissociates from the sarcolemma and accumulates in the cytosol of *mdx* muscle. However, Chang *et al.* (1996) noted that both the particulate and soluble nNOS levels were greatly reduced in *mdx* mice and nNOS mRNA levels were reduced in *mdx* muscle. Oestrogen treatment of Ovx rats has been shown to increase nNOS expression in hindlimb skeletal muscles (Fadel, Zhao and Thomas 2003). Therefore, it is feasible that, via oestrogen-mediated increased nNOS and NO expression, blood flow to and function of skeletal muscles can be improved in the *mdx*.

It is known that an oestrogen-mediated increase in nNOS production can lead to an increase in NO expression. NO generated by skeletal muscle is believed to have a direct role in the regulation of force production. Recent work by Pouvreau and Jacquemond (2005) suggested that NO exerts an inhibitory control of Ca^{2+} release channels possibly via the RyR (Pouvreau and Jacquemond 2005). This would limit Ca^{2+} entry into *mdx* muscle cells (which are known to be in a state of Ca^{2+} overload and impaired Ca^{2+} handling) and may potentially have beneficial effects on skeletal muscle

force production. Increased NO could regulate excess Ca^{2+} influx into Ca^{2+} overloaded cells (in the *mdx* mouse) and restore calcium handling to more functional levels.

In 1972 Rudman *et al.* proposed that there may be therapeutic potential for oestrogen in DMD due to oestrogens peripheral inhibition of the catabolic effect of endogenous GH in dystrophic muscle cells (Rudman *et al.* 1972). In 2003, a GH deficient patient (who was clinically asymptomatic in terms of DMD) was reported by Ghafoor *et al.,* GH replacement therapy was initiated with the resultant emergence of clinical DMD symptoms including proximal muscle weakness and increased fatigability. Serum CK levels subsequently confirmed a diagnosis of DMD and it was concluded that GH exacerbated the pathology of DMD (Ghafoor, Mahmood and Shams 2003). In 2004 Leung *et al*. showed that oestrogen inhibits GH action (in a dose-dependent manner) by suppressing GH receptor function (Leung *et al.* 2004). In this study, oestrogen treatment resulted in an increase in muscle contractility (EDL and SOL muscles), no anabolic effects (as would typically be evidenced by GH) with either no change in cell diameter (SOL muscles) or a decrease in cell diameter (EDL muscles), both of which are consistent with the effects of suppressed GH receptor function and inhibited GH action.

The composition of skeletal muscle fibres is not static, and fibres are capable of adapting their molecular composition by altering gene expression. This altered gene expression results in muscle fibre-type switching which involves morphological and biochemical changes that produce altered contractile properties and endurance capacities of the muscle (Zebedin *et al.* 2004). Riley *et al.* (1982) showed that (in female rats) the onset of puberty elicited a muscle type switch of the SOL muscle from type-I (fast-glycolytic) to type-II (slow-oxidative), concluding that the sex hormone oestrogen plays a key role in skeletal muscle fibre-type switching (Riley, Ellis and Bain 1982).

Despite some merit in the mechanisms discussed above (in terms of their possible role in improving skeletal muscle function in the *mdx* mouse) a more likely explanation of the results of this trial potentially involve the effects of oestrogen on skeletal muscle calpain activity. A 20-fold increase in the levels of the protease calpain is evident in DMD skeletal muscles (Laine and Montellano 1998) and Spencer, Croall, and Tidball (1995) reported elevated

calpain levels in both four and 14 week old *mdx* mice (Spencer, Croall and Tidball 1995). Carnwath and Shotten (1987) showed that at the peak of *mdx* muscle necrosis, ~7% of the muscle cross-section (in EDL and SOL muscles) is occupied by degenerating fibres (Carnwath and Shotton 1987). Spencer (1995) further argued that, if the majority of the increased calpain activity occurs in these fibres, then the actual increase in calpain activity may be as high as 20 to 30-fold in degenerating *mdx* fibres (Spencer, Croall and Tidball 1995).

It has been hypothesised that calpains (ubiquitous calcium-dependent cysteine proteases) may be responsible for initiation of the proteolytic cascade leading to cell death in dystrophic muscle (Tidball and Spencer 2000). Elevation of Ca^{2+} levels in unaffected muscle leads to elevation in calpains and initiates proteolysis (Zeman *et al.* 1985). Also, Z-disc disruption is an early event in muscle degeneration in DMD and *mdx* mouse muscles (Cullen and Jaros 1988) and Z-disc proteins are particularly susceptible to the proteolytic effects of calpains (Goll *et al.* 2003). In 2002, Spencer and Mellgren generated an *mdx* mouse line with muscle-specific over-expression of calpastatin (the endogenous inhibitor of calpains) and showed that transgene expression ameliorated muscle necrosis and reduced the size of muscle lesions. In addition, they concluded that the beneficial effects occurred without reducing the efflux of Ca^{2+} into muscle fibres, which indicated that calpain-mediated pathology in *mdx* muscle is an event that occurs downstream of membrane damage (Spencer and Mellgren 2002).

A 2001 study by Tiidius *et al.* demonstrated that oestrogen attenuated post-exercise muscle calpain activity in Ovx rats (Tiidus *et al.* 2001). The results of this trial support the theory that oestrogen can reduce calpain activity. This trial showed two important results; (a) oestrogen increased skeletal muscle force generation in EDL (non-significant) and SOL muscles, indicating reduced muscle damage and (b) oestrogen treatment resulted in a trend of decreased percentage of CNFs in both EDL (non-significant) and SOL muscles, further suggesting a possible reduction in calpain activity.

Furthermore, it has been noted that oestrogen treatment can increase nNOS expression in Ovx rats (Fadel, Zhao and Thomas 2003).

nNOS is a calpain-sensitive protein, therefore, oestrogen potentially has the dual benefit of decreasing both the proteolytic effects of calpains together with limiting further calpain-mediated decreases in nNOS in dystrophic muscles .

Histological results showed oestrogen treatment produced a decreased percentage of CNFs in both EDL (non-significant) and SOL muscles. A dose of 0.16 mg/kg/day oestrogen resulted in a 41% decrease ($p<0.05$) in SOL CNFs. Percentage of CNFs and specific force production (sPo) results were not mirrored by cell diameter results. Oestrogen treatment resulted in a trend towards decreased EDL cell diameters (significant for a dose of 0.16 mg/kg/day only). No differences in SOL cell diameters with oestrogen treatment was consistent with the findings of McCormick *et al.* (2004) who conducted a study of seven week old female rats and showed that Ovx had no effect on BW or SOL muscle fibre diameter, further suggesting that oestrogen may actually inhibit skeletal muscle growth when it is the only ovarian hormone present (McCormick *et al.* 2004). This effect may actually be advantageous in DMD where muscle hypertrophy potentially leads to increased sarcolemmal damage and exacerbation of the vicious cycle (Figure 2-13) of muscle regeneration and degeneration typical of the disease. All muscles cells in the study had diameters less than 25 μm but cell diameter did not reflect a specific functional benefit in either EDL or SOL muscles.

This unique study produced a number of results which indicate that further research into the potential therapeutic role of oestrogen in DMD be undertaken. It is clear that oestrogen treatment could increase both basal and maximum FOC in the LA of *mdx* male mice and that oestrogen also improves responsiveness to exogenous Ca^{2+} in *mdx* left atria. In terms of skeletal muscle responses, type-I fast-twitch (EDL) and type-II slow-twitch (SOL) muscles responded positively to oestrogen treatment. Oestrogen produced higher (albeit non-significant) specific forces in EDL muscles and significantly higher specific forces in SOL muscles. The positive effects of oestrogen on skeletal muscle function may have been due to antioxidant/antiinflammatory processes, nNOS/NO mediated effects, GH inhibition, fibre-type switching, and/or reduction in calpain activity.

Furthermore, oestrogen does not compete for glucocorticoid sites in skeletal muscle (Snochowski, Dahlberg and Gustafsson 1980), unlike testosterone which displaces glucocorticoids from the glucocorticoid receptor (Danhaive and Rousseau 1986; Danhaive and Rousseau 1988), thus suggesting the potential for concomitant oestrogen and glucocorticoid therapy in DMD.

7 Influence of age trial

7.1 Influence of age on body weight

Both mouse strains showed a general increase in BW with increasing age although 330 day old *mdx* had a 9% lower BW than 180 day old *mdx*. In only five of the ten age groups studied were there significant differences in BW between strains (Figure 7-1). At ages 19 ($p<0.01$), 21 ($p<0.05$), and 23 ($p<0.05$) days old C57s were significantly heavier than *mdx*. This trend continued up to 90 days of age when the situation reversed and *mdx* mice were heavier than C57s (significant at 90 days ($p<0.05$) and 330 days ($p<0.01$)).

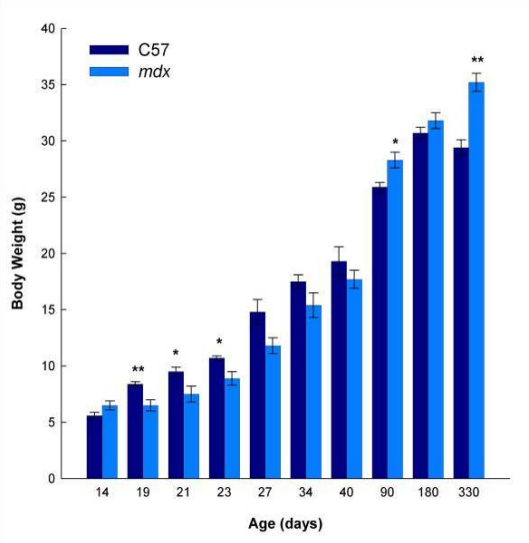


Figure 7-1 **Body weight comparison between C57 and *mdx* male mice at varying ages. * $p<0.05$, ** $p<0.01$. n = 5-10 mice.**

7.2 Influence of age on testes weights

In both mouse strains there was an increase in testes weight with age from 14 days old until 180 days old, with 330 day old mice having lower testes weights than 180 day old mice (Figure 7-2). In all ages, C57 testes were heavier than *mdx*, however, this was only significant at 21 days, 23 days, and 27 days of age ($p<0.05$).

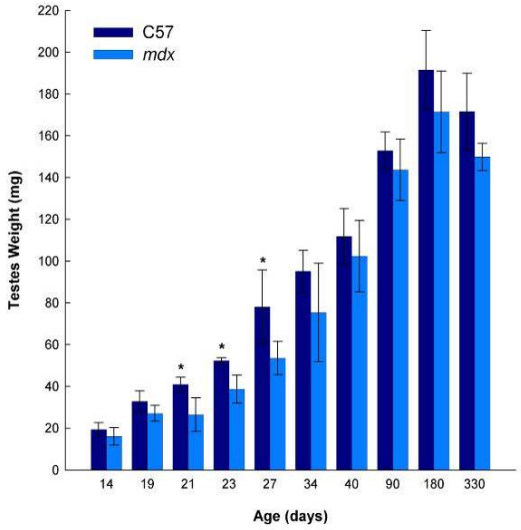


Figure 7-2 Influence of age on testes weights across varying ages in male mice. Mouse strain comparison; * p<0.05, ** p<0.01. n = 5-10 mice.

7.3 Influence of age on cardiac characteristics

7.3.1 Cardiac morphometry

At 14 days of age there were no differences in body and cardiac weights between strains (Table 7-1).

Table 7-1 Body and cardiac weights of 14 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	5.61±0.30	6.49±0.41
HW (mg)	31.77±3.08	33.81±2.68
HW% (%)	0.56±0.02	0.52±0.02
RA weight (mg)	1.27±0.26	0.8±0.17
LA weight (mg)	0.47±0.06	0.61±0.07
RV weight (mg)	7.09±0.62	8.16±0.58
LV+S weight (mg)	22.94±2.33	24.24±2.17

*** p<0.05, ** p<0.01 between strain comparison. n = 7-8 mice. [1]This information is described in Figure 7-1.**

At 19 days of age significant differences began to emerge between strains. BW (p<0.01), HW (p<0.01), and LV+S weight (p<0.05) were 23%, 18%, and 20% lower in the *mdx* compared to C57s, respectively (Table 7-2).

Table 7-2 Body and cardiac weights of 19 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	8.43±0.25	6.53±0.53**
HW (mg)	50.24±1.62	41.44±2.36**
HW% (%)	0.60±0.02	0.65±0.04
RA weight (mg)	1.84±0.26	1.38±0.15
LA weight (mg)	1.09±0.10	0.84±0.20
RV weight (mg)	9.29±0.61	8.80±0.79
LV+S weight (mg)	38.03±1.53	30.43±2.32*

*** p<0.05, ** p<0.01 between strain comparison. n = 7-8 mice. [1]This information is described in Figure 7-1.**

The differences between strains observed at 19 days of age continued at 21 days old with the addition of a 34% lower LA weight in *mdx* compared to C57s ($p<0.05$). At 21 days *mdx* body, heart, and left ventricles were 21% ($p<0.05$), 19% ($p<0.05$), and 22% ($p<0.01$) lower than C57s, respectively (Table 7-3).

Table 7-3 Body and cardiac weights of 21 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	9.53±0.45	7.49±0.75*
HW (mg)	53.33±2.04	42.94±3.75*
HW% (%)	0.56±0.02	0.58±0.02
RA weight (mg)	1.93±0.10	1.51±0.23
LA weight (mg)	0.89±0.08	0.59±0.10*
RV weight (mg)	9.38±1.14	8.87±0.85
LV+S weight (mg)	41.14±0.89	31.97±2.70**

*** p<0.05, ** p<0.01 between strain comparison. n = 7-8 mice. [1]This information is described in Figure 7-1.**

The differences in LA weight observed at 21 days of age were no longer evident at 23 days old. However, body, heart, and left ventricle weights continue to be significantly lower in the *mdx* by 17% ($p<0.05$), 20% ($p<0.01$), and 18% ($p<0.05$), respectively. At 23 days a difference in RV weight emerges with *mdx* RV weight being 30% ($p<0.01$) (Table 7-4).

Table 7-4 Body and cardiac weights of 23 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	10.74±0.24	8.89±0.65*
HW (mg)	58.29±1.02	46.51±3.71**
HW% (%)	0.55±0.02	0.52±0.02
RA weight (mg)	1.31±0.11	1.45±0.19
LA weight (mg)	0.98±0.10	0.76±0.10
RV weight (mg)	14.18±0.78	9.99±0.84**
LV+S weight (mg)	41.83±0.84	34.31±2.89*

*** p<0.05, ** p<0.01 between strain comparison. n = 8 mice. [1]This information is described in Figure 7-1.**

There was no significant difference in the body and cardiac weights between strains in 27 day old mice (Table 7-5), 34 day old mice (Table 7-6), or 40 day old mice (Table 7-7).

Table 7-5 Body and cardiac weights of 27 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	14.76±1.14	11.82±0.81
HW (mg)	67.35±4.78	61.98±2.51
HW% (%)	0.46±0.01	0.53±0.02
RA weight (mg)	2.20±0.18	1.88±0.27
LA weight (mg)	0.80±0.13	0.90±0.11
RV weight (mg)	14.33±1.08	13.16±1.58
LV+S weight (mg)	50.03±3.78	46.04±3.09

*** p<0.05, ** p<0.01 between strain comparison. n = 5-8 mice. [1]This information is described in Figure 7-1.**

Table 7-6 Body and cardiac weights of 34 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	17.53±0.64	15.40±1.18
HW (mg)	74.04±2.26	68.45±6.83
HW% (%)	0.42±0.01	0.44±0.03
RA weight (mg)	2.25±0.20	1.75±0.21
LA weight (mg)	0.99±0.08	0.93±0.09
RV weight (mg)	15.89±0.64	16.01±1.92
LV+S weight (mg)	54.91±1.98	49.76±5.02

***p<0.05, ** p<0.01 between strain comparison. n = 8 mice. [1]This information is described in Figure 7-1.**

Table 7-7 Body and cardiac weights of 40 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	19.30±1.36	17.68±0.88
HW (mg)	90.01±6.96	79.18±3.65
HW% (%)	0.47±0.00	0.45±0.02
RA weight (mg)	2.79±0.23	2.61±0.22
LA weight (mg)	1.06±0.06	1.46±0.36
RV weight (mg)	19.58±1.92	15.93±1.33
LV+S weight (mg)	66.59±5.54	59.18±2.63

*** p<0.05, ** p<0.01 between strain comparison. n = 8 mice. [1]This information is described in Figure 7-1.**

At 90 days of age significant differences between strains re-emerged albeit with a reversal of trend. At 90 days old *mdx* body, LA, and LV+S weights were heavier than C57s by 8% ($p<0.05$), 33% ($p<0.01$), and 6% ($p<0.05$), respectively (Table 7-8).

Table 7-8 Body and cardiac weights of 90 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	25.93±0.43	28.33±0.77*
HW (mg)	109.22±0.90	114.79±2.90
HW% (%)	0.42±0.01	0.41±0.01
RA weight (mg)	2.63±0.22	3.06±0.16
LA weight (mg)	0.96±0.08	1.43±0.12**
RV weight (mg)	22.74±0.93	21.95±1.62
LV+S weight (mg)	82.89±0.79	88.35±1.95*

*** p<0.05, ** p<0.01 between strain comparison. n = 8-9 mice. [1]This information is described in Figure 7-1.**

Despite no significant difference in the BWs of 180 day old mice, *mdx* mice had significantly lower HW (-13%, $p<0.05$), HW% ($p<0.01$), and LV+S ($p<0.01$) weights compared to C57s (Table 7-9).

Table 7-9 Body and cardiac weights of 180 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	30.74±0.54	31.82±0.76
HW (mg)	139.24±6.58	121.10±4.46*
HW% (%)	0.45±0.01	0.38±0.01**
RA weight (mg)	3.45±0.30	3.34±0.26
LA weight (mg)	1.06±0.11	1.12±0.13
RV weight (mg)	25.31±1.62	25.82±1.51
LV+S weight (mg)	109.42±6.12	90.81±3.28*

*** p<0.05, ** p<0.01 between strain comparison. n = 9-10 mice. [1]This information is described in Figure 7-1.**

At 330 days old *mdx* mouse BW was 16% ($p<0.01$) higher than C57s. As there was no difference in HWs between strains, the difference in BW may account for the observed difference in HW% observed, in which *mdx* mice were 10% ($p<0.01$) lower than C57s. In addition, the *mdx* RV was 18% heavier than C57s ($p<0.05$) (Table 7-10).

Table 7-10 Body and cardiac weights of 330 day old male mice.

mouse strain	C57	*mdx*
BW (g) [1]	29.44±0.73	35.16±0.82**
HW (mg)	142.73±1.89	153.08±4.44
HW% (%)	0.49±0.01	0.44±0.01**
RA weight (mg)	3.12±0.31	3.68±0.39
LA weight (mg)	1.55±0.20	1.96±0.19
RV weight (mg)	25.05±1.38	30.49±1.33*
LV+S weight (mg)	112.97±3.14	116.95±3.26

*** p<0.05, ** p<0.01 between strain comparison. n = 6-8 mice. [1]This information is described in Figure 7-1.**

7.3.2 Cardiac function

At 14 days basal FOC of LA (normalised for LA weight) was significantly ($p<0.05$) higher in C57s than *mdx*. This trend reversed from 19 to 23 days of age where *mdx* forces were higher than C57s (non-significant). Subsequently, from 27 to 180 days of age, C57 forces were consistently higher than *mdx* (significant at 90 days, $p<0.05$). At 330 days, *mdx* forces were higher than C57s (non-significant) (Figure 7-3).

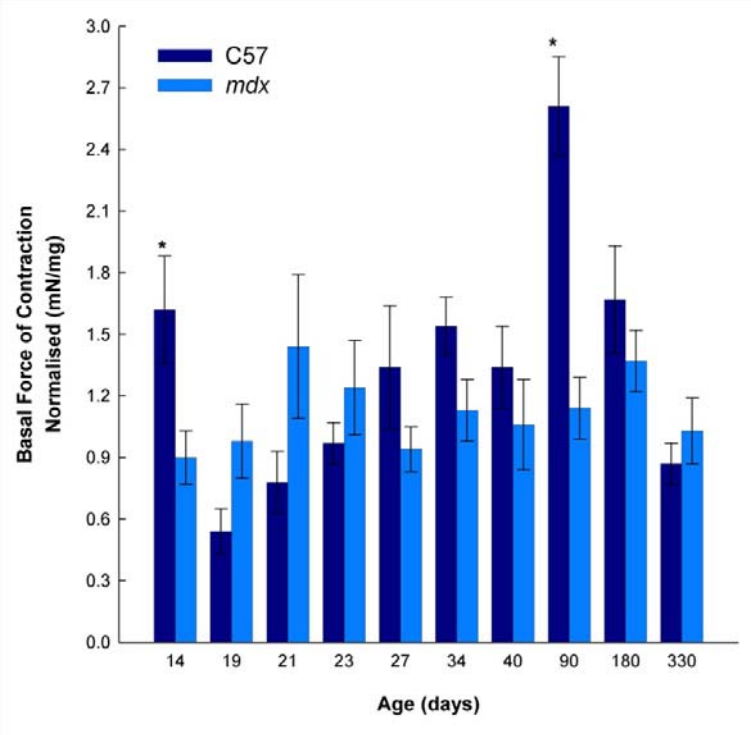
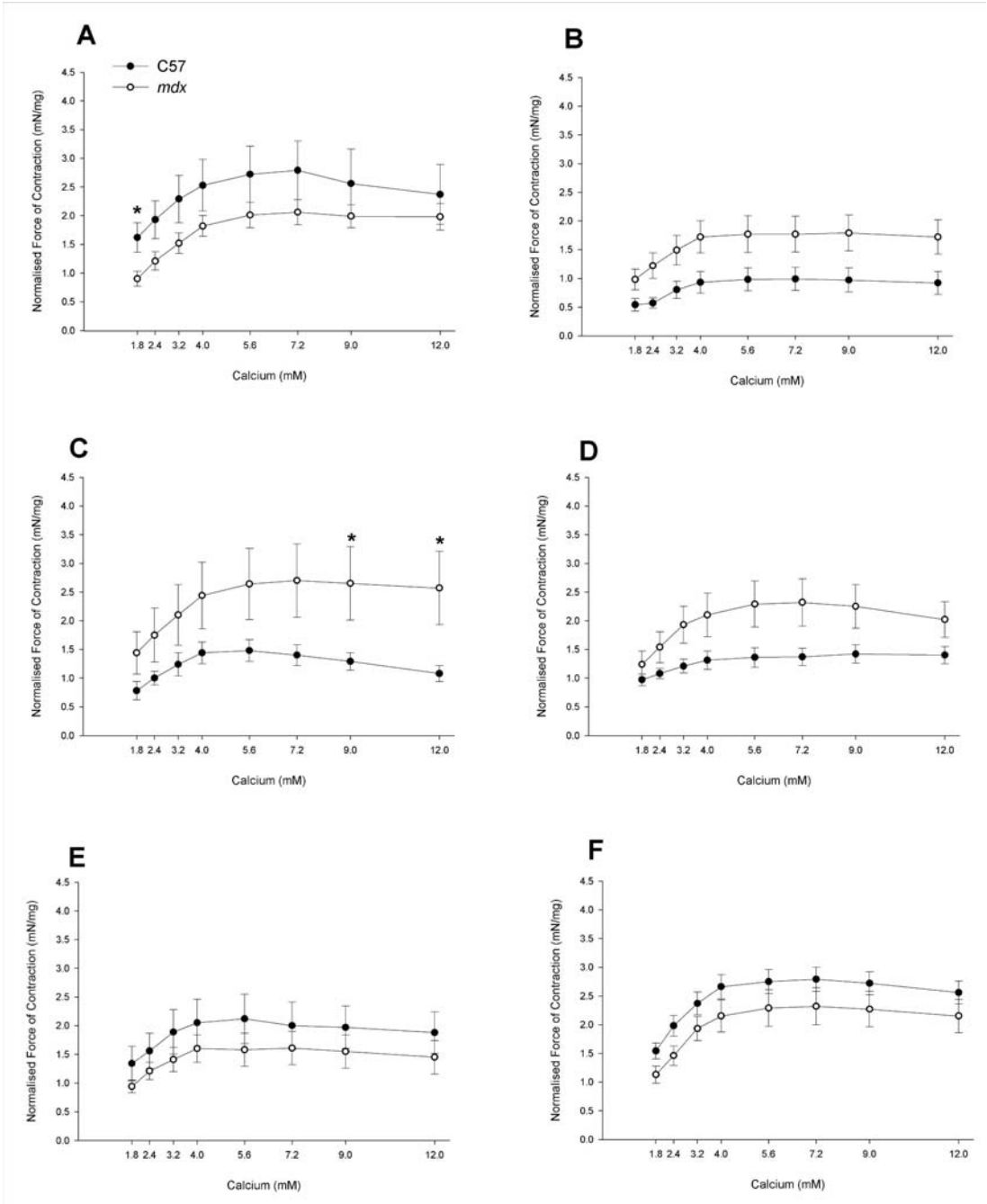


Figure 7-3 **Basal FOC of the LA across ages in male mice.**
*** p<0.05, ** p<0.01 between strain comparison. n = 5-10 mice.**

Ca^{2+} CRCs showed no significant differences in response to exogenous Ca^{2+} from basal forces in 14 day (Figure 7-4A), 23 day (Figure 7-4D), 27 day (Figure 7-4E), 34 day (Figure 7-4F), 40 day (Figure 7-4G),180 day (Figure 7-4I), and 330 day old mice (Figure 7-4J) between strains. However, the trend was not consistent, with C57 forces being greater than *mdx* force s at 14, 27, 34, 40, 90 and 180 days of age whereas at 19, 21, 23 and 330 days of age, *mdx* forces were generally greater than C57s (non-significant). At 21 days of age (Figure 7-4C) all C57 forces were higher than *mdx* forces but only significant at Ca^{2+} concentrations of 9.0 mM and 12.0 mM ($p<0.05$). Figure 7-4H specifically shows that at age 90 days, C57 values were significantly greater than *mdx* values across all Ca^{2+} concentrations.


A
C57
mdx
Normalised Force of Contraction (mN/mg)
Calcium (mM)
1.8 2.4 3.2 4.0 5.6 7.2 9.0 12.0
B
C
D
E
F

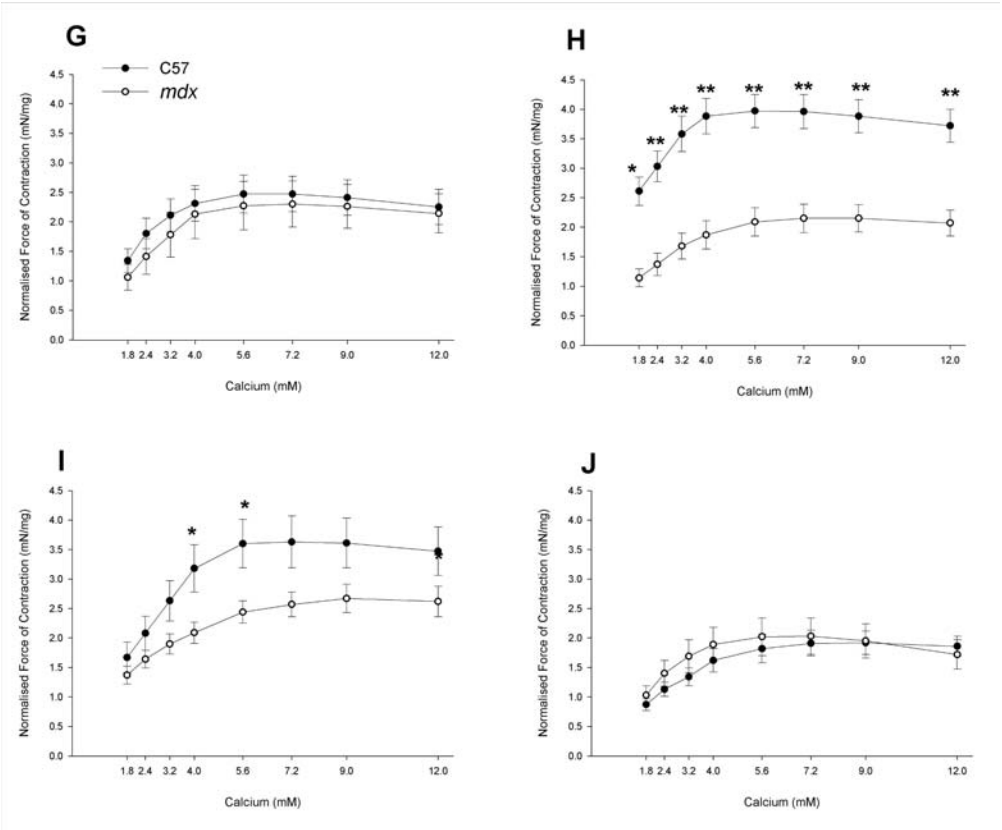


Figure 7-4 Concentration response curves to calcium across varying ages in male mice.

A = 14 days old, B = 19 days old, C = 21 days old, D = 23 days old, E = 27 days old, F = 34 days old, G = 40 days old, H = 90 days old, I = 180 days old, J = 330 days old.

*** p<0.05, ** p<0.01 between strain comparison. n = 5-10 mice.**

At 14 days ($p<0.05$) and 90 days ($p<0.01$) of age, maximum FOC of the LA (normalised for LA weight) was greater in C57s than *mdx* mice. Conversely, at 21 days of age, *mdx* force was greater than C57s ($p<0.05$). Over the ages studied there was less variability in values for *mdx* than C57s (Figure 7-5).

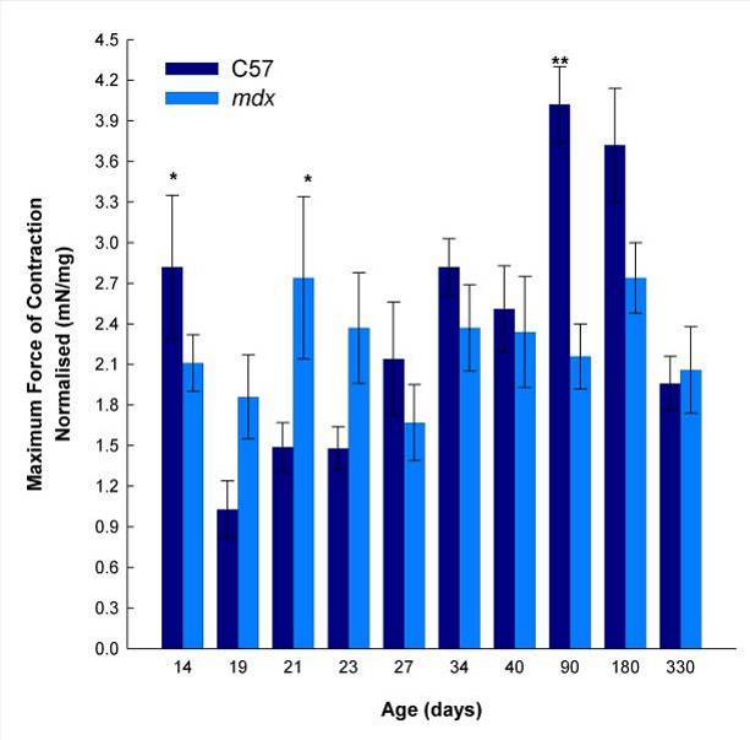


Figure 7-5 Maximum FOC of the LA across ages in male mice.

*** p<0.05, ** p<0.01 between strain comparison. n = 5-10 mice.**

7.4 Influence of age on skeletal muscle morphometry

There were no differences in the parameters measured between strains at 14 days of age (Table 7-11).

Table 7-11 Skeletal muscle morphometry of 14 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	5.61±0.30	6.49±0.41
EDL weight (mg)[2]	1.44±0.25	1.97±0.23
EDL Lo (mm)	6.00±0.58	6.73±0.41
EDL CSA (mm^2)[3]	0.51±0.05	0.63±0.06
SOL weight (mg)[4]	1.52±0.31	1.86±0.25
SOL Lo (mm)	7.19±0.47	6.60±0.97
SOL CSA (mm^2)[5]	0.27±0.04	0.37±0.03

*** p<0.05, ** p<0.01 between strain comparison. n = 3-5 mice (EDL), n = 5 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

At 19 day of age, significant differences between strains emerge. *Mdx* EDL weights were 23% lower than C57s which was consistent with the overall difference in BW between strains (Table 7-12).

Table 7-12 Skeletal muscle morphometry of 19 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	8.43±0.25	6.53±0.53**
EDL weight (mg)[2]	3.09±0.08	2.39±0.22*
EDL Lo (mm)	7.72±0.27	6.99±0.48
EDL CSA (mm^2)[3]	0.86±0.04	0.74±0.06
SOL weight (mg)[4]	1.97±0.25	2.31±0.33
SOL Lo (mm)	6.60±0.31	7.10±0.32
SOL CSA (mm^2)[5]	0.39±0.05	0.44±0.07

*** p<0.05, ** p<0.01 between strain comparison. n = 7-8 mice (EDL), n = 7 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

At 21 days of age, *mdx* EDL weight and CSA remain lower than C57s (non-significant) whereas EDL Lo was 6% lower than C57s ($p<0.05$). *Mdx* SOL Lo is greater than C57s (non-significant) whilst SOL weight and SOL CSA were 20% and 22% respectively lower than C57s ($p<0.01$) (Table 7-13).

Table 7-13 Skeletal muscle morphometry of 21 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	9.53±0.45	7.49±0.75*
EDL weight (mg)[2]	3.29±0.18	2.72±0.22
EDL Lo (mm)	7.46±0.07	7.01±0.17*
EDL CSA (mm^2)[3]	0.95±0.05	0.83±0.07
SOL weight (mg)[4]	3.16±0.10	2.53±0.17**
SOL Lo (mm)	7.00±0.11	7.22±0.33
SOL CSA (mm^2)[5]	0.60±0.02	0.47±0.04**

*** p<0.05, ** p<0.01 between strain comparison. n = 6-8 mice (EDL), n = 7-8 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

At 23 days of age, all measured EDL parameters were significantly lower in *mdx* compared to C57s. EDL weight, Lo, and CSA were 27% ($p<0.01$), 9% ($p<0.05$), and 21% ($p<0.05$) respectively lower than C57s. Interestingly, there was no significant difference between strains with respect to SOL muscle parameters measured (Table 7-14).

Table 7-14 Skeletal muscle morphometry of 23 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	10.74±0.24	8.89±0.65*
EDL weight (mg)[2]	3.97±0.14	2.89±0.28**
EDL Lo (mm)	7.95±0.17	7.21±0.25*
EDL CSA (mm^2)[3]	1.07±0.04	0.85±0.06*
SOL weight (mg)[4]	2.90±0.10	3.04±0.55
SOL Lo (mm)	7.44±0.11	7.17±0.30
SOL CSA (mm^2)[5]	0.52±0.02	0.55±0.08

*** p<0.05, ** p<0.01 between strain comparison. n = 8 mice (EDL), n = 7-8 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

There were no differences in the parameters measured between strains at 27 days of age (Table 7-15).

Table 7-15 Skeletal muscle morphometry of 27 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	14.76±1.13	11.82±0.81
EDL weight (mg)[2]	5.35±0.43	4.30±0.21
EDL Lo (mm)	8.12±0.23	7.81±0.45
EDL CSA (mm^2)[3]	1.41±0.10	1.19±0.05
SOL weight (mg)[4]	4.24±0.37	3.80±0.32
SOL Lo (mm)	8.21±0.17	8.79±0.37
SOL CSA (mm^2)[5]	0.69±0.06	0.59±0.08

*** p<0.05, ** p<0.01 between strain comparison. n = 5-8 mice (EDL), n = 5-8 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

At 34 days of age, despite no significant difference in EDL weight, *mdx* EDL Lo is 15% longer ($p<0.05$) than C57s whilst CSA is reduced 26% ($p<0.01$) compared to C57s. There were no differences in SOL muscle parameters measured (Table 7-16).

Table 7-16 Skeletal muscle morphometry of 34 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	17.53±0.64	15.40±1.18
EDL weight (mg)[2]	5.99±0.21	5.00±0.40
EDL Lo (mm)	7.58±0.08	8.73±0.39*
EDL CSA (mm^2)[3]	1.69±0.05	1.25±0.12**
SOL weight (mg)[4]	4.61±0.28	4.43±0.60
SOL Lo (mm)	8.04±0.13	8.37±0.38
SOL CSA (mm^2)[5]	0.76±0.04	0.70±0.09

*** p<0.05, ** p<0.01 between strain comparison. n = 7-8 mice. ⊗ described in Figure 8-1. [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

Any significant differences observed in skeletal muscle parameters between strains of mice aged 14 to 34 were no longer evident in mice at 40 days of age (Table 7-17).

Table 7-17 Skeletal muscle morphometry of 40 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	19.30±1.36	17.68±0.87
EDL weight (mg)[2]	6.66±0.86	6.79±0.49
EDL Lo (mm)	9.07±0.33	8.44±0.49
EDL CSA (mm^2)[3]	1.55±0.16	1.73±0.09
SOL weight (mg)[4]	5.58±0.47	5.66±0.64
SOL Lo (mm)	8.80±0.35	9.16±0.39
SOL CSA (mm^2)[5]	0.83±0.04	0.81±0.07

p<0.05, ** p<0.01 between strain comparison. n = 8 mice. [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.

At 90 days of age clear and significant differences emerge between strains. *Mdx* EDL muscles were 31% heavier than C57s with a CSA 22% greater than C57s (p<0.01). The *mdx* SOL weight, Lo, and CSA were 50% (p<0.01), 18% (p<0.05), and 25% (p<0.01) greater than C57s (Table 7-18).

Table 7-18 Skeletal muscle morphometry of 90 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	25.93±0.43	28.33±0.77*
EDL weight (mg)[2]	10.09±0.17	13.21±0.40**
EDL Lo (mm)	9.78±0.35	10.43±0.30
EDL CSA (mm^2)[3]	2.23±0.09	2.72±0.08**
SOL weight (mg)[4]	6.83±0.18	10.26±0.49**
SOL Lo (mm)	8.76±0.54	10.31±0.23*
SOL CSA (mm^2)[5]	1.06±0.06	1.33±0.07*

*** p<0.05, ** p<0.01 between strain comparison. n = 8-9 mice (EDL), 7-8 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

The general trends observed at 90 days of age continued at 180 days of age. *Mdx* EDL weight, Lo, and CSA were 30% (p<0.01), 1% (non-significant), and 27% (p<0.05) higher than C57s. *Mdx* SOL weight remains significantly heavier than C57s (23%, p<0.05) whilst, although non-significant, *mdx* Lo and CSA were 5% and 16% greater than C57s, respectively (Table 7-19).

Table 7-19 Skeletal muscle morphometry of 180 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	30.74±0.54	31.82±0.76
EDL weight (mg)[2]	12.79±0.66	16.66±0.99**
EDL Lo (mm)	10.19±0.35	10.30±0.25
EDL CSA (mm^2)[3]	2.73±0.18	3.46±0.18*
SOL weight (mg)[4]	10.47±0.52	12.89±1.03*
SOL Lo (mm)	9.21±0.21	9.68±0.28
SOL CSA (mm^2)[5]	1.52±0.08	1.76±0.11

*** p<0.05, ** p<0.01 between strain comparison. n = 9-10 mice. [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

The greatest difference between strains was observed at 330 days of age where all skeletal muscle parameters measured showed *mdx* values significantly greater than C57s (p<0.01). EDL weight, Lo, and CSA were 73%, 16%, and 49% greater than C57s whilst equivalent SOL parameters measured were 113%, 22%, and 75% higher, respectively. Both skeletal muscle weight differences (percentage wise) were greater than BW difference between strains (Table 7-20).

Table 7-20 Skeletal muscle morphometry of 330 day old male mice.

mouse strain	C57	*mdx*
BW (g)[1]	29.44±0.73	35.16±0.82**
EDL weight (mg)[2]	10.80±0.21	18.73±0.41**
EDL Lo (mm)	10.14±0.22	11.80±0.22**
EDL CSA (mm^2)[3]	2.29±0.06	3.41±0.11**
SOL weight (mg)[4]	7.48±0.50	15.94±0.85**
SOL Lo (mm)	9.24±0.30	11.27±0.28**
SOL CSA (mm^2)[5]	1.08±0.07	1.89±0.13**

*** p<0.05, ** p<0.01 between strain comparison. n = 6-8 mice (EDL), 5-8 mice (SOL). [1]described in Figure 7-1. [2]described in Figure 7-8. [3]described in Figure 7-9. [4]described in Figure 7-6. [5]described in Figure 7-7.**

From 14 to 23 days of age *mdx* SOL were heavier (significant at 21 days, p<0.01) than C57 SOL muscles. However, at 27 and 34 days of age the *mdx* SOL has a lower weight than that of C57s (non-significant). Then, from 40 to 330 days of age the situation is reversed and the *mdx* SOL were consistently heavier than that of age matched C57s (significant at 90 (p<0.01), 180 (p<0.05), and 330 (p<0.01) days of age) (Figure 7-6).

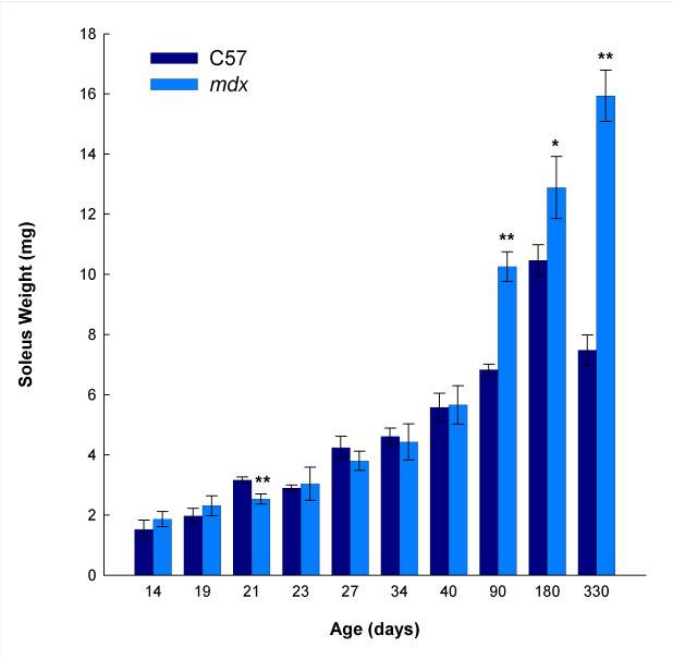


Figure 7-6 SOL weight between strains across varying ages in male mice.

*** p<0.05, ** p<0.01. n = 5-10 mice.**

Figure 7-7 shows that at 14, 19, and 23 days *mdx* SOLs have a higher CSA than age matched C57s (non-significant). However, at 21 days, *mdx* SOL have a significantly ($p<0.01$) lower CSA than C57s. At 27, 34, and 40 days *mdx* CSA is lower (non-significant). The situation reversed from 90 days when *mdx* CSA were consistently higher than age matched C57s (significant at 330 days of age, $p<0.01$).

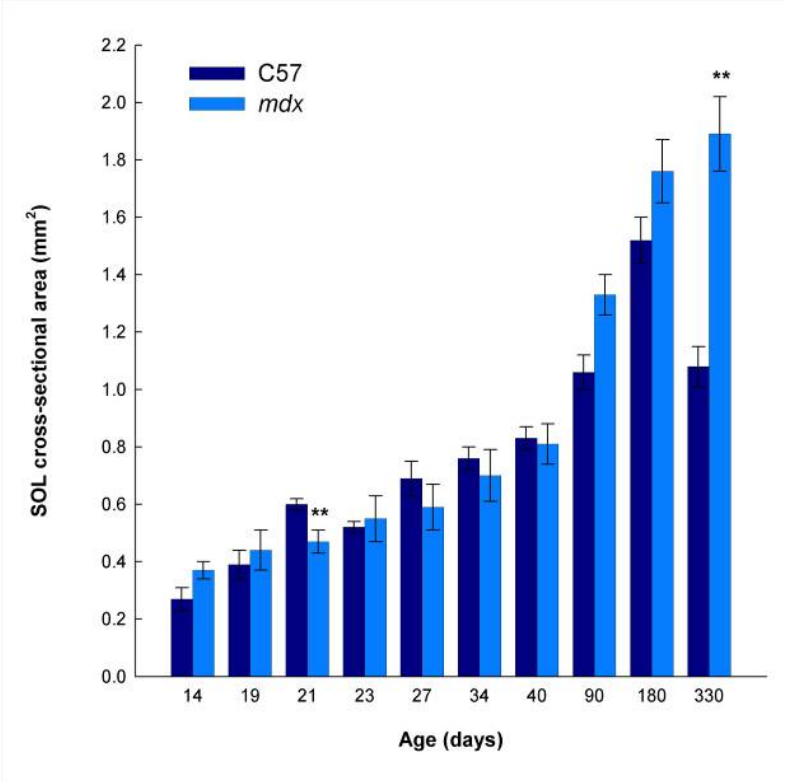


Figure 7-7 SOL CSA between strains across varying ages in male mice. * p<0.05, ** p<0.01. n = 5-10 mice.

Figure 7-8 shows that at 14 days of age *mdx* EDL were heavier (non-significant) than C57 EDL. However, from 19-34 days of age the *mdx* SOL had a lower weight than that of C57s (significant at 23 days, $p<0.01$). Then, from 40-330 days of age the situation was reversed and the *mdx* EDL were consistently heavier than that of age matched C57s (significant at 90, 180, and 330 days of age, $p<0.01$).

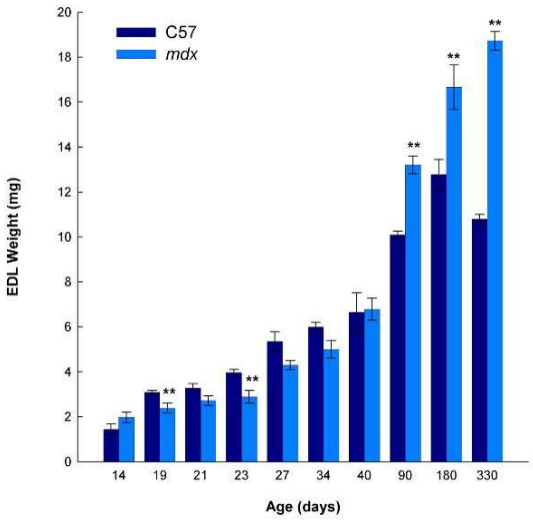


Figure 7-8 EDL weight between strains across varying ages in male mice. * p<0.05, ** p<0.01. n = 6-10 mice.

At 14 days of age *mdx* EDL had a larger CSA (non-significant) than C57 EDL. However, from 19 to 34 days of age the *mdx* EDL had a lower CSA than that of C57s (significant at 23 days ($p<0.05$) and 34 days ($p<0.01$)). Then, from 40 to 330 days of age, the situation was reversed and *mdx* EDL were of consistently higher CSA than that of age matched C57s (significant at 180 days ($p<0.05$) and 330 days ($p<0.01$) of age) (Figure 7-9).

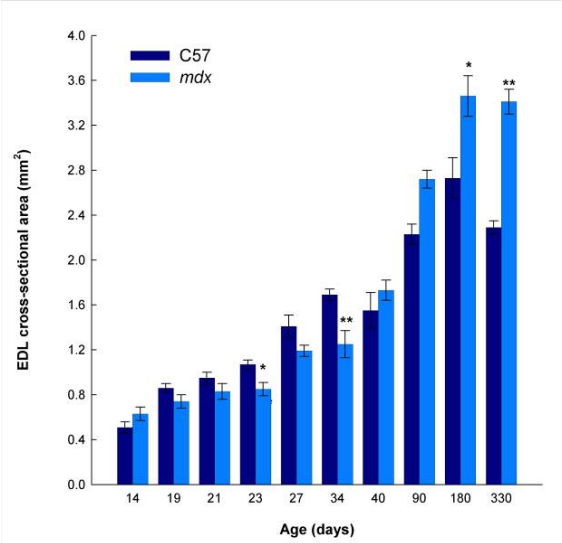


Figure 7-9 EDL CSAs between strains across varying ages in male mice. * p<0.05, ** p<0.01. n = 6-10 mice.

7.5 Influence of age on EDL characteristics

7.5.1 EDL function

Only at 14 days of age *mdx* twitch force s (normalised) were 63% greater than C57s (non-significant). However, at 19, 21, 23, 27, 34, 40, 90, 180, and 330 days of age *mdx* force s were 12%, 43% ($p<0.01$), 62% ($p<0.01$), 52% ($p<0.01$), 17%, 18%, 1%, 16%, and 30% ($p<0.01$), respectively lower than C57s (Figure 7-10).

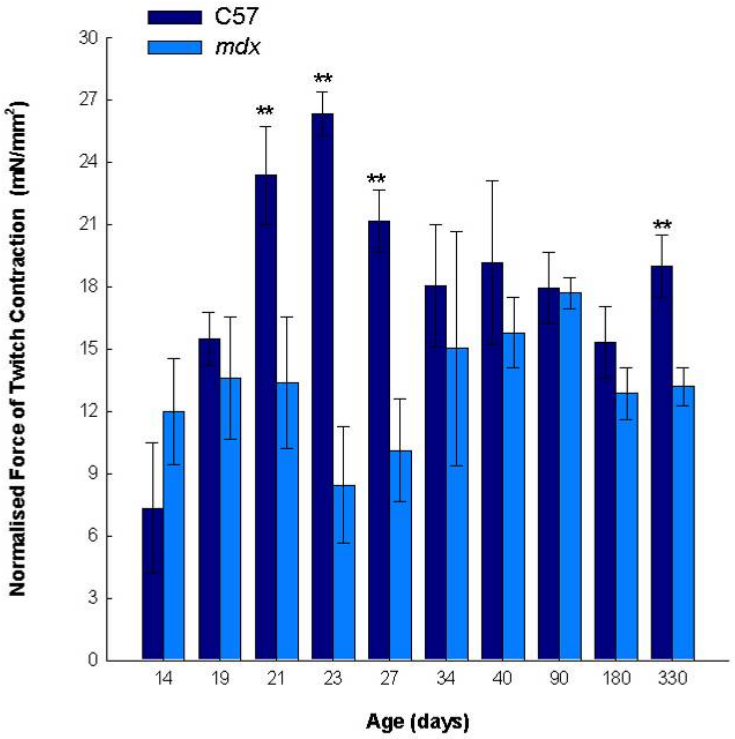


Figure 7-10 EDL sPt across varying ages in male mice.
*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

The results of EDL twitch force generated were somewhat mirrored in the results of tetanic forces in which 14, 34, and 90 day old *mdx* mice produced higher forces than C57s (non-significant). At 19, 21, 23, 27, 40, 180 and 330 days of age, *mdx* EDL tetanic force s were 36% ($p<0.05$), 42% ($p<0.01$), 67% ($p<0.01$), 56% ($p<0.01$), 24%, 11%, and 33% ($p<0.01$) lower than C57s (Figure 7-11).

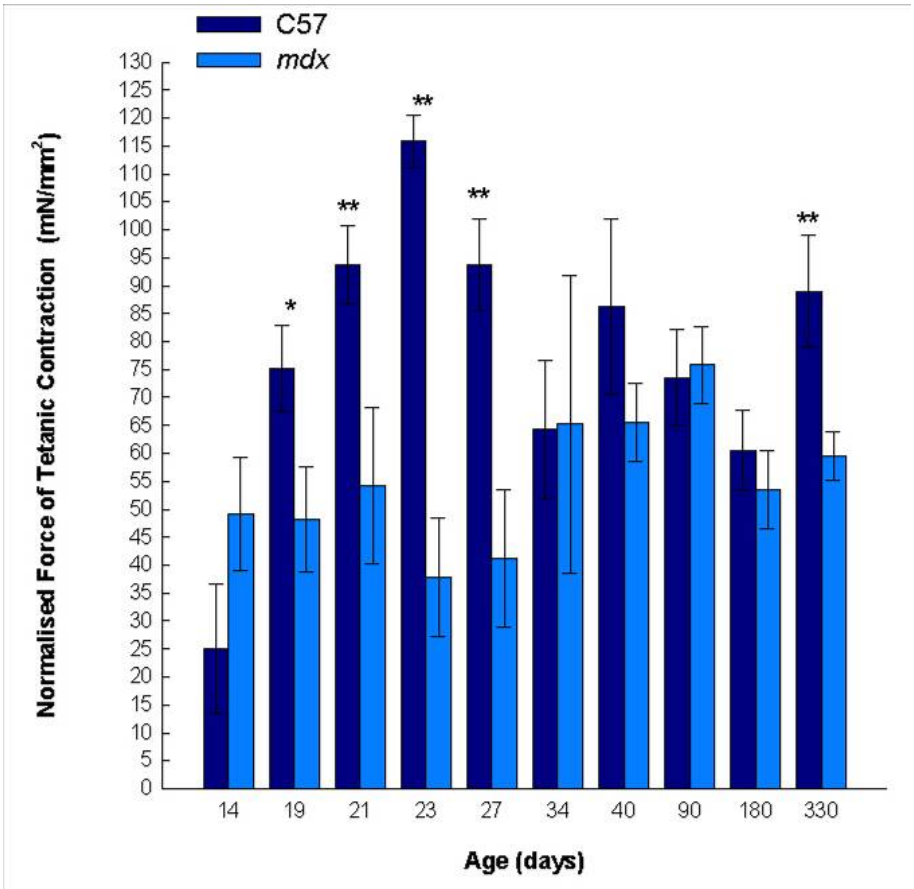


Figure 7-11 EDL sPo across varying ages in male mice.

*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

7.5.2 EDL histology

At all ages younger than 27 days (14, 19, 21, and 23 days of age), the percentage of EDL CNFs did not vary significantly between strains. However, from 27 days of age *mdx* mice had significantly higher ($p<0.01$) percentages of EDL CNFs compared to C57s. The trend in EDL CNF percentage differences between strains was not mirrored in the comparison of sPo values (Figure 7-12).

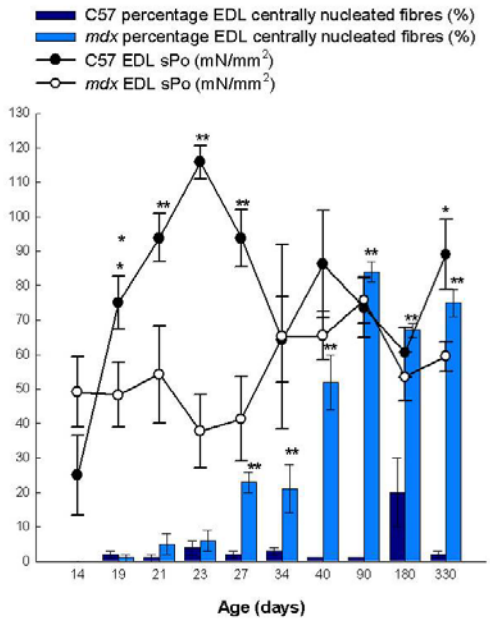


Figure 7-12 EDL percentage of CNFs and sPo across varying ages in male *C57* and *mdx* mice.

*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

Mdx EDL cell diameters were larger than C57s in 330 day old mice. At all other ages there was no difference between strains (Figure 7-13).

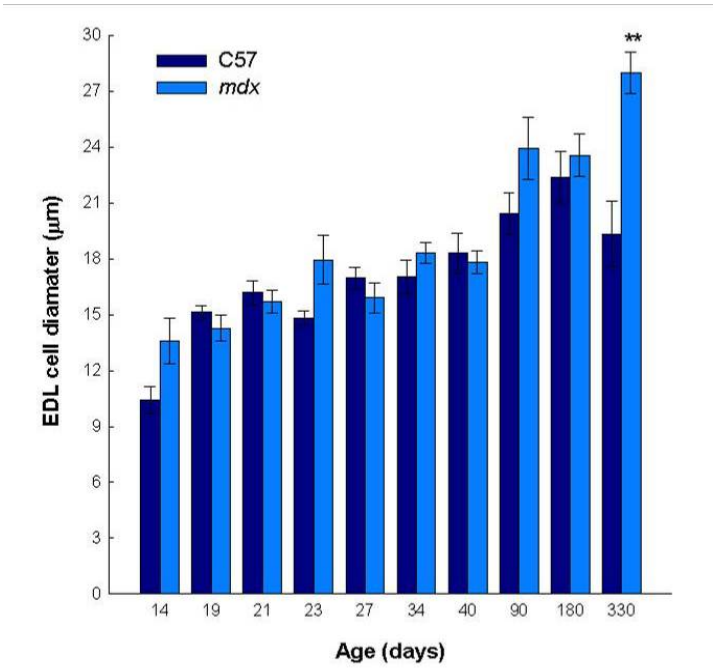


Figure 7-13 EDL cell diameters across varying ages in male mice.

*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

7.6 Influence of age on SOL characteristics

7.6.1 SOL function

At four specific ages, 19 days ($p<0.01$), 21 days ($p<0.05$), 23 days ($p<0.01$), and 330 days ($p<0.01$) *mdx* sPt forces were 45%, 40%, 82%, and 31% respectively lower than C57s (Figure 7-13). At 14 days of age *mdx* forces were lower than C57 forces albeit non-significant. At 27, 40, 90, and 180 days of age *mdx* SOL sPt were actually greater than C57s (non-significant).

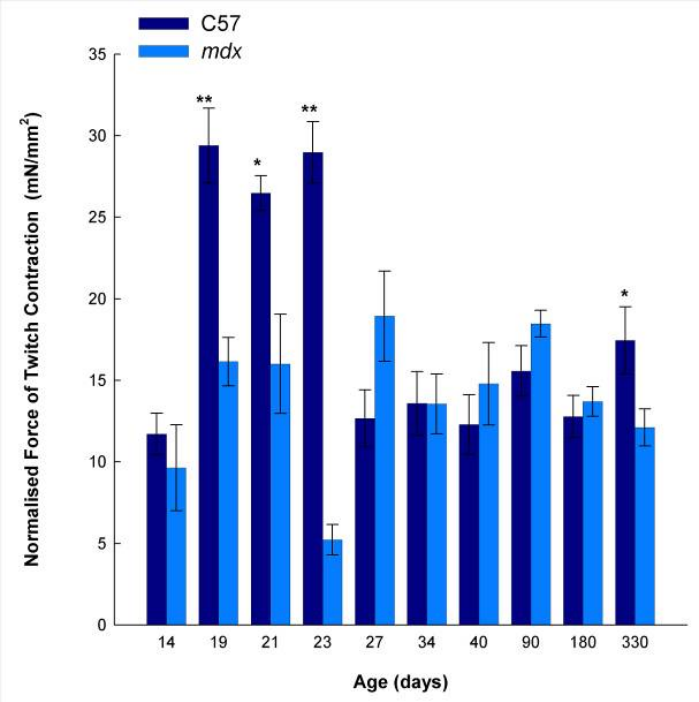


Figure 7-14 SOL sPt across varying ages in male mice.
*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

The only significant ($p<0.01$) differences in SOL sPo were specifically at three ages (19, 21, and 23 days of age) when *mdx* forces were 53%, 49%, and 85% lower than C57 equivalents. At all other ages (both younger and older) there were no significant differences in sPo between strains (Figure 7-15).

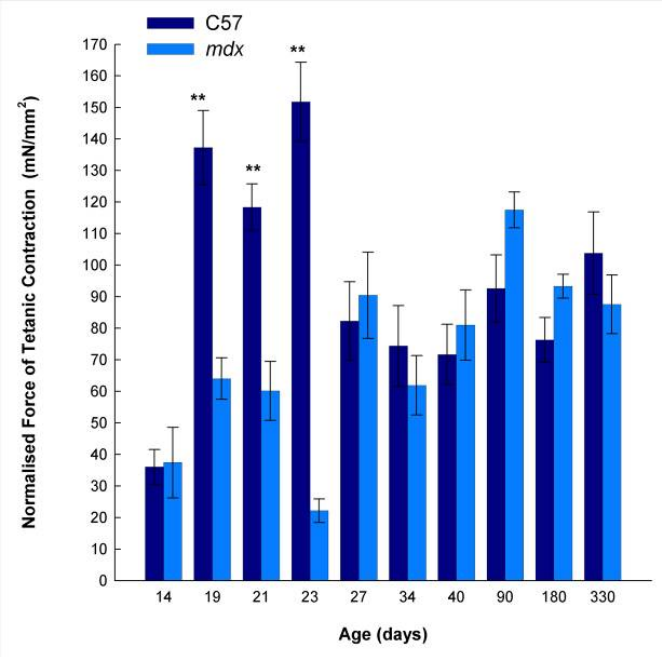


Figure 7-15 SOL sPo across varying ages in male mice.

*** p<0.05, ** p<0.01 between strain comparison. n = 6-10 mice.**

7.6.2 SOL histology

The percentage of SOL CNFs was relatively constant across the ages in C57s. In contrast, *mdx* mice showed relatively constant CNF percentages from 14 to 23 days of age, a significant increase in CNF percentages from 23 days old to 27 days old and a subsequent further increase in CNF percentages with increasing age.

There was no difference in the percentage of centrally nucleated SOL fibres between strains at or younger than 23 days of age (14, 19, 21, or 23 days of age), whilst *mdx* mice from 27 days old (27, 34, 40, 90, 180, and 330 days of age) had 10.5-, 32-, 50-, 65-, 2.58-, and 36.5-fold increase in CNFs than age matched C57s ($p<0.01$). The trend in percentage of CNFs was not mirrored in the tetanic force results for either mouse strain (Figure 7-16).

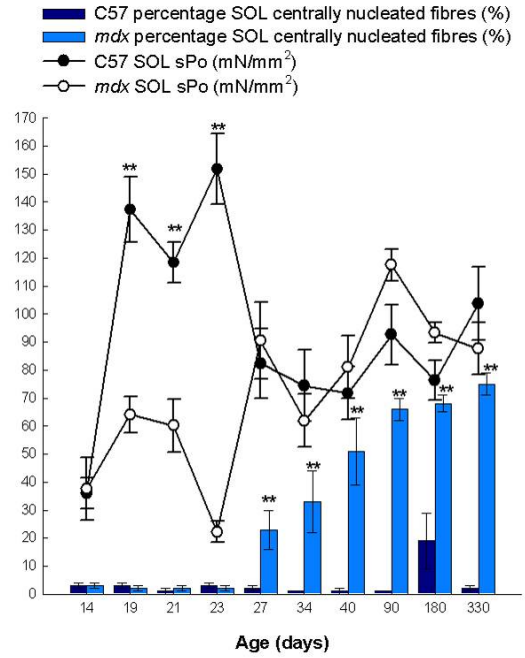


Figure 7-16 SOL percentage of CNFs and sPo across varying ages in male mice. Mouse strain comparison; * p<0.05, ** p<0.01. n = 6-10 mice.

Mdx SOL cell diameters were larger than C57s at 330 days of age. At all other ages studied there was no significant difference in cell diameters between strains (Figure 7-17).

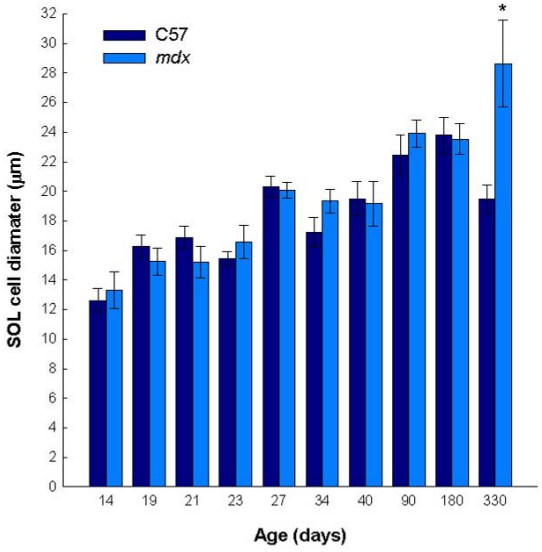


Figure 7-17 SOL cell diameters across varying ages in male mice. Mouse strain comparison; * p<0.05, ** p<0.01. n = 6-10 mice.

7.7 Discussion

Both mouse strains showed a generalised age-related increase in BW from 14 to 180 days of age. At 19, 21, and 23 days old, C57s were significantly heavier than age-matched *mdx*. The results for 21 day old mice are consistent with the findings of Laws (2005) however, these results (albeit for male mice only) were in contrast to Dupont-Versteegden and McCarter (1992) who found no significant differences between *mdx* and C57s in terms of BW in male and female mice ranging in ages from 14 days to two years of age (Dupont-Versteegden and McCarter 1992).

This study found no difference in BW at 180 days of age which concurred with the findings of Pastoret (1995) (Pastoret and Sebille 1995b) and Lynch *et al.* (2001). Age had a differential effect in strains beyond 180 days of age. Lynch *et al.* (2001) found *mdx* mouse BWs declined linearly beyond 180 days of age and showed a continuing increase in weight for corresponding ages in C57s. Conversely, Hayes and Williams in a 1998 study of male mice aged eight to 10 months old, found no difference in BW between *mdx* and C57s. Laws (2005) found that from 12 months of age *mdx* showed declining weight relative to C57s, and this study showed *mdx* being significantly heavier than C57s at 330 days of age. At 330 days of age, *mdx* males were heavier than controls, whereas C57s showed a decrease in BW from 180 to 330 days old.

The BW results were not mirrored in the testes weight results. At all ages, C57 testes were heavier than corresponding *mdx* testes (significant at 21, 23, and 27 days of age). At 19 days of age C57s were heavier than *mdx* but there was no difference in testes weight. At 21 and 23 days of age both BW and testes weights were higher in C57s compared to *mdx* and, conversely, at 27 days of age C57 testes weight was heavier than *mdx* despite no difference in BW (Figure 8-2). No comparative studies were found with respect to testes weights across the ages in *mdx*.

In 1836 the first case describing clinical evidence of cardiac disease in DMD was reported (Nigro *et al.* 1990). Subsequently, extensive research has been conducted into the cardiac manifestations of the disease in humans and it is currently agreed that both the incidence and severity of cardiac

abnormalities in DMD increase progressively with age. There are significant discrepancies between the *mdx* mouse model and the human disease form and Grady *et al.* (1997) argued that the *mdx* mouse model was not a sound model of cardiomyopathy in DMD (Grady *et al.* 1997). Despite widespread use of the *mdx* mouse model in DMD research limited electrophysiological data is available on this species, and the relationship between age and cardiac manifestations has not been clearly defined. Therefore, the objective of the cardiac work in this study was to characterise LA function and responsiveness to Ca^{2+} throughout the lifespan of both the *mdx* and control C57 mouse strains with a view to improving the use of the *mdx* mouse model as DMD cardiac research tool. This study encompasses 10 age groups ranging from 14 days to 330 days of age.

Basal FOC of LA (normalised for LA weight) was only significantly different between strains at two specific ages, namely 14 days and 90 days of age (Figure 7-3). C57 forces at 14 and 90 days of age were greater than *mdx* forces; however, at 21 days of age (an age corresponding to reportedly the peak necrotic period in skeletal muscles in *mdx*) *mdx* forces were actually greater than C57s (non-significant). At 21 days old, *mdx* LAs had a 34% lower weight than C57s, which may account for the increased normalised forces in *mdx*.

Lu and Hoey (2000) studied atrial function in 12 week old *mdx* and C57 males and showed significantly altered contractile activity and efficacy to Ca^{2+} between strains (Lu and Hoey 2000b).

This study of *mdx* mouse cardiac function did not show a linear age related decline in function as is generally reported in human DMD research findings. In fact, at three ages studied (19, 21, and 330 days) *mdx* maximum FOC of LA (normalised for LA weight) values were actually greater than C57s (in direct contrast to human reports in which cardiac function in DMD patients is age-related and consistently lower than age-matched controls).

Skeletal muscle weights and CSAs were reflective of BW trends in both mouse strains. At two specific ages (19 days and 23 days) C57 SOLs were significantly heavier than *mdx* muscles. This situation was reversed at 90, 180, and 330 days of age where *mdx* EDL muscles were heavier than C57s. Results suggested atrophy of fast-twitch (EDL) muscles of *mdx* at

younger ages and hypertrophy at older ages. *Mdx* EDL CSAs were significantly lower than C57s at 23 and 34 days of age but higher at 180 and 330 days of age.

Slow-twitch (SOL) muscles showed a similar growth pattern to EDL muscles in that 21 day old C57s had heavier muscles than *mdx* whilst 90,180, and 330 day old *mdx* had heavier EDL muscles. CSAs were only significantly different between strains at 21 days (C57s heavier than *mdx*) and 330 days (*mdx* heavier than C57s).

Hayes and Williams (1998), in a study of male mice aged eight to 10 months old, found no difference in SOL or EDL weights between *mdx* and C57s, this is in contrast to the results of this study which showed both EDL and SOL skeletal muscles being heavier in 330 day old *mdx* mice compared to C57 controls.

Reports vary amongst researchers as to the period of peak necrosis in the male *mdx* mouse. Figure 7-16 is a schematic representation of indicators of peak skeletal muscle necrosis in *mdx* mice; Granchelli *et al.* (1995) reported histological evidence of necrosis in gastrocnemius (mixed type-I and type-II muscle fibres) muscles in *mdx* from two to eight weeks of age, Muntoni (1993) showed spontaneous motility and treadmill motor activity was grossly affected from the end of the second week of life up to the fifth week (Muntoni *et al.* 1993), Karpati (1988) observed that the number of CNFs in *mdx* limb muscles rises sharply from 20 to 60 days of age (Karpati, Carpenter and Prescott 1988), Spencer's group (1995) showed a significant rise in muscle calpain levels at four weeks of age (Spencer, Croall and Tidball 1995), Ridge (1994) noted that *mdx* muscle fibres displayed extensive disruption of surface (myotendinous junction) features at four weeks of age (Ridge *et al.* 1994), and in 2004 Salimina *et al.* concluded that skeletal muscles of male *mdx* mice show increased sarcolemmal permeability, numerous inflammatory foci, and marked deposition of the extracellular matrix components (ECM) type-I collagen and laminin at six weeks of age (Salimena, Lagrota-Candido and Quirico-Santos 2004).

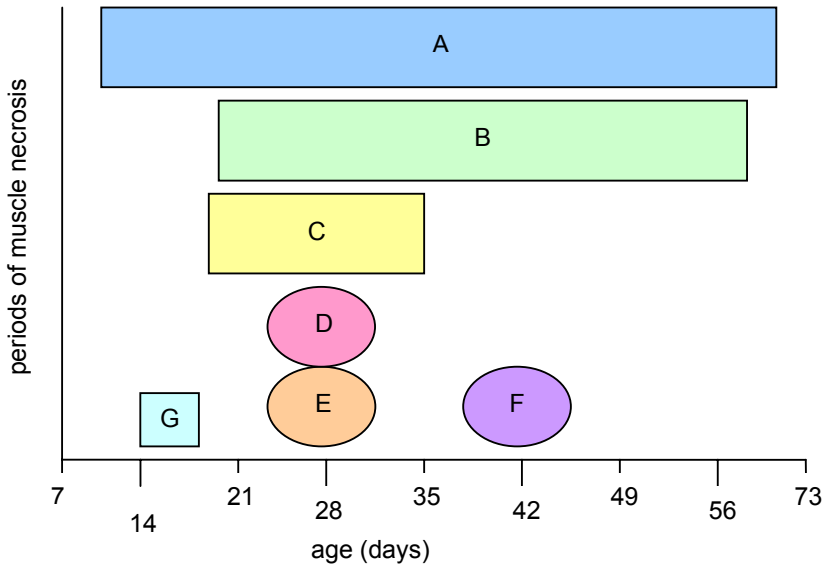


Figure 7-18 Periods of peak skeletal muscle necrosis in the *mdx* mouse.

(A) (Granchelli, Pollina and Hudecki 1995) (B) (Muntoni *et al.* 1993) (C) (Karpati, Carpenter and Prescott 1988) (D) (Spencer, Croall and Tidball 1995)(E) (Ridge *et al.* 1994) (F) (Salimena, Lagrota-Candido and Quirico-Santos 2004) and (G) (Roma *et al.* 2004)

Roma (2004) found there was temporal correlation between the decline of utrophin at 15 days of age and the onset of muscle necrosis. Conversely, reappearance of utrophin, with a peak around 2 months of age, was followed by a progressive decline of necrosis. They concluded that, a lineal correlation between utrophin and β-dystroglycan levels, not seen in C57 controls, indicated that functional improvement in *mdx* may be due to utrophin binding to dystrophin-associated glycoproteins (Roma *et al.* 2004).

Lefaucheur *et al.* (1995) maintained that in dystrophanopathies, predominantly slow-twitch 'postural' skeletal muscles such as the SOL are affected to a greater degree than fast-twitch 'locomotion' muscles such as the EDL (Lefaucheur, Pastoret and Sebille 1995).

Lynch (2001) proposes that the measurement of power output of limb muscles in the *mdx* mouse is arguably the most important physiological measurement of skeletal muscle function. However, it is extremely difficult to make direct comparisons with the work conducted by other researchers due to the lack of consistency in experimental protocols (between researchers), particularly in skeletal muscle function studies, the problem being further

escalated by the fact that there is limited research data available on force generation in *mdx* skeletal muscles (Deconinck *et al.* 1998; Lynch, Hinkle and Faulkner 2000).

Dangain (1984) maintained that muscle necrosis in the *mdx* was followed by subsequent regeneration and restoration of function (Dangain and Vrbova 1984). Pastoret and Sebille (1995) concluded that in the EDL muscles of *mdx* mice up to 12 months of age, necrosis is compensated for by vigorous regenerative processes (Pastoret and Sebille 1995a; Pastoret and Sebille 1995b). Therefore, one would expect to observe no significant differences in EDL contractile forces between strains within this age frame (and would encompass all experimental ages tested in this trial).

Dupont-Versteegden and McCarter (1992) found no age-related changes in the isometric contractile properties of SOL or EDL muscles in *mdx* or control C57 mice. However, the study was conducted using both male and female mice of ages 14 days to two years of age, thus not allowing for potential sex or age differences within strains (Dupont-Versteegden and McCarter 1992). Anderson *et al.* (1988) found that in *mdx* EDL at three weeks, twitch and tetanus tensions were significantly less, and time-to-peak twitch tensions were significantly faster than in control EDL. By 32 weeks, *mdx* EDL twitch and tetanus tensions expressed relative to muscle weight continued to be significantly lower than in age-matched controls, despite normal absolute tensions (Anderson, Bressler and Ovalle 1988).

This study found that at only five of the 10 ages tested (for EDL muscles) and four of the 10 ages tested (for SOL muscles) there were significant differences in specific force production (sPo) between strain. Fast-twitch (EDL) sPo forces were significantly greater in C57s compared to *mdx* specifically at 19, 21, 23, 27, and 330 days of age. This suggests that the use of the *mdx* model for functional studies on EDL muscle most appropriately would utilise mice at these ages.

Anderson *et al.* (1988) found that isometric twitch and tetanic tensions in SOL were significantly less than in controls at 4 weeks, but by 32 weeks, values were not different from age-matched controls. They also found that isometric twitch and tetanic forces produced by the SOL of *mdx* and controls at 32 weeks of age were not significantly different (Anderson,

Bressler and Ovalle 1988). This trial showed that slow-twitch (SOL) sPo forces were significantly higher in C57s at 19, 21, 23, and 330 days of age. This also suggests that the use of the *mdx* model for functional studies on SOL muscle is most appropriate at those specific ages. SOL results were not consistent with Anderson *et al.* (1988) who found no force production differences between strains in four week old mice. This study also reiterates the importance of choosing an appropriate age for studies in the *mdx* mouse and that specific ages may or may not be appropriate for the study of specific skeletal muscles.

Research reports of the histological features of *mdx* muscle across the ages vary; at 180 days old Lynch (2001) found that C57s had normal structure (peripherally located nuclei) whilst *mdx* EDL and SOL muscles had ~60% centrally located nuclei. This study concurred with Lynch (2001) on C57 structure and showed 67±2% and 68±3% CNFs in EDL and SOL muscles of *mdx,* respectively. Lynch (2001) also reported an increase in the number of CNFs in *mdx* mice over 180 days of age that was not evident in C57 controls. Laws and Hoey (2004) demonstrated significantly higher percentages of CNFs in the latissimus dorsi, longissimus dorsi, diaphragm, and intercostal muscles of 17 month old *mdx* mice compared to controls (Laws and Hoey 2004).

The most overwhelming histological finding in this study was the observation of high percentages of CNFs in *mdx* EDL and SOL specifically from 27 days of age through to the oldest mice studied at 330 days of age. This is consistent with other researchers including Bulfield *et al.* (1984), Carnwath and Shotton (1987), Coulton *et al.* (1988), Zacharias and Anderson (1991) and Pastoret and Sebille (1995).

Cell diameters between strains were not significantly different for either EDL or SOL muscles except at 330 days of age when *mdx* cell diameters were larger than C57s in both muscle types. This was inversely related to muscle function where C57s produced significantly greater specific forces (for both EDL and SOL) than *mdx* mice at the same age. The only group in which SOL and EDL muscles cell diameters were greater than 25 μm was 330 day old *mdx* mice. All other groups in both mouse strains and for both EDL and SOL muscles had cell diameters below 25 μm. This study (as

with all other trials in this project) was unable to support the theory of Karpati (1986) which proposed that there is a functional benefit in muscle cells below 20 to 25 microns in diameter.

The results of this ageing study support the hypothesis that the appropriateness of the *mdx* mouse model of DMD (and its use in comparative studies with the C57 control strain) is limited to specific muscles at specific ages. It may be argued that the *mdx* mouse model at 14 and 90 days of age is an appropriate model for atrial contractility studies; that from 19 to 27 days of age and at 330 days of age are the most appropriate ages for fast-twitch (EDL) functional studies; and that 19 to 23 days and 330 days are useful ages for studying slow-twitch (SOL) muscle function in the *mdx* mouse.

8 Conclusions and future directions

Duchenne muscular dystrophy (DMD) is an X-linked, recessive genetic disorder affecting one in 3500 live male births across all human cultures and socio-economic groups. The disease affects cardiac and skeletal muscles of the body, is progressive and ultimately fatal, and no cure is currently available. DMD is caused by mutations in the gene encoding the protein dystrophin, which is currently the largest identified human gene, and the precise mutation is specific to each individual patient. A unique clinical case involving a boy with idiopathic growth hormone deficiency and DMD showed an uncharacteristically benign progress of the disease. Furthermore, it is known that clinical disease symptoms of DMD are exacerbated with the onset of puberty (coinciding with a surge in circulating sex hormone levels) and clearly raises the question as to the specific role of sex hormones in the progression of the disease.

A number of both naturally occurring and engineered animal models of DMD exist, however they differ considerably in terms of their suitability for research of the human disease. Arguably, the *mdx* mouse remains the preferred DMD experimental animal model due to low cost, speed of reproduction, and wide availability, and was subsequently the model of choice in this research project. The implementation of standardised protocols for the assessment of cardiac function, skeletal muscle function, histological parameters, and treatment strategies using the *mdx* mouse model is imperative to the successful future DMD research.

This dissertation examined the role of sex hormone status in cardiac and skeletal muscle function in the *mdx* mouse model of DMD and is the first study to co-quantify muscle-, sex-, and age-specific differences in the *mdx* mouse. Initially, the effects of testosterone-treatment were examined, followed by an investigation of sex (gender) differences, determination of the effects of surgical castration, in addition to the effects of oestrogen treatment in both the *mdx* and C57 control strains. Furthermore, cardiac and skeletal muscle function was characterised across a range of ages in order to improve the applicability of the *mdx* mouse model for research.

Representative muscles included the left atrium (cardiac muscle), extensor digitorum longus (fast-twitch skeletal muscle, type-I), and soleus (slow-twitch skeletal muscle, type-II).

Cardiac muscle studies revealed that, at 35 days of age, *mdx* left atrial basal forces were 29% lower than C57s (when normalised for left atrial weight) however, results were not significant due to large variability in values (and subsequent standard errors) for *mdx* compared to C57s. These results show that left atrial basal contractility is compromised in the *mdx* mouse. Maximum left atrial forces generated by C57s were greater than *mdx* forces for controls, 1.0, 1.5, and 2.0 mg/kg/day testosterone-treated mice (despite testosterone not altering maximum left atrial contractility within either strain). Calcium concentration response curves revealed a varied pattern of response between strains with *mdx* showing a more flattened curve (rather than the traditional sigmoid curve shown in C57s) and implying a dampened responsiveness to calcium in the *mdx*. Most importantly, testosterone treatments (1.0, 1.5, and 2.0 mg/kg/day) caused left atrial hypertrophy in the *mdx* (which was not evident in C57s) that did not translate to increased force generation. Sex (gender) differences in the *mdx* mouse model were investigated in order to provide research information directly applicable to female DMD carriers and to further elucidate the role of sex hormones (both male and female) in the disease. Cardiac studies found that left atrial forces in the *mdx* strain were not different between males and females at any age examined (90, 180, 330 days of age) and that both male and female *mdx* had a dampened left atrial responsiveness to calcium, suggesting that *mdx* mice of both sexes from 90 to 330 days of age have cardiac abnormalities relative to C57 control mice. Surgical castration of male mice (castration at 42 days of age, euthanasia at six months of age) caused no significant functional cardiac effect between strains whereas differences within strains were evident. C57 castrates showed higher left atrial forces than entires and yet *mdx* castrates showed consistently lower forces than entires. Having determined that testosterone-treatment caused left atrial hypertrophy without subsequent force increases, the fact that surgical castration (and the resultant sex hormone removal) produced decreased left atrial forces in the *mdx*, and that left atrial forces were not different between male and female

mdx, the cardiac effects of oestrogen-treatment in *mdx* males was examined. Oestrogen showed a significant and positive effect on left atrial contractility. Basal left atrial forces were increased at all oestrogen doses (significant at 0.08 and 0.16 mg/kg/day), maximum left atrial forces mirrored basal forces (significant at 0.16 mg/kg/day), and calcium concentration response curves showed a highly significant increase in left atrial forces for all calcium concentrations in *mdx* treated with a dose of 0.16 mg/kg/day oestrogen. This research showed that, in the *mdx*, cardiac function is compromised compared to C57s, that testosterone-treatment produces left atrial hypertrophy without functional benefit, that both male and females have reduced cardiac contractility without sex (gender) differences within the strain, and that oestrogen-treatment can produce improved responsiveness to calcium and increased left atrial contractility.

Skeletal muscle functional studies using extensor digitorum longus (fast-twitch, type-I) muscles and soleus (slow-twitch, type-II) muscles were conducted in the sex (gender), castration, and oestrogen trials. It was imperative that both muscle fibre-types were studied independently as it is known that different skeletal muscle fibre-types have distinctive roles; fast-twitch muscle fibres (as predominating in the EDL) are involved in locomotion and ambulation whereas slow-twitch fibres (as predominating in the SOL) are responsible for maintaining posture.

Both male and female *mdx* EDL muscles were hypertrophic compared to C57s but this muscle hypertrophy did not translate to increased force generation. Surgical castration (and the resultant removal of circulating sex hormones including testosterone) resulted in significantly larger EDL tetanic forces in both *mdx* and C57s (irrespective of lowered muscle weights). Despite a lack of statistical significance, results were encouraging in terms of the potential of oestrogen treatment to improve force production in *mdx* EDL muscles with oestrogen-treated *mdx* showing increases of between ~15% and ~34% compared to *mdx* controls.

The gender (sex) trial results for SOL muscles mirrored that of EDL muscles with *mdx* males and females showing muscle hypertrophy (compared to C57 equivalents) which failed to translate to greater forces than control C57s. However, surgical castration failed to result in increased force

production in the slow-twitch SOL muscle in the *mdx*. This was in contrast to the effect seen in the EDL muscles of *mdx* castrates and suggests that fast- and slow-twitch skeletal muscles responded differently to surgical castration in the *mdx*. Once again, the results of oestrogen-treatment on SOL muscle function mirrored the effects produced in EDL muscles and an oestrogen treatment of 0.32 mg/kg/day (despite producing no significant differences in muscle weight compared to *mdx* controls) elicited a ~29% increase in SOL specific muscle force compared to un-treated *mdx*.

Few studies specifically characterise the *mdx* mouse model across many different ages. This study determined left atrial, EDL, and SOL muscle function at 10 ages ranging from 14 to 330 days of age with a view to improving the use of the *mdx* mouse model as a DMD research tool. Cardiac function studies showed that basal left atrial forces in the *mdx* were only significantly different between strains at two specific ages, 14 days and 90 days of age. Skeletal muscle studies (quantifying specific force production) isolated significant differences between *mdx* and C57s at 19, 21, 23, and 330 days of age in both EDL and SOL muscles, deeming these ages to be the most appropriate ages for use of the model in skeletal functional studies.

Clearly, this research has revealed a number of important findings. Firstly, testosterone-treatment does not produce functional benefits in cardiac muscle (left atria), fast-twitch (EDL) skeletal or slow-twitch (SOL) skeletal muscles in the *mdx*. It is evident that a hypertrophied muscle is not necessarily a muscle which can generate greater forces, and this challenges the use of androgenic compounds in the pharmacological treatment of DMD. In addition, the studies conducted show that oestrogen-treatment has therapeutic potential in the treatment of DMD through mechanisms which increase force generation in both cardiac and skeletal muscles. However, there are a number of psychosocial considerations that would need to be considered before oestrogenic drugs may be considered appropriate. Rather, future research should focus on the mechanisms underlying the benefits of oestrogenic treatments so that these mechanisms could be targeted effectively. Finally, this project further characterised the *mdx* mouse model concluding that the model can be used for the study of specific muscles most appropriately when it is at specific ages.

"Motion is life….."

Guillaume Benjamin Amand Duchenne de Boulogne

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10 APPENDIX I

10.1 Krebs physiological salt solution (KPSS) recipe

KPSS Stock Solutions (in 1.0L milliQ H_2O)

	Final Concentration (mM)	Weight (g)
STOCK A		
NaCl	118	137.9
KCl	4.7	7
$MgSO_4.7H_2O$	1.16	5.7
KH_2PO_4	1.18	3.2
STOCK B		
$NaHCO_3$	25	42
STOCK C		
$CaCl_2.2H_2O$	2.5	7.35

Working KPSS

STOCK A	100mL
STOCK B	100mL
STOCK C	100mL
Glucose ($C_6H_{12}O_6$)	4.0g

(made up to total volume of 2.0 L with milliQ H_20)

10.2 Tyrode's physiological salt solution (TPSS) recipe

TPSS Stock Solutions

	Final Concentration [mM]	Weight (g)
STOCK A		
NaCl	136.90	160.00
KCl	5.40	8.10
$MgCl_2.H20$	1.05	4.30
$NaHPO_4.2H_20$	0.42	1.30
STOCK B		
$NaHCO_3$	22.60	38.00
STOCK C		
$CaCl_2.2H_20$	1.80	5.30

Working TPSS

Stock A	100mL
Stock B	100mL
Stock C	100mL
Glucose ($C_6H_{12}O_6$)	2.0 g
L- Ascorbic acid ($C_6H_7O_6Na$)	0.2 g
Na_2EDTA (4.5%)	0.8mL

(made up to total volume of 2.0 L with milliQ H_20)

All chemicals were purchased from Sigma Chemical Co., St Louis, U.S.A. unless otherwise specified.

10.3 Haematoxylin stain (Mayer's Haematoxylin) recipe

Combine 0.5 g Mayer's Haematoxylin powder (Ajax Chemicals, Auburn, Australia) with 500 mL ddH_2O. Heat at 50º C - 60º C on a magnetic stirrer until dissolved. Add 25 g aluminium potassium sulphate (AlK $(SO_4)_2.12H_2O$, stir until dissolved. Add 100 mg sodium iodate ($NaIO_3$). Cool overnight. Adjust pH by adding 0.5 g citric acid (HOC (COOH) (CH_2COOH)) (Ajax Finechem, Seven Hills, Australia). Add 10 g chloral hydrate ($CCl_3CH(OH)_2$) (Asia Pacific Specialty Chemicals Limited, Seven Hills, Australia) to preserve solution. Store at 4º C. This volume is sufficient to stain approximately 500 slides (Paul Addison, University of Queensland, *personal communication,* 2003).

10.4 Eosin stain recipe

Make up eosin stock solution by adding 2 g Eosin yellowish (eosin Y) powder (George T Gurr Ltd., London, England) to 40 mL ddH_2O and 160 mL 95% EtOH. Mix until dissolved. To make up working solution add 150 mL eosin stock solution to 450 mL 80% EtOH. Mix well. Store at 4º C. This volume is sufficient to stain approximately 200 slides (Paul Addison, University of Queensland, *personal communication,* 2003).

10.5 Scott's solution recipe

To 1000 mL ddH_2O add 4.5 g magnesium sulphate ($MgSO_4.7H_2O$) and 3.75 g sodium bicarbonate ($NaHCO_3$). Mix well. Store at 4º C to room temperature 22º C - 23º C.