The effect of taurine on dystrophic muscle tissue function

Submitted by

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a lethal X-linked genetic disorder which results in chronic degeneration of skeletal muscle, significantly impacting on the duration and quality of life. Despite the genetic defect and the missing protein dystrophin having been identified and characterised over 20 years ago, curative genetic therapies are still not clinically applicable, and corticosteroids, which are the only significantly beneficial treatment option currently available to DMD patients, are associated with several side-effects. Thus, there is a need for additional therapeutic interventions that can improve skeletal muscle function and delay the onset of severe pathology in dystrophy.

The amino acid taurine is essential for normal skeletal muscle function, and has been shown to act on several factors thought to be key contributors to the development of skeletal muscle pathology in dystrophy. Moreover, as dystrophic skeletal muscle demonstrates a significant decrease in taurine content, it is possible that raising intramuscular taurine stores may preserve muscle function in dystrophy, and thus have potential therapeutic applications. Despite this, only two studies have ever examined the effect of taurine supplementation on dystrophic muscle function.

The purpose of this thesis was to examine the effect of taurine on dystrophic skeletal muscle function, which was performed in three studies using the dystrophic *mdx* mouse as a model for DMD. Study 1 (Chapter 4) investigated the effect of taurine supplementation throughout the early *mdx* lifespan (time-points from day 28 to day 70), where skeletal muscle pathology is more homologous to the human condition, to determine if taurine supplementation could significantly increase skeletal muscle taurine content in the *mdx*, despite the significant muscle degeneration that occurs at this stage. The expression of excitation contraction (E-C) coupling, calcium (Ca^{2+}) handling and contractile proteins were also investigated, to determine if the beneficial effects of taurine supplementation on muscle function are associated with changes in protein expression. Taurine supplementation successfully increased skeletal muscle taurine content in the *mdx* mouse despite a significant depression in taurine transporter

expression, and also increased the expression of the Ca^{2+} handling protein calsequestrin (CSQ) as well as the contractile proteins actin.

Study 2 (Chapter 5) examined the effect of long and short-term taurine supplementation in the 6 month old *mdx* mouse on contractile function and the activity of several key metabolic enzymes. This study found that while taurine supplementation is unable to improve peak tetanic or twitch force, that long-term taurine supplementation significantly reduced fatigue in the *mdx* extensor digitorum longus (EDL) muscle. In addition, taurine supplementation altered the activity of phosphofructokinase (PFK) and beta-hydroxyacyl CoA dehydrogenase (*β*-HAD) in the EDL, and creatine kinase (CK) and citrate synthase (CS) in the soleus (SOL).

The final study (Chapter 6) investigated the effect of altering skeletal muscle taurine content in non-dystrophic and dystrophic fast and slow-twitch muscle on contractile function, assessed using a protocol that utilised stimulation frequencies and a bath temperature that is similar to what the muscles investigated would experience *in vivo*. Taurine effectively increased intramuscular stores of taurine while *β*-alanine significantly depleted them. Despite these alterations in taurine content, no significant differences in peak tetanic or twitch force were observed in control or *mdx* mice, although taurine did significantly reduced susceptibility to fatigue in the EDL of the *mdx* mouse, while *β*-alanine significantly reduced fatigue in both the *mdx* and control mice.

In conclusion, this thesis has demonstrated that taurine supplementation is able to significantly increase the intramuscular stores of taurine in dystrophic skeletal muscle, while *β*-alanine treatment significantly depletes taurine stores. Taurine did not improve peak tetanic or twitch force in the *mdx* mouse, while taurine depletion with *β*-alanine did not reduce muscle function, an effect likely due to beneficial effects of *β*-alanine itself. Taurine did, however, significantly improve resistance to fatigue and recovery in fast-twitch dystrophic skeletal muscle. In the young *mdx* mouse, taurine supplementation increased the expression of contractile proteins and CSQ, while in older (6 month) *mdx* mice taurine supplementation has no significant effect on any of these measures. Thus, it appears that the beneficial effects of taurine supplementation are highly dependent on the age of the mice at experimentation, as well as the level of muscle damage at the time of treatment.

VICTORIA UNIVERSITY

CANDIDATE DECLARATION

"I, Deanna Maree Horvath, declare that the PhD thesis entitled:

The effect of taurine on dystrophic muscle tissue function

Is no more than 100, 000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Full Name Deanna Maree Horvath

Signed …….

Date……..

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LIST OF PUBLICATIONS AND AWARDS

PAPERS

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PRESENTATIONS

Horvath, D. M., Hayes, A., & Goodman, CA. (2010). The effect of taurine and *β*-alanine supplementation on taurine content and contractile properties of skeletal muscle in the *mdx* mouse. *Proceedings of the Australian Physiological Society* (Poster Presentation)

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ABBREVIATIONS

Abs/min	Absorbance per minute
ACS	Analytical consulting services
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ARC	Animal resource centre
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUS	Australia
β	Beta
β-HAD	Beta-hydroxyacyl CoA dehydrogenase
BAL	Beta-alanine
BSA	Bovine serum albumin
$^{\circ}C$	Degrees celsius
c	centimetres
C57BL/10	C57 black ten mouse
Ca^{2+}	Calcium
CAC	Citric acid cycle
$CaCl_2$	Calcium chloride
CAT	Catalase
CCD	Charge coupled device
CK	Creatine kinase
Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide
CoA	Coenzyme A
CON	Control
Cr	Creatine
CS	Citrate synthase
CSA	Cross sectional area

CSQ	Calsequestrin
CXMDJ	Canine X-linked muscular dystrophy (Japan)
DAPC	Dystrophin associated protein complex
ddH_20	Double distilled water
DHAP	Dihydroxyacetone phosphate
DHPR	Dihydropyridine receptor
DIA	Diaphragm
DMD	Duchenne muscular dystrophy
DSHB	Developmental studies hybridoma bank
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
dw/ww	Dry weight per wet weight
EBD	Evan's blue dye
E-C	Excitation-contraction
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
F-1,6-P_2	Fructose 1,6- diphosphate
F-6-P	Fructose-6-phosphate
FAD	Flavin adenine dinucleotide
FMOC	Fluorenylmethyloxycarbonyl
g	Grams
g/cm^3	Grams per centimetres cubed
G-3-P	Glyceraldehyde- 3-phosphate
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GAST	Gastrocnemius
GES	Guanidinoethane sulfonate
GLUT1	Glucose transporter -1
GLUT4	Glucose transporter -4
GRMD	Golden retriever muscular dystrophy
GSH	Glutathione
H^+	Hydrogen free radical
H_2	Hydrogen
H_20	Water

H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HCO_3^-	Bicarbonate
HK	Hexokinase
HOCl	Hypochlorous anion
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor -1
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Intra-peritoneal
I-R	Ischaemia reperfusion
K^+	Potassium
KATP	Potassium adenosine triphosphate
KCl	Potassium chloride
kDa	Kilodaltons
KH_2PO_4	Potassium dihydrogen phosphate
L_f	Optimal muscle fibre length
L_o	Optimal muscle length
L-Tau	Long-term taurine supplemented
M	Molar
m/min	Metres per minute
MANOVA	Multiple analysis of variance
mdx	Mouse muscular dystrophy
MEP	Mitochondrial encoded proteins
mg	Milligram
mg/kg	Milligram per kilogram
$mg.kg^{-1}$	Milligram per kilogram per minute
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_47H_20$	Magnesium sulfate heptahydrate
MHC	Myosin heavy chain
µ/L	Microlitre/litre

µ/ml	Microlitre/millilitre
µl	Microlitre
µmol	Micromole
µmol/g/ww	Micromole per gram of wet weight
µmol/min/g	Micromole per minute per gram
min	Minutes
mM	Millimolar
mN	Millinewton
mN^2	Millinewton squared
MPO	Myeloperoxidase
msec	Milliseconds
MT	Mechanical threshold
Mt-CK	Mitochondrial creatine kinase
MyoD	Myogenic differentiation factor -1
n	Number
N/cm^2	Newton per centimetre squared
Na^+	Sodium
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NAD^+	*β*-Nicotinamide adenine dinucleotide
NADPH	*β*-Nicotinamide adenine dinucleotide phosphate hydrate
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear factor kappa B
nM	Nanomolar
NMR	Nuclear magnetic resonance
nNOS	nitric oxide synthase
NO	Nitric oxide
O_2	Oxygen
O_2^-	Superoxide
Ox-Phos	Oxidative phosphorylation
PBS	Phosphate buffered saline
Pcr	Phosphocreatine
PDN	α-methyl prednisolone

PFK	Phosphofructokinase
PIC	Protease inhibitor cocktail
PLANT	Plantaris
P_o	Maximum tetanic force
P_t	Peak twitch force
P-T	Permeability-transition
½ RT	Half relaxation time
ROS	Reactive oxygen species
RPM	Revolutions per minute
RyR	Ryanodine receptor
SAC	Stretch activated channels
SD	Standard deviation
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase pump
SOD	Superoxide dismutase
SOL	Soleus
sP_o	Maximum specific force
SR	Sarcoplasmic reticulum
S-Tau	Short-term taurine supplemented
TA	Tibialis anterior
Tau	Taurine
TauCl	Taurine chloromine
TauT	Taurine transporter
TBST	Tris buffered saline
TNFα	Tumour necrosis factor-alpha
tRNA	Transfer RNA
TTP	Time to peak
T-tubule	Transverse-tubule
UNT	Untreated
UTRN	Utrophin
v	Volume
V	Volts
v/v	Volume per volume
w/v	Weight per volume

CHAPTER ONE

PERSPECTIVES

1.1 Thesis scope

Duchenne muscular dystrophy (DMD) is a lethal X-linked genetic disorder that results in the absence of the cytoskeletal protein dystrophin and affects one in every 3,500 live male births (Bushby et al., 2010b, Emery, 1995, Koenig et al., 1987). This makes DMD the second most common single gene disorder in Western countries and the most common of the muscular dystrophies (Bogdanovich et al., 2004). The lack of dystrophin leads to chronic degeneration of skeletal muscle with progressive muscle weakness, characteristic atrophy and replacement of myofibres with fat and endomysial fibrosis (Desguerre et al., 2009, Turgeman et al., 2008). The eventual result of this damage for affected boys is severe muscle wasting that necessitates permanent use of a wheelchair by 10-12 years of age, and death from respiratory or cardiac failure by their early twenties (Emery, 1995, Pellegrini et al., 2004).

Despite the fact that the genetic abnormality leading to the development of DMD was identified and characterised more than 20 years ago, the disease remains incurable with very few beneficial treatment options available to sufferers (Mendell et al., 2010). Research continues into molecular therapeutic approaches such as gene replacement therapy (Ohtsuka et al., 1998, Tremblay et al., 1993), stem cell transplantation (Ferrari et al., 1998, Gussoni et al., 1999) and manipulation of myoregulatory growth and development factors (Bogdanovich et al., 2002, Grounds and Torrisi, 2004, Lynch et al., 2001a). However, the widespread use of these techniques as a treatment still seems unlikely in the short term. Currently, the only significantly beneficial treatment available is corticosteroids, commonly prednisolone and deflazacort (Bushby et al., 2010, Griggs et al., 1993, Parreira et al., 2010). While corticosteroids unequivocally improve the quality of life for DMD patients, the harmful side effects that are associated with their use, particularly in young children, limit their application as a long-term therapy for DMD.

For this reason alternative and adjunct treatment options have been sought, such as surgery, physical therapy and nutritional interventions. To date there is some evidence that nutritional compounds such as creatine (Escolar et al., 2005, Louis et al., 2004, Passaquin et al., 2002, Pulido et al., 1998), green tea extract (Buetler et al., 2002, Dorchies et al., 2009), conjugated linoleic acid (Davidson and Truby, 2009) and glutamine (Granchelli et al., 2000, Mok et al., 2008) may be able to alleviate some of the symptoms associated with DMD. However, the search for additional compounds that can specifically target the cellular pathways involved in the development of skeletal muscle pathology in DMD continues (De Luca et al., 2002).

It has recently been suggested that the sulphur-containing amino acid taurine may be a potentially beneficial compound in DMD treatment (Conte Camerino et al., 2004, De Luca et al., 2003, McIntosh et al., 1998a). Taurine, the most abundant free amino acid found in skeletal muscle, has been shown to play a role in cytoprotection, membrane and protein stabilisation, Ca^{2+} homeostasis, antioxidant defence, regulation of inflammation and modulation of E-C coupling (Bakker and Berg, 2002, Conte Camerino et al., 2004, De Luca et al., 2001a, Huxtable, 1992, Schuller-Levis and Park, 2003, Warskulat et al., 2004). Furthermore, significant depletion of taurine in skeletal muscle (as evidenced by the taurine transporter knockout mouse) results in severely reduced exercise capacity, muscle atrophy and cardiac abnormalities (Ito et al., 2010, Warskulat et al., 2007).

Interestingly, many of taurine's actions described above are key therapeutic targets of DMD pathology, and depletion of taurine results in pathological changes within muscle that are consistent with those observed in dystrophic conditions. As the ability to retain taurine appears to be impaired in DMD patients and the *mdx* mouse (a commonly used model for human DMD) (De Luca et al., 2002, McIntosh et al., 1998a), and given the importance of taurine in normal skeletal muscle function, supplementation with taurine could possibly be a new therapeutic strategy for DMD treatment.

Despite the evidence suggesting that taurine may ameliorate DMD pathology there has been very little research specifically investigating the effects of taurine in DMD, although initial results from a few studies have shown promise (Conte Camerino et al., 2004, Cozzoli et al., 2011b, De Luca et al., 1998, McIntosh et al., 1998a). The effect of *in vitro* application of taurine on E-C coupling of the EDL muscle of the *mdx* mouse was examined by De Luca and colleagues in 1998 and again in 2001, with taurine shown to have beneficial effects on membrane stabilisation through changes to mechanical threshold (MT) (De Luca et al., 1998, De Luca et al., 2001a), with the MT shifting towards more positive potentials (a profile associated with sedentary control animals), and maintaining a high level of chloride conductance (De Luca et al., 2003, De Luca et al., 2001a). Furthermore, *in vivo* supplementation with taurine was able to counteract exercise induced loss of forelimb strength (De Luca et al., 2003). Most recently, Cozzoli et al. (2011) evaluated the potential of a combined treatment of taurine and prednisolone on *mdx* muscle function to determine whether synergistic treatment was better than corticosteroid treatment alone. Combined treatment not only improved strength and restored MT to control levels but was also able to decrease the over-activity of a subset of calcium channels thought to be involved in the development of altered Ca^{2+} homeostasis in dystrophic muscle, a key factor in the progression of muscle wasting (Cozzoli et al., 2011b). Given the promising results of the limited studies to date, further investigation is warranted into the potential beneficial effects of taurine on dystrophic skeletal muscle.

Thus, the aim of this thesis was to further investigate the effect of taurine on dystrophic skeletal muscle function. This includes determining if taurine supplementation is able to significantly increase skeletal muscle taurine content in the *mdx* mouse during peak damage, and in adult mice where a chronic but low level of muscle degeneration persists. The protein expression of the taurine transporter (TauT) was also examined, as it has been reported that while taurine content is low in dystrophic skeletal muscle, an increase to 140% of control taurine content has been observed in the plasma of *mdx* mice (De Luca et al., 2001a), suggesting that there may be a alterations in the TauT with dystrophy. Finally, as improvements in muscle function are considered to be an important outcome measure for pharmacological interventions in DMD, contractile function including peak tetanic force, fatigue and recovery are all investigated, as well as the expression of several key E-C coupling and Ca^{2+} handling proteins.

CHAPTER TWO

REVIEW OF THE LITERATURE

2.1 Duchenne Muscular Dystrophy

2.1.1 Dystrophin structure and function

Dystrophin has a large molecular mass of 427kDa and the gene responsible for regulating its expression is the largest in the human genome (Zhou et al., 2006). Structurally, dystrophin is a peripheral membrane protein which is present close to, but not integrated into, the lipid bilayer of the cell, and is responsible for linking the myofibres contractile machinery and associated cytoskeleton to the extracellular matrix (Ozawa et al., 1999). Dystrophin has four distinct domains: 1) the actin domain attaching to the muscle fibre cytoskeleton, 2) a cystein rich domain with two Ca^{2+} binding sites, 3) a rod domain and 4) a carboxy-terminal domain that binds directly to *β*-dystroglycan, α1-syntrophin, nitric oxide synthase (nNOS), calmodulin and α-dystrobrevin forming a link to the sarcoglycans (Niebroj-Dobosz et al., 2001).

Together dystroglycans, sarcoglycans, syntrophin, and dystrobrevin form the dystrophin associated protein complex (DAPC) that is associated with dystrophin expression and is a crucial structural and signalling link across the sarcolemma (See Figure 2.1) (Zhou et al., 2006). In DMD, the absence of dystrophin means this crucial link is not maintained and the expression of the proteins in the DAPC are significantly reduced, with studies in both DMD patients and the *mdx* mouse reporting an 85 % decrease in DAPC constituents and delocalisation of the proteins (Cullen et al., 1994, Ohlendieck and Campbell, 1991, Ohlendieck et al., 1993).

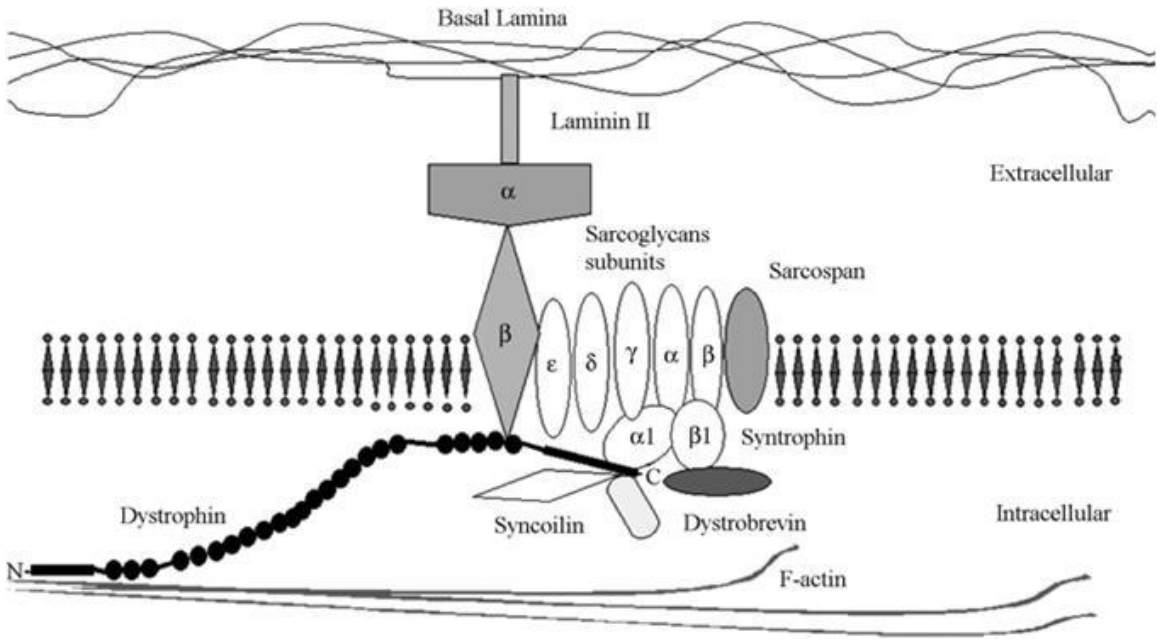


Figure 2.1 The link between dystrophin and the DAPC in skeletal muscle

The figure demonstrates how dystrophin connects the myofibre cytoskeleton to the extracellular matrix via the sarcolemmal DAPC, forming a key structural and signalling component of skeletal muscle. Taken from (Zhou et al., 2006)

The end result of this structural and signalling abnormality in DMD is a cascade of events that increase the fragility of the muscle cell, predisposing the myofibres to damage particularly during contraction (Dellorusso et al., 2001b, Rousseau et al., 2010). This leads to increased membrane permeability and impaired Ca^{2+} homeostasis, which then results in further damage via protease activation and inflammation, impairing contractile function (Deconinck and Dan, 2007). The excess cytosolic Ca^{2+} caused by this disruption also affects the mitochondria which act as sinks for excess Ca^{2+}, causing impaired energy metabolism and an increase in oxidative stress within the muscle (Brookes et al., 2004, Whitehead et al., 2006).

Initially, the muscle of DMD patients is able to repair itself, however, over time the persistent cycles of degeneration-regeneration eventually exhausts this capacity resulting in failure of regenerative processes, with myofibres becoming replaced with adipose and fibrotic connective tissue (Abdel-Salam et al., 2009, Porter et al., 2002, Taniguti et al., 2011). The muscles that are affected depend on the stage of progression of the disease, with some muscles being more susceptible to damage than others. As several mechanisms have been shown to be involved in the development of the DMD pathology, it appears that the cause of the muscle damage is multifactorial and cumulative (see Deconinck and Dan (2007) for a comprehensive review). Some of the major factors thought to be involved in DMD pathogenesis are described briefly below;

2.1.2 Mechanical damage and membrane weakness

As one of the earliest findings in DMD patients was an elevation in muscle specific enzymes (such as CK) in plasma, it was suggested that a lack of dystrophin, and delocalisation of the DAPC, could compromise the integrity of the sarcolemma and thus increase its susceptibility to damage, causing the leak of intracellular proteins (Emery, 2003). Under normal conditions, dystrophin and the DAPC distribute mechanical forces evenly across the sarcolemma ensuring that sarcomere length is uniform across the muscle fibre, thus minimising stress to the sarcolemma (Rousseau et al., 2010, Zhou et al., 2006). In DMD the ability to sustain contraction, particularly eccentric (lengthening) contractions, is significantly reduced (Dellorusso et al., 2001b, Head et al., 1994, Moens et al., 1993) and it has been proposed that the stress imposed during this activity causes the development of membrane tears. Evidence supporting this theory has largely come

from permeability studies using dyes such as Evans blue (EBD) and Procion orange (Deconinck and Dan, 2007, Whitehead et al., 2006). Figure 2.2 demonstrates typical results from this type of study, where EBD appears in dystrophic muscle but is absent from control tissue, indicating increased permeability that is often attributed to tearing (Rooney et al., 2009). Indeed, several studies using limb-immobilisation demonstrate that reducing contractile activity results in significantly reduced signs of dystrophy in young *mdx* mice (Mizuno, 1992, Mokhtarian et al., 1999), while upregulation of the dystrophin homologue utrophin can decrease susceptibility to damage, as evidenced by a lower uptake of EBD, both at rest, and after eccentric contractions (Miura et al., 2009).

However, in direct contrast to this theory it has been suggested that while dystrophic tissue does take up more membrane-impermeable dyes and is significantly more prone to contractile-induced damage, this does not necessary support the theory of an increase in membrane weakness and tearing (Allen and Whitehead, 2011, Whitehead et al., 2006).

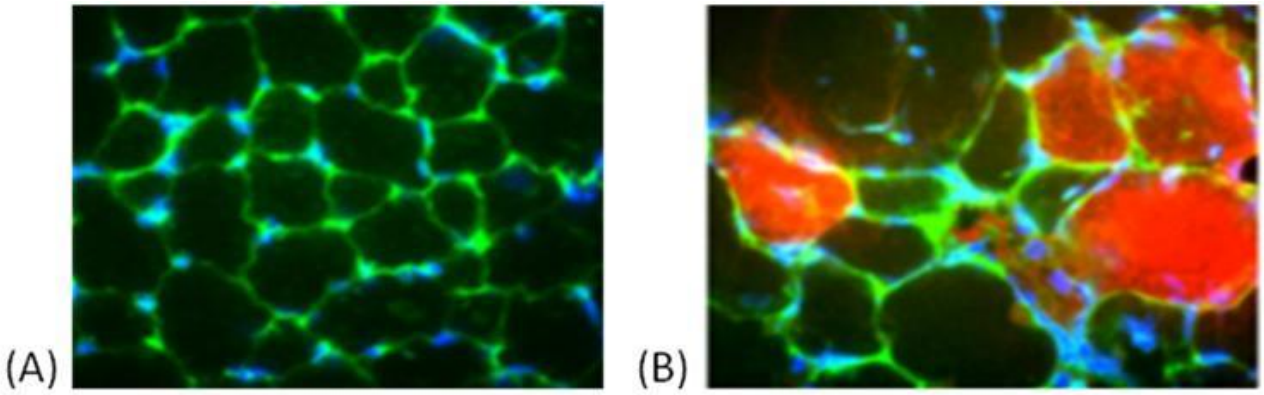


Figure 2.2 EBD in (A) non-dystrophic and (B) *mdx* mouse muscle

Displays the influx EBD into the dystrophic (B) cell (red portions) whereas in non-dystrophic tissue (A), the dye is not seen as it is unable to permeate the membrane as sarcolemmal integrity is maintained. Taken from (Rooney et al., 2009).

Some evidence exists that there is little or no difference in the strength of dystrophic sarcolemma when this is measured directly via suction of membrane patches (Hutter et al., 1991) and actual visualisation of membrane tearing post-contraction has never successfully been observed. In addition, it has been shown that when holes are artificially made in the *mdx* sarcolemma, the repair of this damage occurs in less than one minute, and that the capacity for repair is not different between *mdx* and control muscle (Bansal et al., 2003). These findings lend support to another key theory in dystrophic muscle pathology which suggests that dystrophin may be involved in the aggregation and normal functioning of ion channels in the sarcolemma, with its absence resulting in alterations to normal function. Altered channel activity could lead to increased Ca^{2+} entry into the cell, causing damage to the membrane and thus increased uptake of dyes, rather that dye uptake resulting from tearing in an inherently weak sarcolemma. uptake of dyes in DMD.. Of particular interest are those channels that allow Ca^{2+} entry into the muscle fibre, as altered Ca^{2+} homeostasis has been found in human, canine and murine models of DMD and is thought to be a key factor in the development of necrosis (Bakker et al., 1993, Emery, 2003, Fong et al., 1990, Williams et al., 1990).

2.1.3 Ion channel dysfunction

Alteration in the expression and activity of two main Ca^{2+} channels in skeletal muscle have been investigated as possible mechanisms of the altered Ca^{2+} homeostasis observed in muscle from DMD patients, and various dystrophic animal models. The Ca^{2+} leak channel normally opens in response to calcium depletion of the SR, allowing an increased influx of extracellular Ca^{2+} into the muscle to refill depleted SR stores(Alderton and Steinhardt, 2000, McCarter and Steinhardt, 2000). These store-operated Ca^{2+} channels (SOC) play a key role in the maintenance of normal cytosolic Ca^{2+} concentrations.

As such, In both DMD boys and the *mdx* mouse, Ca^{2+} leak channels appear to be more active and have a greater open probability (McCarter and Steinhardt, 2000). Fong et al (1990) has shown Ca^{2+} leak channels to be more active in resting dystrophic muscle cells compared to controls, while Turner et al (1993) found further increases in Ca^{2+} channel leak activity in association with contractile activity. A consequence of increased Ca^{2+} leak into muscle is the activation of Ca^{2+}-dependant proteases, such as calpains,

leading to myofibre damage (Alderton and Steinhardt, 2000, McCarter and Steinhardt, 2000). Interestingly, Ca^{2+} activated proteolysis and leak channel activity appear to interact with each other in a positive feedback loop creating a self perpetuating cycle of Ca^{2+} entry and protease driven damage, ultimately leading to muscle cell destruction (Alderton and Steinhardt, 2000, Turner et al., 1993, Whitehead et al., 2006). To date, the exact mechanism that leads to this alteration in Ca^{2+} leak channel function in dystrophy remains unclear. However, recent investigations into two major components of SOC channels, the stomal interaction molecules (STIM) and the Ori family of channels, are providing further insight into SOC's involvement in dystrophic muscle pathology (Edwards et al., 2010, Launikonis et al., 2010).

Stretch-activated channels (SAC) are another type of Ca^{2+} channel under investigation as a possible source of excess Ca^{2+} entry into dystrophic muscle (Allen et al., 2010, Rolland et al., 2006, Suchyna et al., 2000, Yeung et al., 2005). In particular, two members of the SAC family have been a key focus, including TRPC1 (transient receptor potential channel 1) and TRPV2 (transient receptor potential V2) (Millay et al., 2009, Rolland et al., 2006, Zanou et al., 2009), These mechanosensitive ion channels are not only more abundant in dystrophic tissue, but have also been shown to have a greater open probability in muscle fibres from *mdx* mice in response to membrane stretch (Vandebrouck et al., 2002, Whitehead et al., 2006). Furthermore, Whitehead et al (2006) showed improved force production, an attenuation of the rise in cytosolic Ca^{2+} concentration and decreased membrane permeability in dystrophic muscle after exercise when investigating two SAC blockers, streptomycin and GcMTx4.

It has also been shown that expression of mini-dystrophin in *mdx* muscles is able to reduce damage associated with stretch contractions, suggesting that dystrophin is essential for normal channel function (Deconinck et al., 1996). These findings indicate that Ca^{2+} entry through SAC leads to alterations in membrane permeability, and thus the initiator of the self-perpetuating cycle of Ca^{2+} entry and protease driven damage, rather than membrane damage being the primary cause of an increase in cytosolic Ca^{2+} (see Figure 2.4). This could also explain why dystrophic tissue uptakes membrane impermeable dyes.

Together, these observations provide strong evidence to suggest that both the Ca^{2+} leak and mechanosensitive SAC channels are a major contributor to the disturbed Ca^{2+} homeostasis observed in dystrophic muscle.

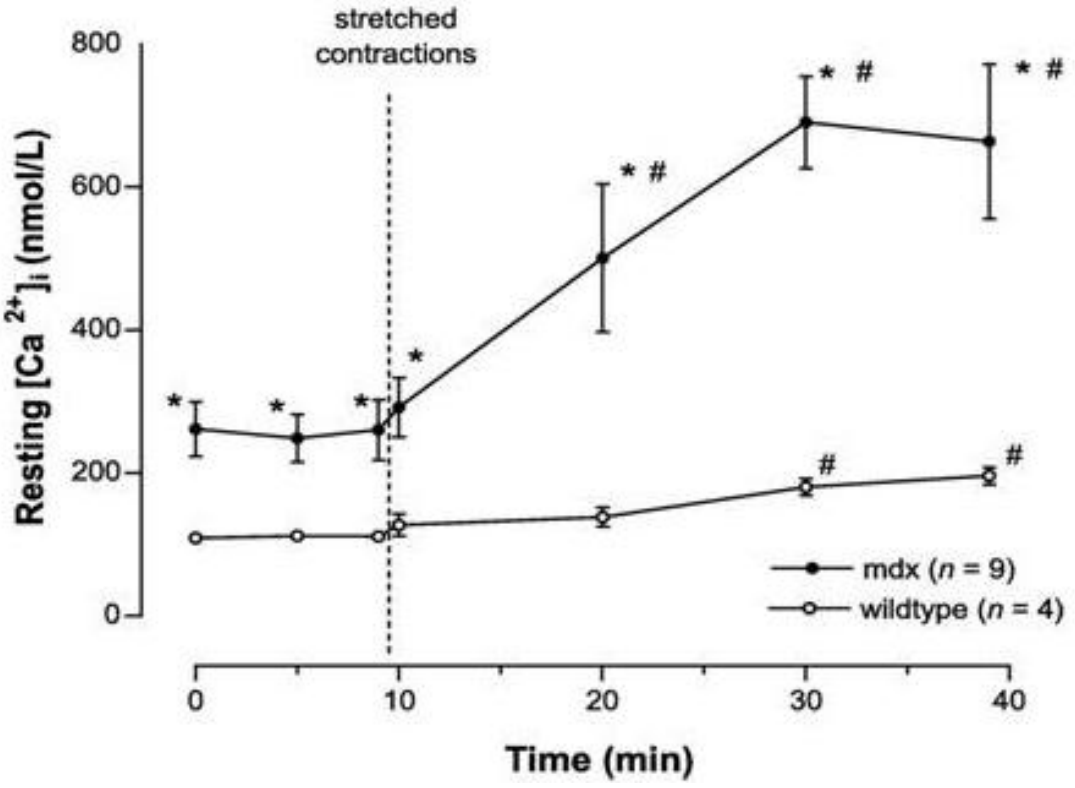


Figure 2.3 Resting Ca^{2+} concentration in control and *mdx* skeletal muscle after exposure to 10 stretch contractions

A higher resting Ca^{2+} concentration is observed in mdx muscle which is then exacerbated following stretch contractions. *,Significant difference at $p < 0.05$ between wild-type and *mdx*; #, significantly different at $p < 0.05$ from control period before stretched contractions. (Allen et al., 2010)

2.1.4 Impaired Ca2+ handling

Despite conflict in the literature regarding the source, it is well accepted that dystrophic skeletal muscle has increased cytosolic concentration of Ca^{2+} and that this results in the activation of several downstream processes that result in myofibre damage and eventually necrosis (Bakker et al., 1993, Emery, 2003, Fong et al., 1990, Williams et al., 1990, Yeung et al., 2005). Elevated cytosolic Ca^{2+} causes a redistribution of Ca^{2+} into organelles, such as the SR and mitochondria, leading to further complications such as impaired metabolism and an increase in oxidative stress (Ruegg et al., 2002). Figure 2.4 demonstrates some of the key complications associated with impaired Ca^{2+} handling in dystrophic myofibres.

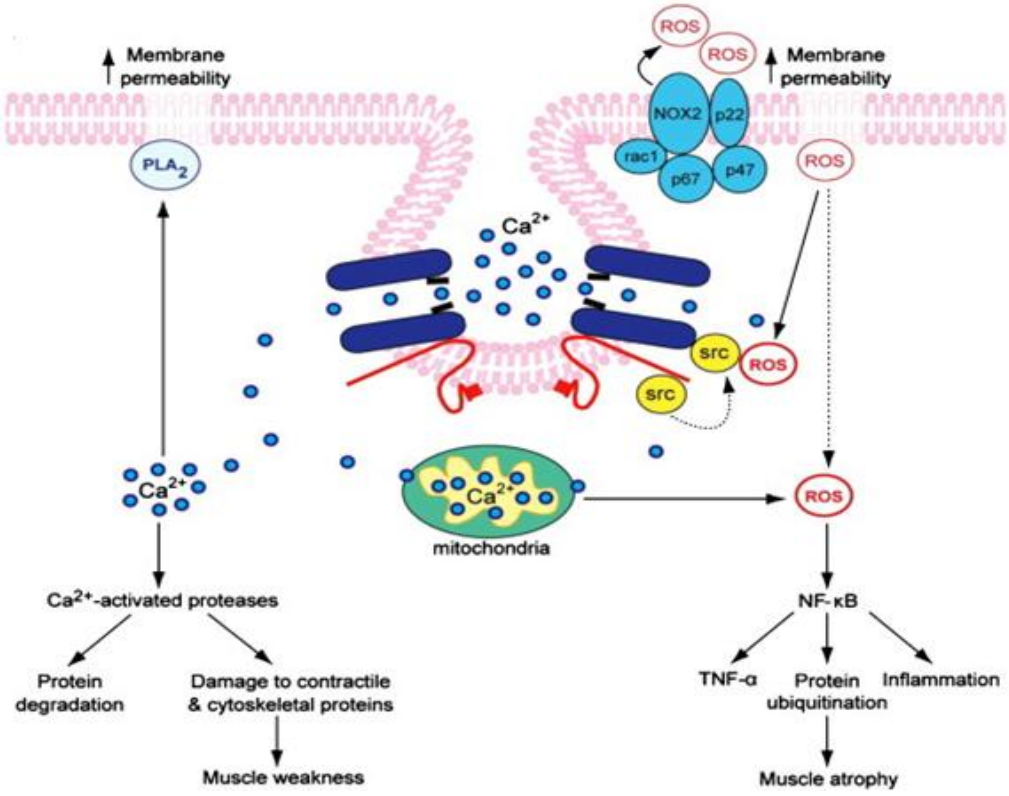


Figure 2.4 Schematic of the multiple pathways involved in DMD pathogenesis that are activated by Ca^{2+}

Increased cytosolic Ca^{2+} triggers multiple pathological events such as protein degradation, inflammation, increased ROS production and impaired ATP synthesis. Figure sourced from (Allen and Whitehead, 2011).

2.1.5 Oxidative stress

Oxidative stress was proposed as a mechanism for muscle injury in DMD as the pathological changes observed are similar to what is seen under conditions of increased oxidative stress, and many of the associated biochemical markers have been found in dystrophic muscle (Disatnik et al., 1998, Hauser et al., 1995, Murphy and Kehrer, 1989). In addition to this, dystrophic muscle cells seem inherently more susceptible to cellular injury when exposed to oxidative stress (Rando et al., 1998), and increased free radical-induced injury have been found in skeletal muscles of DMD patients and the *mdx* mouse (Hauser et al., 1995, Haycock et al., 1996, Murphy and Kehrer, 1989, Rando, 2002). While often considered to be a downstream effect in response to other pathological changes (such as impaired Ca^{2+} handling and necrosis) there is some suggestion that oxidative stress may be a primary rather than secondary cause of degeneration in DMD.

Disatnik et al (1998) found that in the pre-necrotic state, *mdx* muscle showed signs of lipid peroxidation and the induction of several antioxidant genes despite no active cellular necrosis being present. This suggests that elevated oxidative stress precedes the onset of muscle damage, and that the damage observed could be the direct result of increased membrane lipid peroxidation (Disatnik et al., 1998). This theory is supported by McArdle et al (1994) who demonstrated that the increased membrane permeability that is characteristic of dystrophic tissue is not evident in young (14 day old) *mdx* mice, indicating that this pathology is secondary to the loss of dystrophin and not a primary result of its absence.

Be it primary or secondary in nature, increased oxidative stress undoubtedly plays a key role in muscle tissue pathogenesis in dystrophy and is perpetuated by other aspects of DMD pathology, in particular, excessive Ca^{2+} entry. As seen in Figure 2.6, Ca^{2+} overload in the mitochondria can stimulate the generation of excessive ROS which have been linked to the development of increased membrane permeability and damage (Brookes et al., 2004, Whitehead et al., 2006). Interestingly, there appears to be a close and self-perpetuating relationship between calcium entry and ROS production. ROS can not only promote the release of Ca^{2+} from the SR through its actions on the RyR, but has also been shown to increase activity of SAC, establishing an intracellular positive feedback loop for damaging Ca^{2+} entry and overproduction of ROS (Allen et al., 2005, Stamler and

Meissner, 2001).Oneof the primary sources of oxidative damage in dystrophy is NADPH oxidase, with as much as a 2-fold increase in NADPH oxidase protein expression being reported in pre-necrotic *mdx* muscles (Whitehead et al., 2010).(Cozzoli et al., 2011a, Whitehead et al., 2008).

Not surprising given the elevated levels of oxidative stress, antioxidant systems of skeletal muscle are also altered in dystrophy, with significantly elevated antioxidant enzymes and antioxidants being found in DMD patients and the *mdx* mouse (Austin et al., 1992, Dudley et al., 2006). Despite this adaptive change, it appears that this is insufficient to compensate for chronic exposure to elevated oxidative stress that is characteristic of dystrophy, as several studies show decreases in damage and improved muscle function in association with antioxidant treatment (Dorchies et al., 2009, Dudley et al., 2006, Selsby, 2011).

Forexample, Selsby (2011) recently demonstrated a 30-45% decrease in contraction induced damage and a 25% reduction in the fatigability of *mdx* EDL muscle with catalase (enzyme involved in the breakdown of hydrogen peroxide radicals) over-expression. Similarly, supplementation with green tea extract significantly improved antioxidant potential, delayed necrosis and decreased fatigue in fast twitch *mdx* muscle (Dorchies et al., 2009). It appears that antioxidant therapy can also reduce stretch induced increases in membrane permeability, preventing excess Ca^{2+} entry and therefore the cascade of events that then leads to increased muscle necrosis (Whitehead et al., 2008).

Although significant functional improvements associated with antioxidant treatment are seen in mouse models of dystrophy, findings from human trials attempting to attenuate disease progression have been largely unsuccessful (Backman et al., 1988, Fenichel et al., 1988). A possible explanation for this discrepancy is that human trials have utilised patients already experiencing skeletal muscle degeneration and impaired function, whereas successful trials in murine models have initiated treatment before significant muscle damage has occurred (Selsby, 2011). Also, depending on the ROS that is being produced, some of the antioxidants may be less effective in preventing oxidative damage (Whitehead, 2006). Despite the conflicting results from animal and human trials, prevention of oxidative stress though decreasing sources of ROS production or by

increasing antioxidant availability may protect against disease progression in dystrophic muscles. However, more research is required to further explore this potential therapeutic target.

A final crucial point to make about ROS involvement in dystrophic pathology is the link between ROS production and stimulation of the inflammatory response. ROS are known to activate the ubiquitous nuclear factor kappa B (NF-κB) pathway (see Figure 2.6), which is responsible for regulating the inflammatory response through the expression of several pro-inflammatory cytokines (Acharyya et al., 2007). As chronic inflammation and impaired repair mechanisms are considered to be a key feature of dystrophy, inflammation is considered separately in the following section however, it should be noted that there is a distinct link between excessive ROS production and chronic inflammation in dystrophy.

2.1.6 Chronic inflammation

Skeletal muscle of DMD patients consistently exhibit inflammatory changes such as upregulation of pro-inflammatory genes, increases in inflammatory mediators and infiltration of inflammatory cells, all of which contribute to a state of chronic inflammation and myofibre necrosis (Acharyya et al., 2007). The infiltration of inflammatory cells was originally considered to be a non-specific response to muscle fibre damage; however it appears that aberrant intracellular signalling cascades precede disease onset in dystrophic skeletal muscle, and contribute substantially to pathology (Evans et al., 2009). This may be due, in part, to the loss of the DAPC that has now been established to have a key signalling role within muscle (Zhou et al., 2006). Interestingly, corticosteroids, which are the most successful and commonly used drug in DMD treatment, have potent anti-inflammatory effects (Barnes, 1998, Grounds and Torrisi, 2004) and several other immunosuppressive therapies have been shown to significantly ameliorate muscle wasting when administered prior to disease onset (De Luca et al., 2005, Hodgetts et al., 2006, Radley and Grounds, 2006).

While there has been several inflammatory response pathways investigated in dystrophic tissue, two of the most commonly researched are the transcription factor NF-κB and the inflammatory mediator tumour necrosis factor alpha (TNF-α) (Acharyya et al.,

2007, Grounds et al., 2008a, Haslett et al., 2002, Hnia et al., 2008). NF-κB regulates the expression of a plethora of genes involved in the inflammatory response such as cytokines, chemokines, immunoreceptors and inflammatory enzymes (Hnia et al., 2008), while TNF-α has been implicated in several muscle wasting disorders and is a potent inducer of the inflammatory response (Evans et al., 2009, Figueras et al., 2005, Hnia et al., 2008). Increased NF-κB activity has been found in the both hind limb and diaphragm muscle of the *mdx* mouse, with defective signalling also evident before the onset of muscle degeneration (Acharyya et al., 2007, Kumar and Boriek, 2003). Interestingly, inhibition of NF-κB activity has been shown to reduce macrophage infiltration by up to 80% in *mdx* mice coupled with a 77 % reduction in membrane lysis of muscle tissue (Acharyya et al., 2007), as well as improving the morphological appearance of muscle cells by limiting necrosis (Messina et al., 2006b). It has been proposed that some of the effects seen with antioxidant therapy are due to modulation of NF-κB activity, as increased oxidative stress and reduced antioxidants are both key activators of this pathway (Whitehead et al., 2006).

TNF-α is perhaps the most potent activator of NF-κB, both of which are increased in DMD patients and the *mdx* mouse (Hodgetts et al., 2006, Kumar and Boriek, 2003, Messina et al., 2006a). TNF-α is primarily released from activated macrophages and monocytes, although skeletal muscle is also capable of synthesising this cytokine intrinsically (Ramos et al., 2004). TNF-α is known to reduce contractile function, activate proteolytic pathways, increase ROS production and induce muscle wasting (Acharyya et al., 2007, Evans et al., 2009). Not surprisingly, blockade of TNF-α has been successful in attenuating the contraction-induced loss of muscle force in the *mdx* mouse (Piers et al., 2011) and can delay the onset of muscle pathology (Grounds and Torrisi, 2004). The results from these and several other studies suggest that further investigation into the role both TNF-α and NF-κB play in the initiation and progression of DMD pathology is required, and that inflammation may be one of the key therapeutic targets to prevent DMD pathology.

2.1.7 Impaired metabolism

It has been suggested that DMD results in an impairment of mitochondrial function causing a compromised cellular energy status (Jongpiputvanich et al., 2005, Tseng et al., 2002). As significant energy is required for muscle contraction, regeneration and repair, as well as the activity of key ionic pumps within skeletal muscle, and given that this demand is even greater under dystrophic conditions, it is likely that impairments to mitochondrial function contribute significantly to dystrophic pathology (Ge et al., 2003, Jongpiputvanich et al., 2005). The main source of this mitochondrial dysfunction is thought be due to excess Ca^{2+} accumulation in dystrophic muscle. Despite Ca^{2+} being a positive stimulator of oxidative metabolism under normal conditions, increased mitochondrial Ca^{2+} (reported as anywhere between two to six fold higher in dystrophy), when coupled with pathology can induce excessive ROS formation, the opening of the permeability transition pore (PT pore) and apoptosis of muscle cells (Brookes, 2004). Several studies have demonstrated abnormalities in mitochondrial oxidative metabolism in muscular dystrophy (human and mouse) including decreases in the activity of respiratory chain enzymes (Chinet et al., 1994, Even et al., 1994, Kuznetsov et al., 1998, Onopiuk et al., 2009) and a lower rate of maximum oxygen consumption, although other groups have reported no significant changes (Braun et al., 2001, Faist et al., 2001).

While research investigating changes in oxidative metabolism have yielded conflicting results, it seems that alterations to creatine metabolism are widely accepted as a key feature of dystrophic pathology. DMD patients have a higher urinary excretion of creatinine coupled with significant reductions in total creatine within muscle tissue (Fitch and Moody, 1969, Sharma et al., 2003). As PCr is a key energy source for SERCA and the contractile filaments, it is likely that reduced PCr stores will impact muscle function and as such creatine supplementation has been investigated as a potential therapy for DMD. In healthy individuals, creatine has been shown to increase lean tissue mass, muscle fibre area, strength and endurance capacity (Volek et al., 1999) and studies in DMD have yielded similar results. Specifically, supplementation with creatine has been shown to delay the progression of necrosis until day 34 in the *mdx* mouse, and improve mitochondrial respiration by 25% (Passaquin et al., 2002). In addition, creatine supplementation increases myotube survival and can improve intracellular Ca^{2+} handling in dystrophic muscle (Pulido et al., 1998). Positive results such as these from animal

studies have lead to several clinical trials using creatine supplementation in DMD patients in an attempt to enhance muscle function. Interestingly, while creatine demonstrates several beneficial effects in the short-term on muscle strength and function, to date there is no evidence that long-term creatine treatment is beneficial, or that treatment has any positive effect on patient lifespan (Banerjee et al., 2010).

Glycolytic metabolism has also been shown to be affected in DMD (Onopiuk et al., 2009, Wehling-Henricks et al., 2009b). A likely candidate for this impairment is the rate-limiting enzyme PFK, with activity reported to decrease by 45% in type II fibres of DMD patients (Chi et al., 1987). Interest in this enzyme has recently been reignited due to the apparent regulation of PFK by nitric oxide (NO), which is altered in DMD due to the lowered nNOS that results from dislocation of the DAPC (Zhou et al., 2006). NO is known to be an important signalling molecule in healthy skeletal muscle and is reported to play a role in mitochondrial biogenesis, glucose transporter type 4 (GLUT-4) translocation and expression (improving glucose uptake) and modulating neuromuscular transmission (Wehling-Henricks et al., 2009b). Interestingly, nNOS itself has been found to directly bind to PFK, suggesting that nNOS may be able to regulate enzyme activity independently of NO (Wehling-Henricks et al., 2009b). Taken together, these results suggest that the loss of nNOS and altered NO signalling may be the source of altered glucose metabolism frequently reported in dystrophy, and a possible contributor to the disease pathology (Wehling-Henricks et al., 2009b). Collectively, research into metabolic function in DMD suggests that there is a link between the loss of dystrophin and impairments in metabolism, which may contribute to disease pathology and impairments to muscle function, albeit through indirect mechanisms.

2.1.8 Muscle function impairments

i) E-C coupling

While it is well accepted that dystrophic muscle displays significant impairments to whole muscle function, the mechanisms responsible for the impairments observed in E-C coupling and contractile function are currently poorly understood. Intact EDL muscle fibres from *mdx* mice have been shown to contract at more negative potentials relative to age matched controls, and take longer to reach threshold for contraction indicating

changes in Ca^{2+}regulation (De Luca et al., 2001a). Plant and Lynch (2003) also found that when exposed to repeated depolarisations, *mdx* muscle fibres display earlier E-C uncoupling between the DHPR-RyR, and reduced force production relative to healthy controls. Interestingly, this study did not observe any differences in the rate of SR loading or SR Ca^{2+} leak, which contrasts with more recent studies. Head (2010) demonstrated that intracellular Ca^{2+} kinetics are depressed in fast-twitch *mdx* muscle, with this depression most likely attributable to alterations in the function of the RyR and SERCA (Head, 2010). Moreover, Hollingworth et al. (2008) found that Ca^{2+} release from the SR in *mdx* muscle is depressed by 18% during E-C coupling, confirming a similar finding from Wood et al. (2004), which suggests that some of the muscular weakness reported in the *mdx* mouse is likely due to lower SR Ca^{2+} release.

In addition to lower Ca^{2+} release, the RyR also appears to be leakier in dystrophic muscle (Bellinger et al., 2009). Indeed, increased leak from the RyR has been observed in dystrophic muscle, and excessive ROS production has been shown to increase the RyR's sensitivity to Ca^{2+}, further increasing Ca^{2+} leak from the SR (Bellinger et al., 2009, Terentyev et al., 2008, Ullrich et al., 2009). Despite some findings suggesting that SERCA is unaffected in *mdx* muscle, it has been shown that over-expressing muscle specific SERCA in dystrophic tissue results in reduced serum CK, tissue necrosis and myofibre damage (Goonasekera et al., 2011).

ii) Whole muscle function

Both DMD males and dystrophic *mdx* mice exhibit significant alterations to normal muscle function, such as lowered force production, increased fatigability and reduced power output (Emery, 1995). In humans, DMD affects the proximal muscles of the lower limbs, followed by the shoulder girdle musculature, distal limb muscles and ultimately respiratory muscles such as the intercostals and diaphragm (Emery, 1995). Patients often present with abnormal gait (waddling), hypertrophy of calf musculature and exaggerated lumbar lordosis (Yiu and Kornberg, 2008). Weakness in the neck flexors, difficulty getting up off the ground, frequent falls, difficulty walking up stairs and a limited ability to run are also common signs of skeletal muscle impairment in DMD boys (Yiu and Kornberg, 2008).

While tests of strength (e.g. hand held dynamometry) evidence weakness in DMD patients, it appears that timed functional tests are the best indicator of disease progression, with dystrophic children consistently performing poorly in this type of assessment even if force deficits are yet to manifest (Beenakker et al., 2005). In addition to lowered force output, DMD patients also exhibit greater susceptibility to fatigue, with some human studies showing as much as a four-fold decrease in the ability to sustain moderate contraction of limb muscles in DMD patients when compared to normal healthy boys (Frascarelli et al., 1988). Exercise intolerance and increased susceptibility to contraction-induced injury are also key features of DMD pathology. When tested with a six-minute walking test, DMD boys walk significantly less distance than healthy controls (McDonald et al., 2010) . The loss of functional capabilities, strength and endurance necessitates the use of a wheelchair that further impairs skeletal muscle function through the development of contractures at the hip, knee and elbow that are strongly related to the onset of wheelchair dependence (Jansen et al., 2010).

Further to the impairments in limb musculature are detrimental changes to muscles of the respiratory system and cardiac tissue that are frequently the ultimate cause of death in DMD patients. As the disease progresses, respiratory muscles weaken and endurance capacity is reduced, thus requiring many patients to use supportive ventilation, intermittently at first and eventually on a constant basis. Weakening of the diaphragm is particularly evident during sleep where upper airway resistance is increased, resulting in hypoventilation and sleep apnoea (Finsterer, 2006). The development of spinal deformity exacerbates this condition further, and increases the difficulty of breathing, resulting in marked increases in energy consumption which can cause further weakening of respiratory muscles due to malnutrition (Finsterer, 2006). Cardiac muscle involvement is evident from approximately 10 years of age with ECG abnormalities reported in more than 90% of cases (Finsterer, 2006). The heart undergoes progressive fibrosis revealing myocardial wall thickening, wall motion abnormalities, dilation of cardiac cavities and valve abnormalities (Finsterer, 2006). Irregularities in heart rate, ejection fraction and blood pressure are also frequently evident (Finsterer, 2006).

While deficits in skeletal and cardiac muscle are well established in human DMD, significantly less information is available in the literature on exercise capacity and

tolerance. This is due to the implications that strenuous exercise can have on DMD muscle. For this reason a significant proportion of muscle testing for exercise protocols and therapeutic interventions are performed using models of DMD such as the *mdx* mouse. Muscle function deficits in this model are a little less clearly defined than they are for the human condition, although they have been reported to exhibit similar characteristics such as lower force output, increased fatigability, decreased exercise tolerance and excessive damage when exposed to eccentric exercise (De la Porte et al., 1999). The details of muscular impairment for the *mdx* mouse are considered in section 2.3.3, and contrasted with the human condition.

2.2 Mammalian models of dystrophy

The ethical implications associated with research involving DMD patients due to their age and fragility prevent widespread testing of potential therapeutic modalities in humans. This is particularly due to the invasiveness of analytical techniques such as blood collection and muscle biopsies, which have the potential to exacerbate the dystrophic condition, as well as cause further complications and psychological stress to young children. For this reason the bulk of research into DMD is performed using animal models of the disease with dystrophic cats, dogs and mice all available, although the canine and mouse models are the most frequently used and thus will be reviewed here. The *mdx* mouse will be the main focus, as this is the animal model used for all experimental work in this thesis.

2.2.1 Canine muscular dystrophy

Two canine models of DMD exist including the golden retriever (GRMD) and the beagle (CXMDJ) (Lanfossi et al., 1999, Shimatsu et al., 2003). The GRMD model follows a similar disease profile to that of the human condition with dogs demonstrating progressive muscle weakness, atrophy, fibrosis and the development of contractures that impair gait (Ambrosio et al., 2009, Willmann et al., 2009). By six months of age, as ultimately occurs in the human condition, kyphosis and respiratory impairments develop as well significant cardiac involvement, markedly shortening the life span with few living past one year of age (Lanfossi et al., 1999, Thibaud et al., 2007). The CXMDJ (canine X-linked muscular dystrophy in Japan) model was developed by breeding a GRMD affected dog with a beagle, and although they do present with progressive muscle wasting, this model is far less well characterised at present than the GRMD model (Shimatsu et al., 2003). CXMDJ dogs develop a milder degree of muscle wasting with a slower rate of progression and significantly less cardiac involvement (Shimatsu et al., 2003). The benefit of this model is that it has an improved survival rate, is smaller than the golden retriever and is a preferred breed for drug development (Shimatsu et al., 2003). Despite canine models potentially being the most analogous with the human DMD phenotype, research using this model is also somewhat restrictive. The ethical implications, availability and significant costs involved in maintaining colonies makes using the canine models both problematic and expensive (Kornegay et al., 2011). For

these reasons significantly more researchers preferentially use the mouse models of DMD.

2.2.2 Mouse muscular dystrophy

The *mdx* mouse is a naturally occurring, dystrophin-deficient mouse, that has a similar but not identical phenotype to human DMD, and was first discovered in 1981 in a colony of black 10 (C57BL/10) mice (Bulfield et al., 1984). Since then several variants of the *mdx* mouse have been identified, and the *mdx* background has been crossed with knockout mice that are deficient in proteins such as utrophin, MyoD and α-dystrobrevin, to produce a more severe phenotype than is present naturally in the *mdx* mouse (Willmann et al., 2009). Despite the availability of other models, the *mdx* mouse remains the most widely used and well characterised of the DMD mouse models. For a comprehensive review of the *mdx* mouse and its research applications, see Grounds et al (2008).

2.2.3 *Mdx* mouse

Like human DMD, the *mdx* mouse results from a naturally occurring genetic mutation in C57BL/10 mice that leads to the absence of the protein dystrophin and associated skeletal muscle pathology (Bulfield et al., 1984). Despite having the same genetic abnormality, the disease progression in the *mdx* mouse is significantly different to that observed in DMD boys (Bulfield et al., 1984). *Mdx* muscles exhibit early necrosis from approximately day 5 of life in muscles of the head, trunk and girdle (De la Porte et al., 1999). Limb muscles are affected later, where there is an acute onset of skeletal muscle degradation and necrosis at 3-4 weeks of age, with necrosis levels reported to peak at around 24-28 days of age (Grounds et al., 2008b). The specific timing of muscular degeneration is highly variable within the literature, although it is generally agreed upon that maximum degeneration is experienced between the 3rd and 10th week of life (DiMario et al., 1989, Karpati et al., 1990, Nagel et al., 1990) before decreasing to negligible levels in adult mice. During the acute degenerative stage skeletal muscles present with increases in satellite cell activation, a number of newly differentiating myofibres and centralised nuclei (McGeachie et al., 1993). Serum CK is also elevated preceding this damage period, and continues to remain high until this first cycle of

degeneration is complete, making it a frequently used qualitative indicator of muscle damage (Radley et al., 2007). Interestingly, although significant damage is evident, muscle fibrosis is far less pronounced than that of the human condition, and after this initial wave of degeneration the disease follows a far more benign progression, maintaining a chronic but low grade of damage after 8 weeks of age (Grounds et al., 2008b). While *mdx* mice do have a slightly shorter life span than their non-dystrophic C57BL/10 counterparts, loss of muscle tissue is in general very slow, with significant muscle weakness not being observed until late in life (Willmann et al., 2009). In fact, skeletal muscles of adult *mdx* mice tend to undergo hypertrophy from 6-12 months of age (De la Porte et al., 1999, Hayes and Williams, 1998) with increases in both muscle mass and body weight frequently reported (Vilquin et al., 1998). This compensatory hypertrophy slowly declines from 12 months of age, with old (+18 months) mice exhibiting marked skeletal muscle atrophy, fibrosis and impaired contractile function that is similar to that of human DMD (Hakim et al., 2011, Hayes and Williams, 1998, Pastoret and Sebille, 1993). All muscle groups are not affected at the same time, or to the same extent, with these discrepancies attributable to differences in positioning, utilisation and fibre-type (Kaminski et al., 1992, Karpati et al., 1988, Marques et al., 2007, Vilquin et al., 1998). Fast twitch fibres are preferentially, but not exclusively affected (Vilquin et al., 1998) while muscle fibres of small diameter, such as those in extraocular muscles, are relatively spared from damage, demonstrating only mild pathology (Kaminski et al., 1992, Karpati et al., 1988, Marques et al., 2007).

i) Muscular involvement

While the head and trunk exhibit dystrophic signs early in the *mdx* lifespan, few papers report involvement of limb musculature before 21 days of age (Grounds et al., 2008b). At 24-26 days there is an acute onset of myofibre necrosis, where anywhere between 20-80% of limb musculature is involved (Grounds et al., 2008b). The fast-twitch EDL is severely affected with only 5% of its original fibres surviving to 26 weeks of age (De la Porte et al., 1999). Destruction of other hind limb muscles such as SOL and Gastrocnemius (GAST) are delayed when compared to the Tibialis anterior (TA), which has been reported to exhibit as much as 90% of muscle fibre involvement during the acute stage of damage (De la Porte et al., 1999). Proliferation of satellite muscle cells starts at approximately week three and remains elevated throughout the acute stage

before decreasing, yet persisting until 12-18 months of age (McGeachie et al., 1993). After the initial wave of degeneration *mdx* muscles only demonstrate low levels of damage and regeneration that persists until 18 months of age, after which time the regenerative processes become exhausted and they begin to show signs of muscle atrophy, weakness and fibrosis similar to that of DMD (Hakim et al., 2011, Hayes and Williams, 1998). It is thought that the milder phenotype observed in limb musculature of the *mdx* mouse is due to enhanced regenerative capacity, and to some extent, functional compensation due to upregulation of the dystrophin homologue utrophin (van Putten et al., 2010).

The diaphragm shows more severe pathology than limb musculature including marked degeneration and fibrosis, conveying a phenotype far closer to human DMD (Louboutin et al., 1993, Stedman et al., 1991). This increase in patholology is thought to be due to the intrinsic characteristics of diaphragm structure and function, such as it consisting of large proportions of fast twitch fibres with a large diameter, being involved in life-long constant activity, and undergoing forced lengthening with each contraction (Gillis, 1996). Like human DMD, loss of muscle function in the *mdx* diaphragm is progressive, with ten times more collagen density than that of limb musculature by six months of age (Stedman et al., 1991). In response to this progressive degeneration, the diaphragm undergoes a fibre type shift towards a slower phenotype, thereby preserving contractile function and enhancing survival of muscle fibres (De la Porte et al., 1999). Cardiac involvement also occurs in the *mdx* model leading to myocardial fibrosis, necrosis, reduced ejection fraction and infiltration with inflammatory cells (Van Erp et al., 2010).

ii) Muscle function

Functional insufficiency of skeletal muscle within the *mdx* mouse has been reported to mimic some of the characteristic features of human DMD, including weakness, increased susceptibility to exercise-induced damage, impaired exercise tolerance and an increase in fatigue (Brussee et al., 1997, Selsby, 2011, Vilquin et al., 1998). Impairments in functional measures such as grip strength, hanging capability and cage activity have also been found by some authors (van Putten et al., 2010). While there is little dispute that during the acute damage phase *mdx* muscles show significant necrosis, are weaker and more susceptible to fatigue than those of control animals, the

degree of functional impairment in adult mice is unclear (Lynch et al., 2001b). In addition to the wide variety of experimental methods used to evaluate the *mdx* mouse, factors such as breeding, housing conditions and timing of experimentation could go part way to explaining the variability in results that is frequently observed (Grounds et al., 2008b). Inherent variability within the *mdx* mouse itself and the age at which animals are experimented on can also explain the incongruency of results, especially with regard to the muscles chosen, as the degree of damage and the onset of pathology is variable in different muscles of the hind limb (Grounds et al., 2008b). The following section reviews the key aspects of functional insufficiency in the *mdx* mouse with regard to force development, fatigue and exercise tolerance.

iii) Force development

Isometric force development has been extensively studied in the *mdx* mouse, although considerable controversy still exists as to whether muscle mass and force production in *mdx* mice is different relative to age matched controls (Harcourt et al., 2007, Lowe et al., 2006, Lynch et al., 2001b). Generally, twitch kinetics are reported to be similar to that of control animals (Harcourt et al., 2007) although some groups have reported a slowing of the TTP and ½ RT, whereas others have shown the opposite effect (Watchko et al., 2002). A recent study by Lowe et al. (2006) investigated contractile dysfunction in the EDL muscle of young (28 day old) *mdx* mice and determined that the dystrophic mice displayed significantly less twitch force that control animals, which supports previous research from several other groups (Petrof et al., 1993, Quinlan et al., 1992). In direct contrast, Harcourt et al. (2006) demonstrated no difference in TTP, ½ RT or rate of force development in 8 week old *mdx* mice (Harcourt et al., 2007). The discrepancies within the literature are likely due to the variable nature of the contractile protocols, as well as the age of the mice at the time of experimentation.

With regard to absolute tetanic force (P_0), skeletal muscles of *mdx* mice demonstrate forces that are comparable to, or even exceed, that produced by control animals (Lynch et al., 2001b). This is especially common when animals are experimented on between 6-12 months of age, where hypertrophy of limb musculature occurs in the *mdx* mouse (De la Porte et al., 1999, Hayes and Williams, 1998). However, when normalised to cross sectional area to give specific force (sP_0), peak tetanic force is consistently lower in *mdx*

muscle relative to C57BL/10 mice (Brooks, 1998, Corrado et al., 1996, Lynch et al., 2001b, Sacco et al., 1992). Several groups demonstrate relative force decline in order of 20-50% compared to control values (Connolly et al., 2001, Messina et al., 2006b, Raymackers et al., 2003) yet others have not confirmed this finding (Harcourt et al., 2007, Muntoni et al., 1993). Again, this is likely due to the diversity of contractile protocols used, and differences in the age of animals.

iv) Fatigue

Mdx mice have been reported to show an exaggerated exercise-induced fatigue response when compared to control animals. Muscles from DMD patients are also highly susceptible to fatigue, exhibiting lowered endurance, particularly in the diaphragm, causing physical limitations and restricted activities of everyday living (Frascarelli et al., 1988, McDonald et al., 2010). The degree of fatigue in *mdx* mice is variable, and again appears to be highly dependent on the age of the animal, conditions of the stimulation protocol and the temperature at which the experiments are conducted (Gregorevic et al., 2002, Hayes and Williams, 1998, Lynch et al., 2001b, Wineinger et al., 1998).

Connelly et al (2001) examined grip force in the *mdx* mouse across the lifespan from 3 to 24 weeks of age. This study demonstrated strikingly higher fatigue in *mdx* muscles of approximately 40-45% of control values, which was present at all time points and did not change with age (Connolly et al., 2001). Similarly, four minutes of intermittent stimulation of *mdx* muscles fatigued them to 45% of their pre-fatigue force, while control muscles reduced by only 25% (Gregorevic et al., 2002). Moreover, after five minutes of recovery *mdx* muscle still exhibited a 10% decline in force, while control muscle recovered completely within this time (Gregorevic et al., 2002). Several proposed mechanisms for the increase in fatigability of *mdx* muscles have been suggested including elevated ROS production, impaired oxidative metabolism and ionic disturbances (Reardon and Allen, 2009). Recently, it has also been suggested that the decrease in nNOS associated with the loss of dystrophin may also be a possible explanation for the higher rates of fatigue in *mdx* mice. During mild exercise, the loss of nNOS signalling results in impaired vasomodulation in the blood vessels that supply active muscle beds, leading to an exaggerated fatigue response in *mdx* muscle *in vivo* (Kobayashi et al., 2008).

In direct contrast to these findings, a few studies have demonstrated no change, or reduced susceptibility to fatigue in *mdx* mice (Petrof et al., 1993, Sacco et al., 1992), with the differences observed being partially explained by the stimulation frequency used. Sacco et al. (1992) found that *mdx* muscles were in fact less fatigable than that of controls however, the lower relative force at 40Hz in *mdx* muscles would have entailed a lower energy demand, and a therefore a slower rate of fatigue (Sacco et al., 1992). It has also been suggested that enhanced fatigue resistance could be the result of a shift in the MHC phenotype to a slower isoforms (Watchko et al., 2002) as type II fibres are preferentially affected in dystrophy. This fibre type switch would leave *mdx* muscles with a greater oxidative capacity, and a lower myofibrillar ATPase activity, reducing fatigue (Watchko et al., 2002).

The temperature at which fatiguing experiments are conducted can also have an impact in *mdx* mice (Wineinger et al., 1998). A study by Wineinger (1998) examined the effect of age and temperature on *in vitro* contractile characteristics of *mdx* mice using the EDL muscle of 8 and 62 week old animals, at temperatures of 20°C and 35°C. When measured at 20°C the fatigability of the *mdx* EDL was similar to that of control mice however, when the temperature was increased to 35°C, significant age and strain related differences were seen, including increased fatigability of *mdx* muscle (Wineinger et al., 1998). This suggests that there are temperature sensitive mechanisms that make the *mdx* EDL more fatigable at higher (more physiological) temperatures (Wineinger et al., 1998). The larger mass of *mdx* muscle is unlikely to account for this change, as rat studies have previously shown that detrimental effects of temperature in *in vitro* techniques occur with muscles between 70-90mg, and the muscle used in the above example were below 30mg (Segal and Faulkner, 1985, Wineinger et al., 1998). A possible explanation is the increase in ROS production that accompanies increases in temperature, which has been shown to accelerate muscular fatigue (Reardon and Allen, 2009). As *mdx* mice are more susceptible to oxidative stress and have impaired antioxidant systems (Haycock et al., 1996, Rando, 2002, Selsby, 2011), it is possible the increasing temperature more severely affects *mdx* muscles.

v) Exercise tolerance

Like human DMD, *mdx* mice are reported to exhibit impaired exercise tolerance. When allowed to voluntarily run on a wheel or treadmill, *mdx* mice run significantly less distance and at a slower pace than that of controls (Dupont-Versteegden et al., 1994, Hayes and Williams, 1996). They have also been shown to run more intermittently than wild-type mice, suggesting that they are more prone to fatigue or are exhibiting avoidance behaviour (Grounds et al., 2008b, Hara et al., 2002). Despite this apparent decrease in exercise tolerance, low intensity exercise such as swimming has beneficial effects on dystrophic pathology in both the human and mouse condition (Hayes and Williams, 1997).

In contrast, high intensity forced exercise has been used to exacerbate muscle pathology in adult *mdx* mice allowing more rigorous testing of pharmacological interventions (Grounds et al., 2008b). Single bouts of voluntary wheel running can be used to induce damage in *mdx* skeletal muscles, however, forced treadmill running is the most effective method of increasing damage (De Luca et al., 2003, Grounds et al., 2008b). Running on a treadmill for 30 minutes at a speed of 12m/min twice a week at the beginning of the first wave of degeneration increases muscular weakness in the *mdx* mouse, while control muscles remain unaltered (Granchelli et al., 2000, Grounds et al., 2008b). Again, during this type of protocol *mdx* mice demonstrate avoidance behaviour sporadically stopping to rest and needing to be encouraged to restart activity (De Luca et al., 2003).

Of all types of exercise, those involving eccentric contractions are the most damaging to dystrophic skeletal muscle. This may be due to dystrophins role in protecting the sarcolemma against stress-induced damage, with repetitive lengthening contractions in *mdx* muscle decreasing force development and increasing sarcolemmal permeability though SAC (Allen et al., 2010, Suchyna et al., 2000, Yeung et al., 2005). Downhill treadmill running is extremely poorly tolerated by *mdx* mice, with many fatiguing within a minute and in some cases this type of protocol has caused death (Grounds et al., 2008b). As with most measures of muscle function in the *mdx* mouse, the tolerance of eccentric contractions is different between young and old mice (Head, 2010, Piers et al., 2011). A recent study by Head (2009) in young *mdx* animals (<3 months) showed no

difference in susceptibility to eccentric muscle damage respective to control animals however, after 3 months fast-twitch *mdx* muscles are markedly more susceptible to eccentrically-induced damage (Head, 2010). It is suggested that these contradictory findings can be explained by the increase in the number of branched fibres in older *mdx* mice (Head, 2010), as a drop maximal force by 40% associated with eccentric activity has been reported in old *mdx* muscles, where greater than 90% of fibres exhibit branching (Head, 2010).

vi) Limitations of the *mdx* model

Significant inherent variations in *mdx* phenotype exist, with differences in the both the timing and severity of muscle pathology commonly reported (De Luca et al., 2005, Pierno et al., 2007, Radley and Grounds, 2006). This can occur not only between, and within, colonies but also between mice from the same litter, and even between two legs of an individual mouse (Grounds et al., 2008b, Willmann et al., 2011). These inherent variations can be further exacerbated by diverse housing conditions (cage design, frequency of handling, food and water intake, light and dark cycles), exposure to stressors and timing of experimentation (Grounds et al., 2008b, Willmann et al., 2011). These factors can increase variability observed within experiments and necessitates the use of large sample sizes to show statistically significant effects (Grounds et al., 2008b). Moreover, the relatively mild pathology the *mdx* mouse sustained after the initial bout of degeneration makes the phenotype limited in its use for research into pharmacological intervention (Wakeford et al., 1991). This could be circumvented by restricting experimentation protocols to the initial damage period of 3-4 weeks of age, however the extremely small muscle mass of the frequently used muscles (approximately 3-6mg for EDL and 2-5mg for SOL) means many commonly used analytical techniques can not be conducted due to the sheer lack of tissue availability. Larger hind limb muscles can be utilised (such as TA and GAST) however, due to the greater variation in fibre type this may not be ideal for some experimental protocols.

For this reason, methods of worsening the pathology of *mdx* mice have been investigated such as irradiation and exercise interventions, as well as cross breeding to produce knockout mice. While exercise, (particularly that involving eccentric contraction) is certainly capable of worsening the dystrophic condition, protocols must ensure that

this does not have significant detrimental effects on control mice and yet still induces sufficient damage in the *mdx* mouse. Despite exercised *mdx* mice presenting with a more homologous grade of damage to that of the human DMD condition, the equipment required to accurately perform this treatment can be expensive (treadmills) and require mice to be housed alone (wheel running) which is not ideal. In addition, the time requirement for supervision of exercise sessions can be significant, depending on how many animals are required for the experimentation. For these reasons only a limited number of laboratories have adopted the exercised *mdx* mouse as their standard experimental model (De Luca et al., 2003, Spurney et al., 2009).

In the case of the dystrophin-utrophin double knockout mouse, muscle pathology closely mimics that of human DMD with the development of severe muscle wasting, growth retardation, weakness, kyphosis, contractures and connective tissue infiltration that persists throughout the markedly reduced lifespan of 4-20 weeks of age (Deconinck et al., 1997, Grady et al., 1997). While a far superior model to that of the *mdx* mouse in regards to the damage profile, it should be noted that the dystrophin-utrophin knockout mouse no longer shares the same genetic defect as human DMD. Moreover, the limited availability of these animals also restricts its widespread use over the readily commercially available *mdx,* whose muscle pathology is far better characterised than many of the knockout mice available.

Thus, despite its limitations, the *mdx* mouse remains an invaluable resource for DMD research. Even though they present with a milder phenotype than human DMD, the *mdx* mouse has still provided researchers with a far greater understanding of the mechanisms that cause muscle degeneration in dystrophy (Head, 2010) and is still widely used as a model for pre-clinical testing of therapeutic interventions for DMD.

2.3 Current treatment approaches

Despite significant advances in the understanding of the dystrophin gene, its product, and the resulting pathology, the options for DMD treatment remain fairly limited, with the most common pharmacological intervention, corticosteroids, being associated with significant side effects (Angelini, 2007, Biggar et al., 2006, Bushby et al., 2010a). While a cure will undoubtedly come from molecular therapeutic approaches such as gene replacement therapy, currently there are considerable technical hurdles associated with this approach that need to be overcome in order to make this a viable treatment option (Mendell et al., 2010). Although not a key focus of this thesis, current treatment approaches are briefly reviewed in the following sections to highlight the need for additional therapeutic compounds that can target the causes of skeletal muscle impairment in DMD.

2.3.1 Molecular therapies

There are three main treatment approaches in the area of molecular therapy that are currently under investigation: 1) myoblast transplantation, 2) gene therapy and 3) stem cell transplantation (Mendell et al., 2010). Myoblast therapy involves the incorporation of normal muscle precursor cells into dystrophic muscle, resulting in the insertion of a functional copy of the missing dystrophin gene (Brussee et al., 1998, Partridge et al., 1989). However, to date there has only been limited success with this technique in both the mouse and human forms of DMD (Brussee et al., 1998, Kinoshita et al., 1996, Miller et al., 1997, Tremblay et al., 1993). Moreover, clinical trials have shown little benefit from this treatment, with dystrophin expression reaching less than 10% of normal expression at one month post-injection, and undetectable at six months (Miller et al., 1997, Tremblay et al., 1993) This is most likely due to an immune system response causing myoblast death, as immunosuppressive drugs seem to improve survival (Ohtsuka et al., 1998). In contrast, gene therapy aims to deliver the dystrophin gene through viral vectors or plasmid DNA complexes (Wells and Wells, 2002). Although a remarkable degree of progress has been made in this area of research, implementing this method has been hampered as the cloning capacity of viral vectors is exceeded by the size of the dystrophin gene, as it is the largest in the human genome (Wang et al., 2000, Wells and Wells, 2002). This has lead to the development of mini and micro-dystrophin, which

can fit into the best vehicle for gene transfer, the adeno-associated virus (Wang et al., 2000). Trials in the *mdx* mouse result in robust expression of the new gene however, several hurdles are impeding human trials such as previous immunity to the adenovirus and rejection of fibres expressing the new gene (Mendell et al., 2010, Wang et al., 2000). Stem cell transplantation has also been used in an effort to induce dystrophin expression in DMD however, as is the case with myoblast transfer, the efficiency of this process limits its current clinical application (Mendell et al., 2010).

2.3.2 Pharmacological therapies

Several pharmacological therapies with the ability to target specific components of dystrophic pathology have been used in an attempt to ameliorate the disease phenotype in DMD. To date, the most successful and widely used pharmacological treatment for DMD is corticosteroids. Significant improvements in muscle strength and functional measures are consistently reported with corticosteroid use, as well as a delayed loss of ambulation by approximately 1-2 years, drastically improving the quality of life for sufferers (Matthews et al., 2010, Wong and Christopher, 2002). Unfortunately, despite the positive benefits, corticosteroid use is linked to several side effects such as weight gain, growth suppression, behavioural abnormalities, insulin resistance, cushingoid appearance and excessive hair growth (Angelini, 2007, Biggar et al., 2006, Deshmukh, 2007). Interestingly, although corticosteroids are widely used in DMD therapy the actual mechanism of their action is still poorly understood (Deshmukh, 2007). However, suggested mechanisms of action include immunosuppression, altered myogenic and myoregulatory signalling, increased myoblast activity and inhibition of Ca^{2+} influx (Wong and Christopher, 2002).

As impaired Ca^{2+} handling is considered a key player in the progression of muscle damage in dystrophy, the use of several Ca^{2+} channel blockers (such as Dantrolene and Diltiazam) have been investigated with some beneficial results (Johnson and Bhattacharya, 1993, Tutdibi et al., 1999). Downstream effects of Ca^{2+} overload, such as Ca^{2+}-activated proteases have also been targeted by increasing calpastatin (which prevents activation of calpains) expression in *mdx* mice, as well as administering the calpain inhibitor leupeptin, both of which have been shown to decrease muscle necrosis (Spencer and Mellgren, 2002, Turner et al., 1993). In addition to attempting to prevent

damage, other interventions focus on enhancing muscle repair and regeneration such as increasing insulin-like growth factor 1 (IGF-1) and inhibiting the action of myostatin (Haidet et al., 2008, Shavlakadze et al., 2004, Tsuchida, 2008). IGF-1 is a positive regulator of muscle growth and is associated with myofibre hypertrophy and increases in force production, and has been shown to result in functional improvement in *mdx* mice (Schertzer et al., 2006). In direct contrast, myostatin is a negative regulator of muscle mass and blockade of its action causes muscle hypertrophy, hyperplasia and a decrease in body fat (Haidet et al., 2008). Several recent studies have shown improvements in muscle mass, force production and histological profile of dystrophic tissue by blocking the action of myostatin (Dumonceaux et al., 2010, Murphy et al., 2010, Nakatani et al., 2008, Qiao et al., 2008, Tsuchida, 2008). Clinical trials are currently underway in human DMD patients with the human anti-myostatin monoclonal antibody, MYO-029 (Krivickas et al., 2009, Wagner et al., 2008). Another area of intense study is the pharmacological upregulation of the dystrophin homologous protein utrophin, which may be able to serve as a functional substitute for the missing dystrophin (Tinsley et al., 1998). Studies in the *mdx* mouse show increased utrophin expression can improve sarcolemmal integrity and protect against contraction induced injury (Miura et al., 2009).

2.3.3 Nutritional Interventions

At present, there is very little information available on the nutritional management of DMD patients, despite the suggested changes to energy metabolism and expenditure, body composition and gastrointestinal function that is commonly associated with the disease (for review see Davidson & Truby, 2009). Nutritional requirements will undoubtedly be changeable as the disease progresses and can be further complicated by corticosteroid use, which specifically impacts stores of nutrients such as Ca^{2+} and vitamin D (Soderpalm et al., 2007). Delayed growth, short stature, increased rates of obesity, glucose intolerance and muscle wasting impact nutritional status and energy requirements necessitating changes to nutrient intake including modifications to food texture (due to gastro-intestinal tract complications) and macro/micro nutrient composition (Davidson and Truby, 2009). The use of supplements is a growing field of interest in DMD since compounds such as creatine (Escolar et al., 2005, Louis et al., 2004, Passaquin et al., 2002, Pulido et al., 1998), glutamine (Granchelli et al., 2000, Mok et al., 2008), green tea extract (Buetler et al., 2002, Dorchies et al., 2009) and taurine

(De Luca et al., 2003) have shown some benefit in animal models of the disease. Furthermore, combined therapies of corticosteroids and nutritional intervention seem to enhance muscle function to a greater degree than corticosteroid or nutritional intervention alone (Cozzoli et al., 2011b, Payne et al., 2006). A greater focus on nutritional management in the future will be important as multidisciplinary therapy will likely provide the best outcome for patients. Despite the beneficial results achieved in the *mdx* mouse with taurine supplementation alone and in combination with corticosteroids, a greater understanding of taurine's action in DMD is required in order to best understand and utilise this amino acid in dystrophy.

2.4 Taurine

Taurine (2-aminoethanesulfonic acid) is a simple sulphur-containing *β*-amino acid present in virtually all cells throughout the animal kingdom, while only being found in trace amounts within plants and bacteria (Huxtable, 1992). In particular, it is enriched in electrically excitable tissues such as the brain, heart and skeletal muscles (Huxtable, 1992). In fact, skeletal muscle contains the bulk of whole body taurine, accounting for approximately 70% of total stores (Uozumi et al., 2006a). In mammalian tissues, taurine concentration is typically reported in the micro-mole per gram of wet weight (μmol/g/ww) range, with especially high concentrations found within rat and mouse tissue (De Luca et al., 2001a, Huxtable, 1992, Kocsis et al., 1976). Functionally, taurine has been implicated in the maintenance of contractile function, osmoregulation, antioxidant activity, cell signalling, receptor regulation, mitochondrial protein synthesis, inflammation, membrane stabilisation and Ca^{2+} regulation (Bakker and Berg, 2002, Conte Camerino et al., 2004, De Luca et al., 2001a, Huxtable, 1992, Schuller-Levis and Park, 2003, Warskulat et al., 2004). Moreover, taurine has also been reported to have additional biological actions such as modulation of hormone release, bile salt synthesis, stimulation of glycolysis and anticonvulsant properties (Huxtable, 1992). As this extensive list indicates, discussing all aspects of taurine's actions is beyond the scope of this review. As such, the following sections review only the key properties of taurine and biological actions that directly relate to the pathophysiology of DMD, and thus are relevant to the current thesis. For an in depth review of taurine's actions in tissues across several species see Huxtable (1992) and for a review specifically focused on skeletal and cardiac muscle, see Schaffer et al. (2010).

2.4.1 Properties of taurine

Taurine is the most abundant free amino acid in skeletal muscle, although its chemical composition differs greatly from that of classic amino acids (Timbrell et al., 1995). It is a *β*-amino acid, containing a sulphonic acid group rather than a carboxylic amino acid, and is a zwitterion over the physiological pH range, giving taurine the properties of high water solubility and low lipophilicity (Huxtable, 1992). As such, taurine has a limited ability to diffuse across membranes, which assists in maintaining the extraordinarily high concentration gradients that are reported in some tissues such as muscle (Huxtable,

1992, Warskulat et al., 2004). Taurine entry into the cell is therefore controlled by the Na^+-dependant taurine transport system (TauT), with between 1-3 Na^+ ions carried for each taurine molecule exchanged (Huxtable, 1992, Uozumi et al., 2006a, Warskulat et al., 2004). While the bulk of whole body taurine is found in the musculature, and not surprisingly the TauT is also highly expressed in this tissue, little is currently known about the regulation of TauT in skeletal muscle. Park et al (2004) was one of the first to attempt to characterise TauT regulation in skeletal muscle and found that TauT mRNA is upregulated by 55% in response to increased cortisol, and 69% when muscle cells were exposed to a combination of cortisol and IGF1. In addition to mRNA changes, taurine transporter activity increased to 187% of control measures when exposed to high levels of cortisol (Park et al., 2004). Further, Uozumi et al (2006) determined that TauT expression is also upregulated during myogenesis, with MyoD activating TauT expression (Uozumi et al., 2006a). This is interesting from a DMD perspective, as MyoD is a key myogenic regulatory factor involved in not only muscle differentiation, but also regeneration (Uozumi et al., 2006a). Moreover, TauT expression (and therefore taurine accumulation) was able to prevent chemically-induced muscle atrophy, suggesting that taurine also promotes muscle cell viability (Uozumi et al., 2006a).

While taurine accumulation in muscle via the TauT is consistent within different tissues, the ability to synthesise taurine is variable, both within tissues and between species. Cats and humans are unable to produce meaningful quantities of taurine and as such, must rely on dietary intake, while rats and mice are more efficient at production (Schaffer et al., 2010). The liver is the primary site of taurine synthesis, and requires the sulphur-containing amino acids cystine and methionine as precursors. It is important to note that unlike many other amino acids, taurine is unable to be used for ATP synthesis.

2.5 Taurine and the pathophysiology of DMD

2.5.1 Taurine and membrane stabilisation

Taurine has long been suggested to have membrane stabilising effects with several key theories as to this mechanism postulated (Schaffeer et al., 1995, Schaffer et al., 1995, Schaffer et al., 2010). The first proposed mechanism is the antioxidant hypothesis, as one of the major targets of oxidative stress are the fatty acids located on biological membranes, leading to lipid peroxidation (Schaffer et al., 1995). Therefore antioxidants, especially those that are able to reduce lipid peroxidation, may be able to prevent the extensive membrane disruption and cellular malfunction that accompanies this activation (Schaffer et al., 1995). Taurine is known to have indirect antioxidant properties, with several studies in cardiac muscle showing that taurine is able to reduce lipid peroxidation specifically (Chahine and Feng, 1998, Parildar et al., 2008, Sushamakumari et al., 1989). In skeletal muscle, a recent paper by Goodman et al. (2009) demonstrated that taurine administration to rats attenuated F(2)-isoprostane production (a sensitive indicator of reactive oxygen species-induced lipid peroxidation) during a fatiguing stimulation of the EDL muscle. This confirms the findings of previous research by Dawson et al. (2002), who found that taurine administration completely blocked exercise induced increases in lipid peroxidation. As increased membrane lipid peroxidation has been suggested as not only a key factor in the progression of muscle necrosis in dystrophy, but also suggested to be involved in the initiation of pathology, taurine administration to dystrophic *mdx* mice may be able to attenuate lipid peroxidation, and thus improve membrane stability and muscle function. This however, is yet to be investigated.

The second theory of membrane stabilisation relates to taurine's ability to bind directly to phospholipids (Schaffer et al., 1995). It is well established that the interaction between taurine and phospholiipds increases phospholipid binding affinity for Ca^{2+}, resulting from a conformational change in the membrane and opening of additional calcium binding sites (Schaffer et al., 1995). It is proposed that taurine binding further alters membrane architecture, fluidity and other important structural properties (Huxtable, 1992). This could have a number of downstream effects as numerous membrane enzymes and ion transporters are sensitive to changes in membrane lipid structure and fluidity (Schaffer et

al., 1995). One such transporter is the Na^+/Ca^{2+} exchanger, which is a primary source of Ca^{2+} extrusion from the cell (Schaffer et al., 1995). Hamaguchi et al. (1991) demonstrated that taurine is capable of preventing a decrease in Na^+/Ca^{2+} exchanger activity caused by phospholipid N-methylation (methylation alters the phospholipid head structure and therefore membrane spacing), by inhibiting the methylation process. Interestingly, N-methylation of phospholipids has also been shown to increase Ca^{2+} leak from the cardiac SR (Schaffer et al., 1995). As impaired Ca^{2+} regulation is generally agreed upon as one of the most important factors in the development of DMD, and excess Ca^{2+} entry is thought to occur largely through Ca^{2+} leak and SAC, it is possible that if these channels are also sensitive to changes in membrane structure that taurine may be able to alter the activity or accumulation of these channels. This coupled with increased Na^+/Ca^{2+} exchanger activity and decreased Ca^{2+} leak from the SR would significantly decrease Ca^{2+} entry into dystrophic tissue, preventing several of the downstream pathological events that occur as a result of Ca^{2+} overload.

Finally, one of the oldest recognised actions of taurine is osmoregulation, and is likely to be another mechanism by which taurine exerts its membrane stabilising effects. For excitable tissues, this is of particular importance as corrections for changes in cell volume associated with ionic shifts must be accounted for in order to maintain normal cell volume and enhance cell survival (Schaffer et al., 1995). Taurine is a biologically perfect omoregulator, as short-term changes in its concentration does not drastically alter cellular processes such as membrane potential or enzyme activity, while sparing other metabolically important amino acids that unlike taurine, can be coupled to ATP production (Huxtable, 1992). When cells are exposed to a hyposmotic stress a rapid efflux of taurine results in an effort to restore osmotic balance, with the resulting volume decrease serving as a safety valve preventing or minimising damage caused by excessive cell swelling (Roysommuti et al., 2003). Inversely, hyperosmotic stress leads to taurine accumulation within the cell and upregulation of TauT activity (Schaffer et al., 2010). Interestingly, several studies report that dystrophic tissue has an increased susceptibility to osmotic stress (Hutter et al., 1991, Imbert et al., 1996, Menke and Jockusch, 1991) suggesting that normal osmolyte activity is incapable of adequately responding to changes in cell volume. Moreover, taurine content is lowered during the first wave of degeneration in the *mdx* mouse (McIntosh et al., 1998a), providing a possible mechanism for this susceptibility. This also opens up the possibility that taurine

accumulation through supplementation may decrease muscle damage through its osmoregulatory effects.

2.5.2 Taurine and Ca^{2+} regulation

Due to its involvement as a modulator of many Ca^{2+}-dependant processes, the regulation of cellular Ca^{2+} homeostasis is another proposed action of taurine. Much of the interest in this area originated from work that was performed in cardiac muscle tissue, where over 40 years ago taurine was found to protect against Ca^{2+} overload cardiomyopathy, arrhythmias and the Ca^{2+} influx (Ca^{2+} paradox) found during ischemia-reperfusion injury (I-R injury) (Huxtable, 1992). The Ca^{2+} paradox refers to the phenomenon that while reperfusion of ischemic heart tissue is essential to prevent cellular death, necrosis and loss of contractile function, reperfusion itself if not restored within a critical period of time can cause a wide variety of harmful effects such as arrhythmias, contractile dysfunction and ultrastructural damage, in addition to endothelial cell dysfunction and vasoconstriction in the coronary blood supply (Kawabata et al., 2000). This damage is, in part, a Ca^{2+} mediated phenomenon, caused by Ca^{2+} dislocating from the sarcolemma and changing membrane structure, impairing the barrier to Ca^{2+} movement and leading to excessive Ca^{2+} entry during reperfusion (Huxtable, 1992, Kawabata et al., 2000). Early research demonstrated that if taurine was added to the reperfusion medium of cardiac cells exposed to a period of ischemia, the typical necrosis that follows reperfusion was minimised (Kramer et al., 1981). These findings were significant, as they showed that taurine is protective in response to Ca^{2+} induced damage, even when present after injury has already occurred (during reperfusion), is able ameliorate the consequences of this injury on the tissue (Huxtable, 1992).

Taurine has also been found to have beneficial effects on contractile force of skeletal muscle after I-R injury, preserving both twitch and tetanic force output (McLaughlin et al., 2000). Taurine's effects on I-R injury are of particular interest with regard to dystrophy, given that the decrease in nNOS associated with the loss of dystrophin has also recently been associated with recurrent bouts of functional ischemia during muscle contraction, with reperfusion after these bouts causing elevations in oxidative stress (Dudley et al., 2006). Taurine may have a similar protective role via Ca^{2+} regulation in dystrophic tissue

as has been reported in non-dystrophic cardiac tissue, decreasing contraction-induced damage and ROS production while maintaining the force output of skeletal muscle.
The mechanisms by which taurine may be having its Ca^{2+} modulating effects in I-R injury, and in skeletal muscle in general, are thought to be due to taurine's ability to either 1) modify the availability of Ca^{2+} or 2) by modifying the sensitivity to the Ca^{2+} signal or response (Huxtable, 1992). Taurine may be altering the availability of Ca^{2+} through its actions on membrane stability, as was previously discussed in section 2.6.1, which has been shown to alter the function of ion channels. Indeed, through its interaction with the cell membrane taurine is able to alter membrane characteristics, which secondarily influences ion channel activity, including key Ca^{2+} extrusion pumps such as the Na^{+}/Ca^{2+} exchanger.

In addition to this membrane binding, taurine may also modify Ca^{2+} channel kinetics (opening and closing) by directly affecting a hydrophilic site near or on the channel that is also influenced by Ca^{2+} channel antagonists (Huxtable, 1992). Several studies by De Luca and colleagues demonstrate taurine is able to exert direct actions on many ion channels, in addition to those previously mentioned, including chloride (Cl^{-}), Na^{+}, potassium ATP (K_{ATP}) and KCa channels (De Luca et al., 1996a, De Luca et al., 1992, De Luca et al., 2000). During E-C coupling, taurine has been shown to increase Ca^{2+} uptake by the SR through enhancing SERCA activity (Huxtable and Bressler, 1973). This is most likely due to taurine's antioxidant properties, as SERCA has been shown to be inhibited by oxidative stress (Park et al., 1991). More recently, Bakker and Berg (2002) demonstrated that taurine was modulating contractile function in skeletal muscle by augmenting SR Ca^{2+} uptake and release.

The direct effect on taurine of Ca^{2+} handling can also be observed under conditions of taurine depletion, where depletion of taurine using guanidinoethane sulfonate (GES) (a known inhibitor of TauT) has been shown to shift rheobase voltage during E-C coupling towards more negative potentials, which is indicative of increased cytosolic Ca^{2+} (De Luca et al., 1996b), Interestingly, *in vitro* administration of taurine to the GES treated muscle preparation was able to counteract the deleterious effects on Ca^{2+} handling (De Luca et al., 1996b). This apparent modulation of Ca^{2+} handling by taurine may explain why increasing taurine content in skeletal muscle has been associated with improved muscle function, myogenesis and a greater maximum force output (Bakker and Berg,

2002, De Luca et al., 2003, McIntosh et al., 1998b). Interestingly, the effect of taurine on SR accumulation seems to be highly dependent on the precondition of the SR, with the greatest effects demonstrated on preparations with a Ca^{2+} deficient SR (Steele et al., 1990). As several of the pathological features of DMD have been attributable directly (impaired contractile function, muscle weakness, protease activation) or indirectly (inflammation, mitochondrial dysfunction, ROS accumulation) to impaired Ca^{2+} handling, the described effects of taurine on Ca^{2+}-dependant processes could have fundamental importance in limiting dystrophic pathology.

2.5.3 Taurine and oxidative stress

It is generally well accepted that taurine protects cells from oxidative injury, even though it is incapable of directly scavenging many of the classical reactive oxygen species (Aruoma et al., 1988, Schaffer et al., 2010). An early study by Aruoma et al. (1988) demonstrated that taurine does not directly react with superoxide (O_2^-), hydrogen peroxide (H_2O_2) or the hydroxyl radical. Despite this, taurine is capable of having direct effects on a few aspects of oxidative stress. For example, the oxidation process that converts hypotaurine to taurine may serve as a radical trap for hydroxyl radicals, and lipid peroxidation may be reduced by the decrease in malondialdehyde associated with the presence of hypotaurine (Marcinkiewicz et al., 1995, Schuller-Levis and Park, 2004). Taurine is also a natural scavenger of the hypochlorous anion (HOCl) and forms taurochloramine (TauCl) (Marcinkiewicz et al., 1995, Schuller-Levis and Park, 2004). TauCl has beneficial properties that could protect against muscle damage, as it is a powerful antiinflammatory agent with the ability to inhibit several inflammatory mediators such as TNFα, interleukin 6 (IL6) and prostaglandin E2 (Marcinkiewicz et al., 1995, Schuller-Levis and Park, 2004). Aside from these examples, it appears that the antioxidant properties of taurine are indirect, and that taurine is either modulating antioxidant defence systems, or blocking the action of oxidants.

Examples of taurine modulating antioxidant defence are available in the literature, although little evidence exists in mammalian skeletal muscle. A study examining sodium fluoride (NaF) toxicity on hepatocytes extracted from rat livers found that administration of taurine increased cell viability, restored the depleted efficiency of several antioxidant enzymes (glutathione, superoxide dismutase (SOD) and catalyse) and reduced lipid

peroxidation (Das et al., 2008). Similarly, research in cardiac muscle determined that taurine reduced the oxidative stress associated with arsenic toxicity, and reduced myocardial cell apoptosis (Ghosh et al., 2009). It is proposed that taurine's effect in these studies may be the result of reductions in toxicity, rather than a direct upregulation of antioxidant activity. There is however a handful of studies that raise the possibility that taurine may be acting on the antioxidant enzymes directly. Kurz et al. (1998) demonstrated that an upregulation of heat-shock protein by taurine was able to protect *β*-cells against NO toxicity, and Vohra and Hui (2001) found that taurine increased the activity of SOD and glutathione peroxide in neuronal tissue.

Taurine may also be able to reduce ROS formation through its membrane stabilising effects thereby altering membrane fluidity and activity of enzymes increasing the resistance to oxidative damage (Schaffer et al., 2009). In skeletal muscle specifically, taurine has been shown to decrease markers of oxidative stress and increase antioxidant enzymes. Both Goodman et al. (2009) and Dawson et al. (2002) determined that taurine supplementation is able to decrease markers of lipid peroxidation associated with muscle contraction. Silva et al. (2011) also demonstrated that after 15 days of taurine supplementation, eccentrically exercised rats showed decreases in several markers of oxidative stress and muscle damage. This included decreases in superoxide radical production, lipoperoxidation and carbonylation levels with increased total thiol content in skeletal muscle (Silva et al., 2010). Although antioxidant enzyme activity was not altered after exercise, CK concentration was significantly reduced in plasma of the taurine supplementation group (Silva et al., 2010). As oxidative stress is proposed as a mechanism of muscle damage in DMD, the described actions of taurine could possibly ameliorate dystrophic pathology through this pathway. Despite protection largely being attributed to indirect actions of taurine, several of the above examples have merit in skeletal muscle, especially given the decreases in antioxidant activity reported in the *mdx* mouse (Disatnik et al., 1998, Messina et al., 2006a).

2.5.4 Taurine and inflammation

Taurine has been shown to be a powerful modulator of the immune response, with the ability to downregulate the production of pro-inflammatory mediators in both human and animal tissues (Bouckenooghe et al., 2006, Marcinkiewicz et al., 2009, Schuller-Levis

and Park, 2003). Most of taurine's anti-inflammatory actions are attributed to TauCl, which is a relatively stable oxidant that is produced at the site of tissue inflammation through the myeloperoxidase (MPO) pathway (Schuller-Levis and Park, 2004). Both neutrophils and monocytes contain high levels of MPO, that when combined with H_2O_2 form HOCl, which causes tissue damage by directly oxidising carbohydrates, lipids, nucleic acids and amino acids (Schuller-Levis and Park, 2004). Taurine is able to directly scavenge HOCl, forming the far more stable and less toxic TauCl. Further, it appears that TauCl has several biological actions, including the ability to inhibit the formation of many potent regulators of immunity such as TNF-α, NF-κB, IL-6 and IL-8 as well as decreasing NO and O_2^- production (Bhavsar et al., 2010, Bouckenooghe et al., 2006). Through its downregulation of inflammatory mediators and reductions in the amount of ROS produced, taurine has been shown protect tissues from some pathologies (Delic et al., 2010, Schuller-Levis et al., 2009, Schuller-Levis et al., 2003). For example, rat lung tissue treated with the drug bleomycin, which typically causes extensive lung injury including alveolar wall thickening, collapse and scarring, found that taurine was able to significantly reduce the inflammatory response to the drug, and decrease the fibrosis associated with bleomycin treatment (Schuller-Levis et al., 2009, Schuller-Levis et al., 2003). The ability of taurine to reduce lung fibrosis is of particular interest with regard to DMD, as accumulation of fibrotic tissue in dystrophic muscle tissue significantly impacts muscle function and decreases mobility and quality of life in DMD patients (Jansen et al., 2010). If taurine treatment is able to significantly decrease fibrosis in skeletal muscle as has been reported in lung tissue, taurine treatment could have significant beneficial effects in dystrophy.

Interestingly, while taurine has been found to downregulate the production of pro-inflammatory mediators, it also appears to be essential for normal immune system function and important for strengthening host defence capabilities (Delic et al., 2010). For example, taurine has been shown to enhance B-cell proliferation and improve the activity of neutrophils (Muhling et al., 2002, Stapleton et al., 1998) Moreover, when taurine is used in conjunction with chemotherapy it improves tumour inhibition rates by increasing the count and classification of white blood cells, improving lymphocyte proliferation and macrophage phagocytic capabilities, and elevates measures of spleen and thymus function in cancer bearing mice (Wang et al., 2009). Not surprisingly given this role in strengthening host immunity, taurine deficiency in animals with a limited

ability to synthesise the amino acid has been demonstrated to cause significant immune-related abnormalities (Delic et al., 2010, Schuller-Levis and Sturman, 1990). Studies investigating taurine deficiency in cats has found significant leukopenia with depletion of mature and immature lymphocytes, reticular cells (white blood cell storage site in lymph nodes), a decrease in phagocytic capabilities and mild extra-vascular haemolysis (Schuller-Levis et al., 1990). Mice with a TauT deletion, and therefore significant reductions in intracellular taurine content are also more prone to infection, and exhibit increases in inflammatory molecules such as TNF-α and IL1-*β* coupled with greater rates of liver injury (Delic et al., 2010). However perhaps most interestingly, when TauT negative mice are infected with malaria a significant proportion die from multi-organ failure, whereas over 90% of control animals survive (Delic et al., 2010).

As chronic inflammation is considered a significant contributor to the development of dystrophic pathology, and DMD muscle tissue exhibits inflammatory changes such as increases in inflammatory mediators and infiltration of inflammatory cells (Acharyya et al., 2007), it is possible that taurine may be able to ameliorate some of these effects through its immunomodulatory actions. Of particular significance is taurine's ability to downregulate pro-inflammatory mediators such as TNF-α and NF-κB, both of which have been shown to be upregulated in dystrophic human and mouse skeletal muscle (Acharyya et al., 2007, Grounds et al., 2008a, Haslett et al., 2002, Hnia et al., 2008). Taken together, the evidence presented here suggests that taurine may be able to improve dystrophic muscle tissue function, at least in part, due to its effects on immune system-mediated inflammation.

2.5.5 Taurine and metabolism

There is mounting evidence that although taurine is unable to be incorporated into energy production, it may be able to modulate mitochondrial function and energy production via indirect mechanisms (Chang et al., 2004, Schaffer et al., 2009, Sun et al., 2011). It was recently determined that taurine can form a conjugate with mitochondrial transfer RNA's (tRNA) for leucine and lysine, and that a lack of taurine can result in impaired translation of mitochondrial encoded proteins (MEP) (Schaffer et al., 2009). As MEP's are involved in the assembly of respiratory chain complexes, disruption in the translation and expression of MEP's may result in impaired electron transport, and thus

lower energy production (Schaffer et al., 2009). Indeed, defective MEP translation has been shown to reduce electron transport flux, increase formation of O_2^- and lower rates of ATP production (Ricci et al., 2008). This links in well with other examples in the literature where taurine has been shown to reduce mitochondrial O_2^- generation, and increase SOD and catalase activity (Chang et al., 2004, Parvez et al., 2008).

In addition to modulation of oxidative metabolism and MEP expression, taurine has also been shown to enhance glucose uptake in both skeletal and cardiac muscle, thereby possibly improving substrate delivery for oxidation (Carneiro et al., 2009, Ribeiro et al., 2009). Carneiro et al. (2009) examined mice that were fed a diet supplemented with and without taurine for 30 days, and found that taurine regulates the expression of genes required for glucose-stimulated insulin secretion, and enhanced peripheral insulin sensitivity. Similarly, Ribeiro et al. (2009) demonstrated that taurine supplementation in mice improved glucose tolerance, increased glucose uptake and enhanced insulin sensitivity. Control of substrate delivery is not exclusive to glucose, with taurine being found to significantly increase fat oxidation during a submaximal cycling bout in trained athletes, suggesting that taurine can alter substrate selection (Rutherford et al., 2010). Not surprisingly, metabolic alterations are observed with taurine depletion, with significant elevations in lactate and pyruvate production and a concurrent reduction in pyruvate utilisation by the CAC observed in taurine-depleted animals (Schaffer et al., 2010).

Interestingly, in addition to taurine's apparent role in metabolism and substrate availability, taurine has also been shown to protect against mitochondrial-mediated cell death and preserve mitochondrial function when tissues are exposed to stress (Das et al., 2010, Sun et al., 2011). Thus, while taurine would be unable to directly provide energy to dystrophic tissue, it's apparent that the involvement of taurine in several mitochondrial- related processes may assist indirectly in improving energy production and altering substrate utilisation within dystrophic skeletal muscle.

2.5.6 Taurine and contractile function

There is growing evidence that taurine is essential for normal skeletal muscle contraction, as alterations in taurine content can result in contractile dysfunction and decreased performance (Dawson et al., 2002, Hamilton et al., 2006, Warskulat et al., 2007). Depletion of taurine through transport inhibitors, such as GES and *β*-alanine, have demonstrated significant detrimental modifications to MT, Cl^- conductance (Conte Camerino et al., 2004, De Luca et al., 1996b), twitch and sub tetanic force (Hamilton et al., 2006), exercise tolerance (Dawson et al., 2002) and myofilament sensitivity to Ca^{2+} (Cuisinier et al., 2000a). Similarly, the recently developed TauT knockout mouse, which displays reduced muscular taurine content of up to 96% (Ito et al., 2010, Warskulat et al., 2007), exhibit an 80% decrease in total exercise capacity, and are unable to run at even 50% of the speed attained by wild-type animals, when subjected to a downhill running treadmill protocol (Warskulat et al., 2004). Moreover, even at rest the TauT knockout mice had plasma CK concentration double that observed in controls, which may in part explain the decreased exercise tolerance (Warskulat et al., 2004). More recent investigations in TauT knockout mice have confirmed the reduced exercise tolerance during treadmill running, as well as during a weight loaded swimming test (Ito et al., 2010). Conversely, and perhaps not surprisingly, given the results obtained under conditions of taurine depletion, raising skeletal muscle taurine content has been shown to have positive effects on muscle contractile function on a whole body, whole muscle, and single fibre level (Bakker and Berg, 2002, Dawson et al., 2002, De Luca et al., 2003, Goodman et al., 2009, Yatabe et al., 2009b).

Whole body investigations of muscle function commonly examine the effect of taurine supplementation on exercise performance during treadmill running, as taurine has been shown to be depleted with exercise and preferentially lost from fast-twitch muscles (Matsuzaki et al., 2002). Dawson et al (2002) supplemented rats for one month with a solution of 3% taurine via drinking water, and found that supplemented rats demonstrated improved running performance, as well as a decrease in exercise-induced lipid peroxidation within fast-twitch muscle (Dawson et al., 2002). Similarly, Yatabe et al. (2009) found that running time to exhaustion in rats was significantly increased with taurine supplementation, and that the exercise-induced loss of taurine was attenuated with taurine treatment.

On a whole muscle level, several investigations have shown beneficial effects with taurine supplementation. A series of experiments by De Luca and colleagues have shown that through effects on intracellular Ca^{2+} homeostasis, taurine is able to beneficially modulate E-C coupling and contractile function in skeletal muscle (Conte Camerino et al., 2004, De Luca et al., 2000). The MT (membrane potential required for the initiation of contraction) of skeletal muscle indicates the rate at which Ca^{2+} release equals that of Ca^{2+} reuptake. DeLuca et al. (1996) found that GES depletion of taurine results in a shift of MT to more negative potentials, indicating that cytosolic Ca^{2+} concentration had increased. Interestingly, subsequent *in vitro* administration of taurine was able to restore normal MT, demonstrating that the alterations of MT and Ca^{2+} handling was a taurine-dependant effect (De Luca et al., 1996b). Similarly, the age-related decline in taurine content and subsequent alteration to MT in rats is ameliorated by the introduction of taurine, restoring MT to that of adult animals indicating better Ca^{2+} handling and stabilisation of the sarcolemma (Pierno et al., 1998). More recently, Goodman et al. (2009) demonstrated that taurine supplementation improved twitch and subtetanic specific force of rat EDL muscles, while attenuating oxidative stress during a fatiguing stimulation. This was coupled with a 49% increase in CSQ protein content, and significantly enhanced recovery after the stimulation bout (Goodman et al., 2009).

In addition to whole muscle investigations, the utilisation of single fibres has given further insight into taurine's mechanism of action in relation to E-C coupling. Bakker and Berg (2002) demonstrated that taurine enhances force production in skinned muscle fibres by increasing Ca^{2+} release from the SR, despite a small but significant decrease in the Ca^{2+} sensitivity of the contractile apparatus (Bakker and Berg, 2002). The same technique demonstrated that taurine depletion (with GES) decreased twitch force to 23% of control levels, although maximal specific force was unaffected (Hamilton et al., 2006). This study also demonstrated that GES treatment led to reduced force production at stimulation frequencies between 50-100 Hz, and increased resistance to fatigue (Hamilton et al., 2006). Interestingly, results also showed small decreases in contractile filament sensitivity to Ca^{2+}, as was previously shown with elevated taurine by Bakker and Berg's (2002), suggesting that GES may have some taurine-like effects on E-C coupling (Hamilton et al., 2006). Directly contrasting these findings is earlier work by Cuisinier et al (2000) who found that both taurine and GES increased myofilament sensitivity to Ca^{2+}, with no modification to SR Ca^{2+} uptake as measured by flura-2 (Cuisinier et al., 2000a).

Although not necessarily always consistent, the evidence obtained in whole body, muscle and single fibre preparations clearly show that taurine plays a key role in the regulation of skeletal muscle contraction. As taurine concentration has been shown to decrease in dystrophic skeletal muscle and urinary excretion of the amino acid is upregulated in DMD, it is possible that some of the contractile dysfunction associated with this disease may be the result of taurine depletion. Thus, there is potential for taurine as a therapeutic adjunct, as raising taurine concentration in skeletal muscle has shown many positive effects in non-dystrophic tissue. However to date, only two studies have examined the effect of taurine supplementation on dystrophic skeletal muscle.

2.6 Taurine and the *mdx* mouse

To date, there are very few investigations available in the literature examining the effect of taurine on skeletal muscle function in the *mdx* mouse (De Luca et al., 1998, Griffin et al., 2001, McIntosh et al., 1998a). Some studies have utilised *in vitro* application of taurine to muscle preparations (De Luca et al., 1998, De Luca et al., 2001a), while only two investigations have ever supplemented the *mdx* mouse (Cozzoli et al., 2011b, De Luca et al., 2003). The following sections review the available literature that has examined the role of taurine in dystrophic muscle tissue function specifically.

2.6.1 Tissue taurine content in the *mdx* mouse

McIntosh et al. (1998) used proton nuclear magnetic resonance spectroscopy (NMR) to examine *mdx* and C57BL10 tissue throughout the lifespan, to determine if this technique could identify dystrophic from non-dystrophic tissue, and also the stage of repair in the DIA and hind limb musculature. A corticosteroid supplement group was also included within this study, to see if beneficial effects on muscle damage associated with treatment could also be measured. This study utilised three time points for sampling in order to gain information on three key stages of *mdx* dystrophic progression; young (<3 weeks), adolescent (3-6 weeks) and adult (>6 weeks), which respectively corresponds to pre, active and stable levels of muscle degeneration (McIntosh et al., 1998a). Results showed that low taurine content was correlated with the pre and active dystrophy stages, and that concentration of taurine increased with stabilisation of the disease and tissue repair (McIntosh et al., 1998a). Moreover, taurine concentration increased with age in the TA and DIA until ultimately reaching a greater level than that of control muscle (McIntosh et al., 1998a). These results lead the authors to suggest that taurine may be a marker for a large capacity for muscle regeneration (McIntosh et al., 1998a), as is seen in the *mdx* mouse and is thought to be a primary reason for the milder phenotype observed in these dystrophic animals. Interestingly, treatment with corticosteroids resulted in greater amounts of taurine being accumulated in skeletal muscle, especially within hind limb muscles, which are the most acutely affected during this stage of the *mdx* lifespan (McIntosh et al., 1998a).

The apparent correlation between taurine content and the regenerative capacity of skeletal muscle was further investigated by the same research group, examining NMR's capability to distinguish regeneration levels in three different phenotypes of myogenesis (McIntosh et al., 1998b). This included four month old *mdx* mice, MyoD negative mice and double mutant *mdx*/MyoD negative mice. MyoD knockout mice were chosen as while skeletal muscles develop normally, they have a dramatically reduced capacity for regeneration after injury (McIntosh et al., 1998b). Four days after a crush injury to the TA muscle, NMR was used to determine if this technique could distinguish the different degrees of regeneration occurring within the three mouse models (McIntosh et al., 1998b). Taurine was highest in *mdx* muscles, which showed the highest levels of regeneration (McIntosh et al., 1998b). The lowest taurine levels were found in the MyoD knockout which demonstrated the least effective regenerative response, and intermediate taurine levels were observed in the double-mutant mouse (McIntosh et al., 1998b). Griffin et al. (2001) utilised a similar technique to investigate additional tissues in the *mdx* mouse such as cardiac and brain tissue. Again, taurine content was clearly altered in the *mdx* mouse and distinguished dystrophic from control tissue. Moreover, other alterations were also found in markers of glycolysis, *β*-oxidation, CAC cycle and PCr (Griffin et al., 2001). Taken together, these studies clearly show that taurine is altered in dystrophic tissue, and that increases in taurine correlate with regeneration and therefore possibly improvements in muscle function.

2.6.2 Taurine and muscle function in the *mdx* mouse

The *in vitro* application of taurine to skeletal muscle preparations has been shown to improve skeletal muscle function in the *mdx* mouse. For example, De Luca et al. (1998) examined the effect of *in vitro* application of taurine to the *mdx* EDL on E-C coupling measures including MT, with results demonstrating that, similar to taurine depleted rat muscle (De Luca et al., 1996a), *mdx* muscle exhibits a shift towards more negative potentials (De Luca et al., 1998). This indicates that Ca^{2+} handling has been altered in the *mdx* mouse, either through increases in the release of Ca^{2+} or reductions in Ca^{2+} uptake (De Luca et al., 1998). Application of 60mM taurine to the *mdx* muscle preparation significantly restored the MT, suggesting an improvement in Ca^{2+} handling and stabilisation of the sarcolemma (De Luca et al., 1998). In a related study, E-C coupling in the *mdx* EDL muscle was examined at 8-12 weeks and 6-12 months of age,

and also the taurine content of the TA muscle and plasma (De Luca et al., 2001a). The MT was found to be shifted to more negative potentials at both age groups and was associated with a clear trend towards lower taurine content in skeletal muscle (De Luca et al., 2001a). Interestingly, accompanying this trend towards lower taurine content in muscle was a rise in plasma taurine content, such that *mdx* mice displayed a 140% increase in plasma taurine relative to non-dystrophic controls (De Luca et al., 2001a). The authors suggest that the markedly increased plasma taurine may indicate an impaired ability to retain appropriate amounts of taurine in dystrophic skeletal muscle (De Luca et al., 2001a). These results may suggest that TauT expression or activity may be lowered in *mdx* muscle.

2.6.3 Taurine supplementation and the *mdx* mouse

To date, only two studies have investigated the effect of taurine supplementation on *mdx* skeletal muscle function (Cozzoli et al., 2011b, De Luca et al., 2003). De Luca et al. (2003) supplemented *mdx* mice with taurine (10%/weight in chow) before exposing them to a chronic treadmill running program designed to enhance the dystrophic phenotype of the mice. Measures of normalised grip strength showed that taurine treatment counteracted the deleterious effect of treadmill exercise on forelimb force development and moreover, restored MT to more positive potentials, which is a profile associated with sedentary controls (De Luca et al., 2003). A high level of Cl^- conductance in EDL muscles was also maintained with taurine treatment, indicating improved stability of the sarcolemma, although this was not observed in the DIA (De Luca et al., 2003). The authors suggest that the positive effects observed with taurine supplementation could be due to improved Ca^{2+} handling (as evidenced by MT), increased antioxidant activity or potentially taurine's anti-inflammatory effects (De Luca et al., 2003).

Most recently Cozzoli et al. (2011) used taurine in combination with the most beneficial and widely used DMD treatment, glucocorticoids, to determine if any synergistic actions resulted in better outcomes with combined therapy versus glucocorticoid treatment alone. Male control and *mdx* mice at 4-5 weeks of age were subjected to treadmill exercise twice a week for 4-8 weeks in association with three treatments, α-methyl prednisolone (PDN), taurine alone or PDN and taurine therapy combined (Cozzoli et al., 2011b). Groups of sedentary wild type and *mdx* animals were also included for

comparison, in addition to an untreated but exercised *mdx* group (Cozzoli et al., 2011b). While a similar histological profile was observed for both PDN and PDN+Taurine treatment, the beneficial effects of PDN+Taurine combined therapy on MT were better than that of PDN treatment alone (Cozzoli et al., 2011b). Moreover, combined PDN+Taurine therapy was also able to counteract the over-activity of the subset of Ca^{2+} channels that had previously been found to contribute to the altered Ca^{2+} homeostasis observed in dystrophic myofibres (Cozzoli et al., 2011b). The findings of these two studies suggest that it is likely that taurine supplementation would improve additional measures of skeletal muscle function.

2.7 Conclusions

The review of literature presented clearly demonstrates a possible role for taurine in amelioration of skeletal muscle pathology associated with DMD. This is due to taurine's ability to target multiple aspects of DMD damage pathways such as membrane instability, impaired Ca^{2+} handling, increased oxidative stress and chronic inflammation. Despite this, there is very little evidence available in the literature on taurine and dystrophic skeletal muscle function, suggesting that additional investigation into taurine and its possible beneficial effects is necessary. As force production is considered a gold-standard of skeletal muscle function, and one of the hallmarks and key therapeutic targets of DMD is impaired muscular strength, endurance and recovery, the effect of taurine on these parameters should be a key line of enquiry.

2.8 Broad and specific aims of the thesis

The broad aim of this thesis was to examine the effect of taurine supplementation on *mdx* skeletal muscle taurine content and contractile function.

2.8.1 Aims of study one

The purpose of this investigation was to determine if taurine supplementation could elevate muscle taurine content in the *mdx* mouse during the acute phase of degeneration, through to an age showing a more stabilised phenotype. The expression of the TauT was also examined to determine if this is altered in *mdx* muscle, and whether TauT expression is altered with taurine treatment. Several E-C coupling proteins were also investigated to determine if taurine is able to modulate their expression in dystrophic skeletal muscle.

The specific aims of this study were;

i) To investigate the effect of taurine supplementation on skeletal muscle taurine content throughout both the degeneration/regeneration cycles (days 28 to 45) and stabilised phase (70 days) of the *mdx* mouse lifespan.

ii) To characterise the expression of the taurine transporter (TauT) in the *mdx* mouse during degeneration and stabilisation as well as in association with taurine supplementation.

iii) To determine the expression of the major E-C coupling and Ca^{2+} handling proteins (SERCA, DHPR, RyR, CSQ) during the degeneration/regeneration cycle in the *mdx* mouse, and in association with taurine supplementation.

It was hypothesised that taurine supplementation would increase taurine content in hind limb and respiratory muscles of control and *mdx* muscles, and that TauT expression would be lower in *mdx* muscles during peak degeneration, and increase with repair, but that this would be unaltered by supplementation. Similarly, E-C coupling proteins and CSQ would be lower in *mdx* muscles during acute damage but increase with repair. It was also hypothesised that CSQ protein expression would be increased with taurine supplementation.

2.8.2 Aims of study two

The primary aim of this study was to determine if taurine supplementation can improve contractile function in the *mdx* mouse. In an effort to determine the most appropriate timing of taurine treatment, the second aim of this study was to determine if long or short-term taurine supplementation has different effects on contractile function,. The activity of key enzymes involved in glucose metabolism and fat oxidation were also examined, to determine if taurine treatment enhanced enzymatic function.

The specific aims of this study were to;

i) Determine if treatment with taurine improved contractile function in *mdx* EDL and SOL.

ii) To determine if long or short-term treatment is more effective in improving contractile function in the *mdx* mouse.

iii) To determine if taurine treatment reduces muscle damage as measured indirectly via plasma CK concentration.

iv) To determine if taurine treatment affects the activity of some key metabolic enzymes in skeletal muscle, specifically, PFK, CK, CS and *β*-HAD.

It was hypothesised that both long and short-term taurine supplementation would significantly improve contractile function in the *mdx* mouse and significantly decrease plasma CK concentration; although it was expected that long-term supplementation would be more effective than the short-term treatment. It was also hypothesised that both taurine treatments would significantly improve the activity of key enzymes involved in glucose and fat metabolism, and that long-term treatment would be more beneficial.

2.8.3 Aims of study three

The purpose of this study was to determine if 3% w/v taurine supplementation would improve contractile function when measured with a physiological contractile protocol, and if this effect was the same in both non-dystrophic control and *mdx* mice. In addition, to determine if depletion of skeletal muscle taurine, using the competitive transport inhibitor *β*-alanine, would significantly impair contractile function and again, if the degree of this impairment is the same for both control and *mdx* mice. Protein expression of the TauT and several key E-C coupling and Ca^{2+} handling proteins were also examined to determine if taurine or *β*-alanine supplementation alter their expression.

The specific aims of this study were;

i) To determine if 4 weeks of 3% [w/v] taurine or *β*-alanine treatment can significantly increase and decrease taurine content, respectively, in fast and slow-twitch skeletal muscle of 6 month old control and *mdx* mice.

ii) To determine if taurine or *β*-alanine treatment alter contractile function when measured with a physiological contractile protocol, including fatigue and recovery characteristics in the EDL and SOL muscles of control and *mdx* mice.

iii) To determine if 4 weeks of taurine or *β*-alanine treatment alter the expression of the TauT in EDL, and several key E-C coupling and Ca^{2+} handling proteins such as CSQ, RyR, DHPR and SERCA in EDL.

It was hypothesised that taurine supplementation would significantly increase skeletal muscle taurine content and improve contractile function while *β*-alanine supplementation would significantly decrease tissue taurine content and reduce contractile function in both control and *mdx* mice. It was hypothesised that TauT expression would be unaltered with taurine supplementation but would increase with *β*-alanine supplementation, while contractile proteins CSQ and RyR would be altered with taurine and *β*-alanine treatment.

CHAPTER THREE

GENERAL METHODS

3.1 Animals and supplementation

The animals used in this research were male and female dystrophic *mdx* or wild-type C57BL10 mice. All animals in Chapter 4, and all except the short-term supplement group in Chapter 5, were bred at The University of Melbourne animal housing facility from breeding pairs originally obtained from the Animal Resource Centre (ARC) (Western Australia, Australia), due to the requirement of maternal supplementation for some groups in these chapters. Animals used in Chapter 6 were purchased from ARC six weeks before experimental procedures and then housed at The University of Melbourne animal housing facility. This allowed time for the mice to adjust to the new housing conditions as well as for supplementation to be conducted and monitored. All mice were housed at room temperature (20-25°C) with normal light and dark cycles and access to food and water *ad libitum*. Taurine supplementation was administered via drinking water (see Chapters 4-6 for specific details regarding dosage and monitoring procedures). Experimental animals were transported to the Victoria University Exercise Metabolism Unit for data and sample collection. Mice were anesthetised with intra-peritoneal (IP) injections of 40-50 mg/kg of Nembutal (Pentobarbitone Sodium, Rhone Merieux, QLD, Australia) with additional doses given as required to ensure animals remained unresponsive to tactile stimuli. All animal procedures described in this thesis were approved and carried out in accordance with the guidelines of the Victoria University Animal Experimentation Ethics Committee.

3.2 Contractile protocol

3.2.1 Dissection procedures

Skin was removed from the lower limb and under a dissecting microscope (Leica, ZOOM 2000^{TM} Z45V, China) the proximal tendon of the EDL was located by clearing away the surrounding tissues. Fine tipped forceps were then inserted under the proximal EDL tendon and size 2.0 surgical silk (LA-55G Ethicon, Johnson & Johnson, NSW, Australia) was fed through and then tied securely around the tendon to form a “stopper knot”. Size 4.0 surgical silk was then tied to the tendon, distal to the stopper knot, which then formed the ties that would be used to secure the muscle to the contractile setup. The same isolation and tying technique was then performed for the distal EDL insertion. The distal tendon of the EDL was then cut and the muscle was carefully dissected away from the other tissues to the point of the proximal tendon insertion.

Once fully dissected out the muscle was quickly placed into a horizontal custom-built plexiglass muscle bath containing Krebs Henseleit Ringer solution [NaCl 118 mM; KCL 4.75 mM; Na_2HPO_4 1 mM; $MgSO_47H_2O$ 1.18 mM; $NaHCO_3$ 24.8 mM; $CaCl_2$ 2.5 mM and D-Glucose 11.0 mM, pH 7.4]. This HCO_3^- based buffer solution was aerated with carbogen (95% O_2 and 5% CO_2) (BOC gases, Melbourne, Australia). The temperature of the muscle bath was maintained (see individual chapters for specific temperatures) by circulating heated water internally through the plexiglass bath and ensuring constant laboratory temperature, both of which were monitored throughout the experimental protocol. The muscle was then left in the bath to equilibrate while contralateral EDL was dissected out and placed in another muscle bath containing Krebs ringer solution bubbled with carbogen. This muscle was maintained in the bath for the same duration as the contracted. The exposed surgery sites on the mouse were wrapped in saline-soaked gauze, to prevent drying out of the tissues. The level of anaesthesia was monitored periodically while the contractile protocol was being conducted on the EDL, with additional doses of Nembutal being administered as required.

Once the EDL contractile protocol was completed the muscle was removed from the contractile setup, blotted on filter paper (Whatman No. 1, Maidstone, UK) and the silk and excess tendon removed. The muscle was then weighed and snap frozen in liquid nitrogen. The Krebs ringer solution in the bath was drained, the bath was cleaned and fresh solution was added. The SOL muscle was then dissected and tied, using the same technique as was described previously for the EDL. The contralateral SOL was then removed and placed a muscle bath as previously described for EDL, while a sample of blood was taken and additional muscles removed. The Plantaris (PLANT), TA and GAST muscles were all removed and snap frozen before the chest cavity was opened, and a small section of diaphragm was cut close to the rib cage to allow access to the heart. The heart was then pierced with a 22 gauge catheter (Terumo, NSW, Australia) and resulting blood flow was collected (Chapter 6), or cavity blood was collected once the heart was removed (Chapter 5), which was then centrifuged at 12, 000 RPM for 2 minutes. Plasma was carefully removed, placed in a cryule and snap frozen in liquid nitrogen. The diaphragm and heart were then dissected out, blotted on filter paper to remove any excess fluid or blood, and snap frozen. The contractile protocol was then performed on the SOL, with the contracted and contralateral rested muscle being snap frozen at the conclusion of the contractile protocol. All muscle and plasma samples were then stored (-80°C) until further analysis.

3.2.2 Stimulation protocols

The general procedure for the contractile protocol performed in Chapter 4 and 6 was similar, as was the procedure for the different muscles (EDL and SOL). Thus, a description of common procedures is presented within this chapter. Chapters 4 and 6 specifically detail the stimulation frequencies, fatigue and recovery times that were utilised for individual studies.

Once the muscle had equilibrated in the bath, the proximal end of the muscle was tied to a micromanipulator, while the distal end was attached directly to an isometric force transducer (Research Grade 60-2999, Harvard Apparatus, South Natich, MA). This arrangement ensured that the direction of force mimicked that of the muscles natural direction of force development *in vivo*. Within the bath, the muscle was flanked by field-stimulating platinum-plate electrodes attached to a stimulator (Grass S1 stimulator,

Quincy, MA), coupled to an amplifier (CE-1000, Crown Instruments, Elkhart, IN, USA) to ensure supramaximal stimulation. Stimulation of the muscle by the electrodes results in deflection of the force transducer (which was previously calibrated with a calibration weight of known mass), and a measurable electrical signal that is proportional to the force produced. These electrical signals were converted to a digital signal by Powerlab 4510 (ADI Instruments, Castle Hill, NSW, Australia) running Chart, Version 5.02 for Windows.

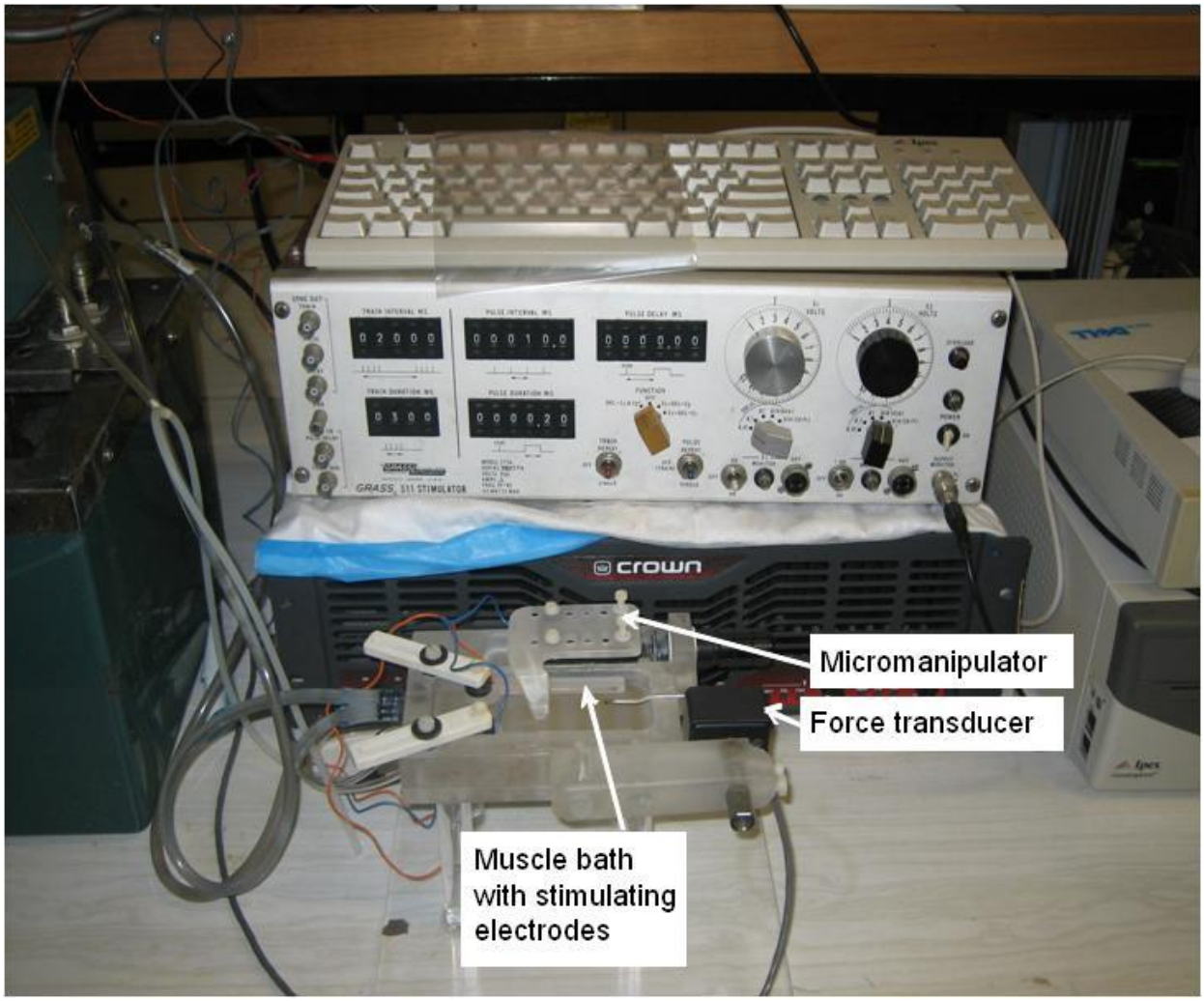


Figure 3.1 Contractile machine setup used for stimulation protocols

Once secured, the optimal length (L_o) of the muscle was determined by eliciting twitch contractions and adjusting the muscle length with the micromanipulator, until the maximum twitch force (P_t) was obtained. The muscle length was then measured and later used to determine the optimal fibre length (L_f) with the previously established L_f to L_o ratio for mouse of 0.44 for EDL and 0.71 for SOL (Brookes and Faulkner, 1988). A single tetanic stimulation at 100 Hz for EDL and 80 Hz for SOL was then performed, to ensure that the knots anchoring the muscle to the contractile setup were secure. If a slip occurred at this point, or at any time throughout the experiment, the muscle length was again adjusted until optimal length was re-established. Following a three minute recovery period, the force frequency relationship was established by stimulating the muscle at frequencies ranging from 10-160 Hz, with 3 minutes rest between stimulations. The P_o was determined from the greatest force produced during the force frequency stimulations. The force produced at each stimulation frequency was then expressed as a percentage relative to the P_o obtained. The sP_o, which accounts for differences in muscle CSA and length was also determined. This was done by first calculating CSA with the equation below;

$$\text{CSA} = \frac{\text{Muscle Mass (mg)}}{\text{Fibre length x density}}$$

The fibre length was determined using the previously established L_f to L_o ratios for EDL and SOL, and density is equal to 1.06 g/cm^3 (Close, 1972).

Peak isometric twitch force was then measured by eliciting three twitch contractions (0.2ms) and calculating the mean value for the factors below;

1. P_t
2. TTP
3. ½ RT

These measures were performed as they can be used as a crude indicator of the rate of calcium release (TTP) and re-uptake (½ RT) into the sarcoplasmic reticulum. Finally, muscles were subjected to a fatigue protocol that consisted of a continuous high frequency, short duration stimulation (see Chapter 5) or intermittent stimulation at more

physiological frequencies with recovery measures (see Chapter 6). On completion of contractile testing, muscles were quickly removed from the muscle bath, blotted on filter paper, and the tendons and surgical silk removed. The muscle was then weighed, snap frozen and stored at -80°C until further analysis.

3.3 Taurine content

3.3.1 Muscle sample preparation

The analysis of taurine content required that the muscle samples be freeze-dried prior to the taurine extraction process. As the typical dry weight to wet weight ratio is 1:4, at least 10-15 mg of muscle was required to ensure sufficient freeze-dried tissue was available for extraction. Prepared muscles were weighed into cryules with vented lids, and placed in a pre-cooled freeze drier (-40 °C) (Edwards Modulyo, Edwards High Vacuum, Britain, England) for a minimum of 48 hours.

After 48 hours muscle samples were removed from the freeze-drier and placed in a desiccator at room temperature for a minimum of 1 hour. Samples were then weighed to determine the dry to wet weight percentage (% dw/ww), as this is a measure of the effectiveness of the freeze drying process and water content of the sample. After the % dw/ww was determined, samples were crushed to powder with a mortar and pestle and then observed under a microscope, so any blood or connective tissue could be removed. The crushing process was kept to a maximum of 10 minutes, to ensure that the sample did not regain excessive moisture. Two milligrams of powdered muscle was then weighed out into an eppendorf tube and stored in a desiccator until all samples were ready for the taurine extraction process. All samples were crushed and extracted on the day of removal from the freeze drier.

3.3.2 Taurine extraction

Taurine extraction was performed on ice with all extraction solutions and tubes pre-cooled to 0 °C. The tube containing 2 mg of powdered muscle was removed from the desiccator and 20 % w/v of sulfosalicylic acid was added to the tube, before the sample was vortexed for 5 seconds and then placed on ice. This process was then repeated for the remaining samples, with no more than 12 samples being extracted at one time. Once all the samples to be extracted were on ice, each sample was vortexed for a further 5 seconds before the tissue was tapped down to the bottom of the tube and the sample placed back into the ice, with this process being repeated for a total of 10 cycles. At the 10 minute mark, the samples were transferred to a pre-cooled centrifuge (0 °C)

and spun at 28,000 RPM for 2 minutes. The samples were then removed from the centrifuge and a 125 µl aliquot of supernatant was carefully isolated without disturbing the pellet, and placed into another pre-cooled tube. To neutralise the sulfosalicylic acid in the extract, 0.4 M Borate buffer was added, and the sample then vortexed for 10 seconds before being placed back on ice for 5 minutes. After 5 minutes, the samples were vortexed again, and then centrifuged as before at 28,000 RPM, 0 °C for 2 minutes. The supernatant (taurine extract) was removed using a pasteur pipette and placed in a labelled cryule, before being stored at -80 °C until further processing for the measurement of taurine content.

3.3.3 Measurement of taurine content

The most common method of measuring taurine content reported in the literature is via HPLC, using taurine extracts that have been derivatised with *o*-phthalaldehyde. Our lab has previously used this method however; several difficulties were experienced with our HPLC setup resulting in variable data when samples were not all analysed within the same run. Due to the large number of samples needing to be analysed for this thesis, this method was insufficient for taurine analysis in this case. Thus, in consultation with a specialist HPLC analysis company, the method of derivatisation with fluorenylmethyloxycarbonyl (FMOC) was employed, as it achieves extremely accurate and reproducible measures of taurine content and improves stability of the sample.

The FMOC derivatisation cocktail consisted of 0.4 mM of FMOC in Acetonitrile, 0.2 M $NaHCO_3$ and millli-Q water, which were mixed into a 5 ml screw cap vial on the day of extraction. Taurine extract (100 µl) was then added to the vial containing derivatisation cocktail, and the solution vortexed vigorously for 30 seconds. The solution was then left to stand for 30 minutes at room temperature (20-23 °C). Spiked samples (samples with a known concentration of taurine standard added) were prepared in the same way, however 10 µl less milli-Q water was added to allow for the addition of 10 µl of taurine standard. After 30 minutes, pentane was added to the vial and then shaken well, to ensure that any unreacted FMOC was removed. The vial was then left to allow the layers of solution to separate (pentane on top, derivatised solution on the bottom). An aliquot of the derivatised solution was then removed using a pasteur pipette, ensuring that no pentane was disturbed, and was placed in a 1 ml HPLC vial which remained

uncapped for 1 minute to allow for any pentane that may have been transferred with the derivatised sample to evaporate. The vial was then capped and stored at 4 °C until delivery to ACS laboratories for HPLC analysis.

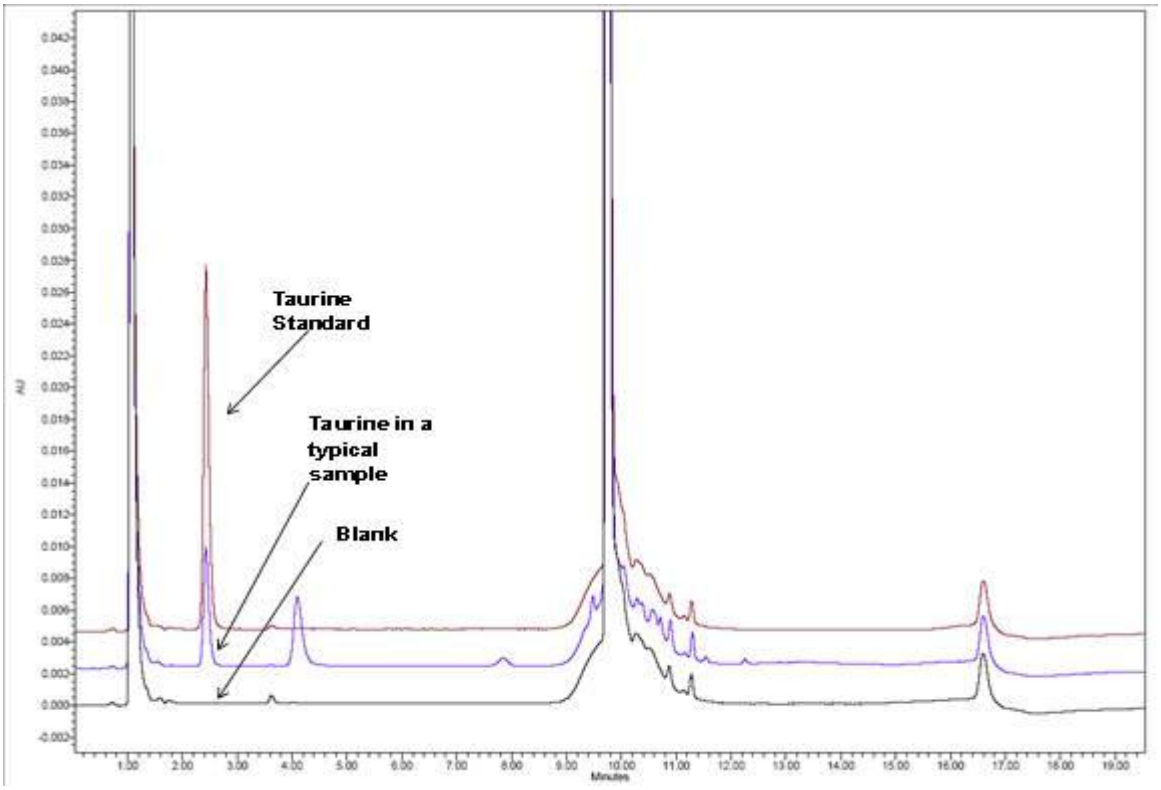


Figure 3.2 Typical HPLC trace for taurine content analysis

Figure shows taurine content as an FMOC derivative in a typical sample, a standard and a blank.

3.4 Enzyme activity

3.4.1 Homogenising procedure

Approximately 10-15 mg of muscle tissue was diluted 1:50 in ice cold homogenising buffer [0.5 M KCl and 5 mM EDTA] and homogenised using a handheld electric tissue homogeniser (Omini, GA, USA) on ice, using the following protocol;

15 sec medium speed, 15seconds off
15 sec medium speed, 15seconds off
10 sec high speed

This protocol was chosen to minimise heat generation by the homogeniser. An aliquot of this homogenate was saved for analysis of CS, while the rest of the sample was further diluted 1:100 in a second buffer [Glycerol 50%; KH_2PO_4 170 mM; BSA 0.02%; Mercaptoethanol 5 mM; EDTA 0.5 mM] which was used for the analysis of the remaining enzymes.

3.4.2 CK (muscle and plasma)

CK is an intracellular enzyme that plays a key role in skeletal muscle energy metabolism while also being a commonly used marker in plasma for skeletal muscle damage (Emery, 1995). Two different isoforms of CK are found in skeletal muscle, one in the cytosol and the other within the intermembrane space of the mitochondria, both of which catalyse the phosphorylation of ADP using ADP and creatine phosphate (Cordain, 1998). This method measures the activity of CK using a plate-based colorimetric reaction.

Method Principle:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-phosphate (G-6-P)}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-P DH}} \text{6-Phosphogluconate} + \text{NADPH}$$

The CK within the sample (plasma or muscle homogenate) first catalyses the conversion of ADP to ATP, which is then utilized to produce glucose-6-phosphate (G-6-P). In the presence of NADP, G-6-P dehydrogenase then catalyses the conversion to form NADP(H). The production of NADPH is then monitored to determine CK enzyme activity.

For analysis, muscle and plasma samples were injected into a clear 96 well plate in triplicate, before reaction cocktail was added to the wells [Imidazole acetate 100 mM; EDTA 2 mM; Creatine phosphate 30 mM; AMP 5 mM; NAD*P 2 mM; N-acetyl-L-cystein 20 mM; Magnesium acetate 10 mM; Glucose 20 mM; Diadenosine-5-pentaphosphate 10 µM; Glucose-6-phosphate dehydrogenase 2 U/ml; Hexokinase 3 U/ml pH 6.1-6.7]. To start the reaction, 0.1 M ADP was added to each well and the plate was shaken on low speed for 20 seconds, before the change in absorbance was measured on a plate reader (x-mark, Bio-Rad, Australia) at 25 °C, for 10 minutes at 340 nm.

CK activity was calculated using the millimolar extinction coefficient of NADPH and the dilution of the sample using the equation below;

CK(U/L) = (Δ Absorbance per min/6.22) x (ml total vol/ ml sample vol) x 1000

3.4.3 PFK

PFK, the rate limiting enzyme in the glycolytic pathway converting F-6-P to fructose 1-6-bisphoshate, is often used as an index of the glycolytic capacity of muscle. This method measures the activity of PFK based on the disappearance of NADH, using a plate-based colorimetric reaction.

Method Principle:

Fructose-6-phosphate (F-6-P) + ATP $\xrightleftharpoons{\text{PFK}}$ Fructose 1,6- diphosphate (F-1,6-P_2)

F-1,6-P_2 $\xrightleftharpoons{\text{Aldolase}}$ Dihydroxyacetonephosphate + Glyceraldehyde- 3-phosphate (G-3-P)

G-3-P $\xrightleftharpoons{\text{Triosphosphate isomerase}}$ Dihydroxyacetone phosphate (DHAP)

DHAP + NADH $\xrightleftharpoons{\text{α- Glycerophosphate DH}}$ Glycerol-3- phosphate + NAD

Two reaction cocktails were required for this assay, one for the microplate wells containing the NADH standards, and the other for the wells containing sample. Both cocktails consisted of [Tris buffer 100 mM; $MgCl_2$ $6H_2O$ 5 mM; ATP 1 mM; KCl 100 mM; BSA 10%; Aldolase 1 U/ml; Triosephosphate isomerise 1 U/ml; α-glycerophosphate dehydrogenase 1 U/ml;pH to 8.0] however, the cocktail used for the sample wells also contained 25 µM of NADH. The samples and reaction cocktail were injected into a microplate before 4 mM of F-6-P was added to start the reaction. The plate was then shaken for 20 seconds and the reaction followed for 5 minutes at 340 nm and 25 °C.

PFK activity was then calculated using the equation below;

$$\text{PFK activity} = \frac{\text{(Change in absorbance) x (Volume in well)}}{\text{(Slope) x (tissue in well) x2}}$$

The change in absorbance between the 2nd and 3rd minute was used to calculate activity as this is more representative of activity than the first (unreliable) and last (rate slows) minute.

3.4.4 *β*-HAD

β-HAD activity was used as a marker for the capacity of fatty acid *β*-oxidation. This method measures the activity of *β*-HAD via the consumption of NADH using a plate-based colorimetric reaction.

Method principle;

$$\text{Acetoacetyl- CoA + NADH +H}^+ \overset{\text{HAD}}{\Longleftrightarrow} \text{L-3-Hydroxybutyryl- CoA + NAD}^+$$

As with PFK, two reaction cocktails were required for this assay. Both contained [Imidazole- HCL (pH 6.0); EDTA 1 mM; BSA 0.05 %] (for NADH standards) while the cocktail to be used with samples contained an additional 100 µM of NADH. Once standards, samples and cocktail were in the appropriate wells, 400 µM of Aceto-acetyl CoA was added to the plate to start the reaction. The plate was then shaken for 20 seconds and the reaction was followed for 5 minutes at 340 nm and 25 °C.

The change in absorbance between the 2nd and 3rd minute was used to calculate *β*-HAD activity with the equation below;

$$\beta\text{-HAD activity} = \frac{(\text{Change in absorbance}) \times (\text{Volume in well})}{(\text{Slope}) \times (\text{tissue in well})}$$

3.4.5 CS

CS is the mitochondrial enzyme that catalyses the synthesis of citrate from acetyl CoA and oxalacetate to start a new cycle of the citric acid cycle. It is neither rate limiting nor membrane bound and is commonly used as measure of mitochondrial density and aerobic capacity.

Method Principle:

$$\text{Acetyl- CoA + Oxalacetate} \xleftrightarrow{\text{CS}} \text{Citrate + CoA- SH}$$

$$\text{CoA- SH + DTND} \longleftrightarrow \text{Co A + DTNB}$$

Homogenized samples underwent two cycles of freeze thawing (freeze in LN_2 and thaw in 25 °C water bath) before being briefly centrifuged at 4 °C, 10,000 RPM to settle cellular debris and produce a clear supernatant. The samples and reaction cocktail [Tris Buffer 100 mM; DTNB 1 mM; Acetyl CoA 3 mM] were then injected into the appropriate wells of a microplate before 10.0 mM oxalacetate was added to start the reaction. The plate was shaken for 20 seconds on low speed and the change in absorbance was followed for 5 minutes, at 412 nm and 37 °C.

The average change in absorbance per minute was calculated after the reaction has reached its maximal steady state. The molar extinction coefficient for DTNB at 412 nm (13,600) and the dilution of the tissue were used in the equation below to determine CS activity.

$$\left(\Delta\text{Abs/ min} \,/_{13.6}\right) \quad \text{x dilution factors muscle} \quad = \mu\text{mol/min/g}$$

3.4.6 Protein determination

Protein quantification was determined colourmetrically using a (Bradford) protein assay kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) standards were prepared ranging from 1,500-25 µg/ml, while samples were diluted to bring them within range of the standard curve. Bradford reagent was allowed to reach room temperature before use and was stirred constantly to ensure any dye-dye aggregates were dispersed. The prepared sample and Bradford reagent were added to the microplate before it was incubated at room temperature for 10 minutes. The microplate was then shaken for 20 seconds on low speed before being read at 595 nm. The total protein concentration of the samples was then calculated using the BSA standard curve and used to express enzyme activity relative to total protein content of the samples.

3.5 Western blotting

3.5.1 Tissue preparation

Tissue samples were homogenised at room temperature using a Polytron homogeniser (Kinematica, Lucerne, Switzerland) for 2x5 seconds in 20 volumes of solution composed of 165 mM Na^+, 1 mM free Mg^{2+} (10.3 mM total Mg^{2+}), 90 mM Hepes, 50 mM ethyleneglycoltetraacetic acid (EGTA), 8 mM ATP, and 10 mM PCr (pH 7.1), with the addition of a protease inhibitor cocktail (PIC, Complete, Roche Diagnostics, Sydney, Australia) and strongly buffered to a very low free [Ca^{2+}] to avoid activating any Ca^{2+}-dependant proteases. Whole muscle homogenates were then diluted in solubilising buffer (0.125 M Tris-Cl [pH 6.8], 4% [w/v] SDS, 10% glycerol, 4 M Urea, 10% [v/v] mercaptoethanol, and 0.001% [v/v] bromophenol blue) and stored at -20 °C until analysis by Western blot.

3.5.2 Gels and transfer conditions

All samples were separated on criterion TGX 4-15 % stain-free gels at 200 volts (V) for 60 minutes, with a mix of the supplementation groups and/or ages on each gel. Gels were imaged on a criterion stain free imager (Biorad, Australia), before the proteins on the gel were wet-transferred onto a nitrocellulose membrane for 60 minutes at 100 V in a circulating ice-cooled bath using transfer buffer containing 140 mM glycine, 37 mM Tris base, and 20% (v/v) methanol. Membranes were then rinsed in double distilled water (DDH_2O) before and after being washed in Miser antibody extender solution (Thermo Scientific, Rockford, IL, USA) for 10 minutes. The membranes were blocked at room temperature for 1 hour in 5% skim milk in Tris buffered saline plus tween (TBST) composed of 50 mM Tris-HCL, 750 mM NaCl, and 0.25 % Tween.

3.5.3 Primary antibodies

After blocking, the membranes were cut into 3 sections and then incubated in primary antibody for 2 hours at room temperature, and then overnight at 4 °C. As the membranes were cut, each section could be probed for multiple proteins of interest without the need for stripping the membrane. All primary antibodies used were diluted in

1 % (w/v) BSA in phosphate-buffered saline (PBS) with 0.025 % (v/v) tween. Specific details on the different primary antibodies used and the order of probing on each section can be found in Table 3.1.

Table 3.1 Antibody supplier and dilution information for proteins investigated

Antibody	Dilution	Probe Number	Supplier
DHPR anti-mouse	1:400	1	Developmental Studies Hybridoma Bank (DSHB)
CSQ1 anti-mouse	1:2000	3	AbCam
RyR anti-mouse	1:2000	1	DSHB
SERCA1 anti-mouse	1:1000	2	DSHB
TauT anti-rabbit	1:1000	1	Alpha Diagnostics and Professor Pow
Tubulin	1:200	2	Invitrogen
Dystrophin	1:200	2	DSHB

3.5.4 Secondary antibodies

Membranes were washed in 5% skim milk in TBST before the secondary antibody was applied for 1 hour at room temperature. Secondary antibodies were diluted in DDH_2O from original stock and used in a dilution of 1 in 20, 000 in 5 % skim milk made with TBST. The goat anti-rabbit IgG (horse radish peroxidase conjugated) antibody was purchased from Thermo Scientific, while the goat anti-mouse IgG (horse radish peroxidase conjugated) antibody was sourced from Pierce Biotechnology. Before imaging, membranes were washed in TBST for 2x 10 minutes.

3.5.5 Imaging and analysis

Images were collected following exposure to Super Signal West Femto (Pierce, Rockford, IL, USA) using a charge coupled device (CCD) camera attached to a ChemiDoc XRS (Bio-Rad). Densitometry was performed using Quantity One software (Bio-Rad).

CHAPTER FOUR

TAURINE SUPPLEMENTATION DURING ACUTE DYSTROPIC PROGRESSION: IMPACT ON TISSUE TAURINE, TAURINE TRANSPORTER AND E-C COUPLING PROTEIN EXPRESSION IN SKELETAL MUSCLE

4.1 Introduction

The amino acid taurine has been shown to act on several of the pathways known to be involved in the development of skeletal muscle pathology in DMD (see Literature review, Section 2.5). Moreover, DMD patients have a higher urinary excretion of taurine, and three studies in the *mdx* mouse demonstrate that taurine content is significantly reduced in dystrophic tissues, including skeletal muscle (Griffin et al., 2001, McIntosh et al., 1998a, McIntosh et al., 1998b). Interestingly, taurine content increases in parallel with regeneration leading to the suggestion that taurine may be a marker for recovery of skeletal muscle function, and that some of the pathology observed in DMD may be the result of the depleted taurine (Griffin et al., 2001, McIntosh et al., 1998a, McIntosh et al., 1998b). In association with decreased tissue taurine, the plasma concentration of the amino acid in *mdx* mice has been reported to be elevated by as much as 140%, suggesting that there may be an impaired ability to retain the appropriate amount of taurine in dystrophic skeletal muscle (De Luca et al., 2001a). Another possible explanation for the increasedconcentration in plasma is a reduction in the expression or activity of the TauT, however to date TauT is yet to be investigated in the *mdx* mouse.

Also of interest is taurine's effect on E-C coupling and Ca^{2+} handling. Taurine has been shown to modulate E-C coupling by restoring the mechanical threshold (MT) for contraction to normal values (De Luca et al, 1996), increasing the sensitivity of the contractile apparatus to Ca^{2+} (Cuisinier et al., 2000b) and enhancing SR Ca^{2+} release (Bakker and Berg, 2002). *Mdx* mice display abnormal E-C coupling with depressed RyR-

mediated Ca^{2+} release (Plant and Lynch, 2003; Woods et al, 2005), accelerated force decline in response to t-tubular depolarisations, reduced repriming rate after depolarisation-induced contractile responses (Plant and Lynch, 2003) and reduced Ca^{2+} release from the SR (Woods et al, 2004). The expression of Ca^{2+} binding proteins is also altered in dystrophy, with CSQ expression significantly decreased in the highly affected limb muscles (Doran et al, 2004; Culligan et al, 2002), while the relatively spared laryngeal muscles demonstrate overexpression of CSQ and SERCA, thereby improving Ca^{2+} handling and preventing necrosis (Ferretti et al, 2009). Interestingly, taurine supplementation has recently been found to increase muscle content of the Ca^{2+}binding protein CSQ by 49% in rat skeletal muscle (Goodman et al, 2009).

To date, few studies have investigated the effect of taurine on dystrophic skeletal muscle (Cozzoli et al., 2011b, De Luca et al., 2003). In fact, no study has investigated whether taurine supplementation during the early degenerative phase of the *mdx* lifespan (which more closely matches the pathology observed in DMD patients) is able to restore skeletal muscle taurine content back to levels found in healthy controls. Also, no study to date has examined TauT protein expression in C57BL10 or *mdx* mice, or the effect of taurine supplementation on TauT expression. Moreover, there is no data examining whether taurine supplementation in young *mdx* mice alters the expression of E-C coupling and Ca^{2+} handling proteins such as DHPR, RyR, SERCA and CSQ, which may help to explain some of the beneficial effects reported with regard to force production, MT and Ca^{2+} handling.

4.2 Aims and hypothesis

The purpose of the investigation was to determine if taurine supplementation could elevate muscle taurine content in the *mdx* mouse during the acute phase of degeneration, through to a more stabilised phenotype. The expression of the TauT was also examined to determine if this was altered in *mdx* muscles, and whether TauT expression is altered with taurine treatment. Several E-C coupling proteins were also investigated to determine if taurine is able to modulate their expression in dystrophic skeletal muscle.

The specific aims of this study were;

i) To investigate the effect of taurine supplementation on skeletal muscle taurine content throughout both the degeneration/regeneration cycles (days 28 to 45) and stabilised phase (70 days) of the *mdx* mouse lifespan.

ii) To characterise the expression of the taurine transporter (TauT) in the *mdx* mouse during degeneration and stabilisation as well as in association with taurine supplementation.

iii) To determine the expression of the major E-C coupling and Ca^{2+} handling proteins (SERCA, DHPR, RyR, CSQ) during the degeneration/regeneration cycle in the *mdx* mouse, and in association with taurine supplementation.

It was hypothesised that taurine supplementation would increase taurine content in hind limb and respiratory muscles of control and *mdx* mice, and that TauT expression would be lower in *mdx* muscle during peak degeneration, and increase with repair, but that this would be unaltered by supplementation. Similarly, E-C coupling proteins and CSQ would be lower in *mdx* muscles during acute damage, but increase with repair. It was also hypothesised that CSQ protein expression would be increased with taurine supplementation.

4.3 Methods

4.3.1 Experimental groups and treatment protocol

Male and female *mdx* and non-dystrophic C57Bl/10 (control) mice (n=115) were bred at Melbourne University animal house using stock obtained from the Animal Resource Centre (WA, Australia). Details on the housing conditions of the animals are available in Chapter 3, section 3.1. All control mice (CON) were untreated and served as an indicator of non-dystrophic tissue taurine content and protein expression. *Mdx* mice were divided into two groups, untreated (MDX) or taurine treated (TAU; 2 % [w/v] via drinking water) initially *in utero* and later via drinking water provided *ad libitum.* Maternal supplementation commenced two weeks before being paired with male mice and continued until breeding pairs were culled, typically after 2-3 months of breeding. Once weaned from supplemented mothers, litters were separated into male and female offspring and immediately placed on taurine water, remaining on the supplement until sacrifice.

4.3.2 Sample collection

Mice from all three experimental groups were sacrificed at day 28, 35, 45 and day 70 (± 1 day) to determine any changes occurring during the first wave of degeneration, through to a more stabilised phenotype at day 70. TA, GAST and DIA muscles were collected under anaesthesia (40-50mg/kg of Nembutal I.P). Due to the small size of EDL and SOL, particularly in the day 28 animals, these muscles were insufficient for analysis of both taurine content and muscle proteins, and therefore could not be used despite the preference for collecting examples of fast- and slow-twitch limb muscles. All muscles were quickly blotted on filter paper, weighed, snap frozen in liquid nitrogen and stored at -80 °C until further analysis.

4.3.3 HPLC determination of taurine content

Taurine content was measured via HPLC using muscle extracts tagged with FMOC. The TA, GAST and DIA muscles at all time points indicated were prepared as is described in Chapter 3, section 3.3. TA and GAST were chosen as both undergo significant muscle damage, however in the GAST, this typically manifests later and the fibre-type between the two muscles are slightly different. While DIA does not show marked pathology at the time points examined, taurine content of this muscle was measured to determine if supplementation was effective in dystrophic skeletal muscle that was not undergoing acute degeneration and thus, could potentially ameliorate the development of pathology typically observed at 6 months of age in the DIA.

4.3.4 Western blot analysis of dystrophin expression

Due to the large number of samples in this study, and the expense of running this analysis, it was decided that TA would be the best indicator of pathology and recovery during the time points chosen, and thus is the only muscle utilised for all Western blot analysis in this study. As such, Western blot analysis at day 28 and day 70 in the TA only was used to confirm the presence or absence of dystrophin in the experimental groups, as ARC does not currently perform this testing on the colonies of animals that are supplied. This also confirmed that the *mdx* genetic defect had not occurred within any of our breeding control animals. Details on the procedure are available in Chapter 3, section 3.5.

4.3.5 Western blot analysis of TauT expression

Taurine transporter expression was measured via Western blot in CON, MDX and TAU supplemented groups at day 28 and day 70 in the TA only to determine if there were any changes from peak damage to low level degeneration. Three primary antibodies for TauT were tested as part of this work. Due to multiple banding observed with the Alpha Diagnostics antibody (which is the most commonly used antibody in the literature) it was decided that this could not be used to accurately determine TauT in mouse muscle. The TauT antibody available from Millipore was also tested; however a single band appeared at 55 kDa which is not within the normal molecular weight range reported for TauT. The

primary antibody that was utilised for the reported results was kindly donated by Professor David Pow from The University of Queensland. Details on the procedure are available in Chapter 3, section 3.5.

4.3.6 Western blot analysis of E-C coupling and Ca^{2+} handling proteins

DHPR, RyR, SERCA and CSQ protein expression was analysed using Western blot in CON, MDX and TAU supplemented groups at day 28 and day 70 in TA only to determine if there was any change from peak damage to low level degeneration or in association with taurine treatment in the *mdx*. Details on the procedure are available in Chapter 3, section 3.5.

4.3.7 Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Taurine content and muscle weight data was analysed using a multiple analysis of variance (MANOVA) on SPSS (Version 17). Protein expression and body weight was analysed using a one-way analysis of variance (1-way ANOVA) and any significant differences examined using a Bonferroni Post Hoc Test on GraphPad Prism 4.1 (GraphPad Software). A probability level of <0.05 was adopted throughout to determine statistical significance.

4.4 Results

4.4.1 Body mass

Taurine treatment significantly increased the body mass of TAU mice compared to MDX animals at day 28 only ($p < 0.05$; Table 4.1), with no significant effects of supplementation observed at any other time point between the groups. No significant difference in body mass between CON and MDX groups was observed, other than at day 70, where the MDX group demonstrated significantly greater body mass. See Table 4.1.

Table 4.1 Comparison of body mass between CON, MDX and TAU groups

	Body Mass (g)			
	Day 28	**Day 35**	**Day 45**	**Day 70**
CON	13.5 ± 1.0	16.0 ± 0.4	17.8 ± 0.9	21.3 ± 0.6
MDX	10.4 ± 0.8	14.3 ± 0.7	17.3 ± 1.6	25.8 ± 1.5 *
TAU	14.5 ± 0.5 ^	14.1 ± 0.5	14.5 ± 0.7	24.2 ± 0.4

* denotes group is significantly different compared to control animals. ^ denotes that group is significantly different compared to MDX mice. (n=7-10)

4.4.2 Muscle mass and relative muscle mass

Alterations to muscle mass and relative muscle mass for each time point are described below for TA and GAST (also see Table 4.2 and 4.3). No data for DIA is shown due to the high variability in the dissection technique for this muscle preventing meaningful comparison of muscle mass values.

i) Day 28

MDX TA and GAST muscle mass and relative muscle mass was significantly lower ($p < 0.05$) when compared to both the CON and TAU groups. Interestingly, taurine treatment appears to effect the early development of muscle mass in the *mdx*, as there was no significant difference in muscle mass between the CON and TAU mice.

ii) Day 35

There was no significant difference in TA or GAST muscle mass between any of the groups at day 35.

iii) Day 45

The MDX group had significantly greater ($p < 0.05$) TA and GAST muscle mass and relative muscle mass when compared to both the CON and TAU groups at day 45. Taurine treatment prevented this gain in muscle mass, with the TAU group having significantly lower mass and relative muscle mass than MDX mice by day 45, whereas there was no significant difference relative to CON mice.

iv) Day 70

Both the MDX and TAU supplemented mice demonstrated significantly greater ($p < 0.05$) TA muscle mass and relative muscle mass when compared to CON animals. Interestingly, unlike previously examined time points, TA muscle/relative muscle mass was not significantly different between the MDX and TAU treated groups suggesting some degree of catch up in muscle growth for TAU between day 45 and day 70 in TA. This effect was not apparent at this time point in GAST, where MDX demonstrated significantly greater ($p < 0.05$) muscle mass and relative muscle mass when compared to the TAU group. As was observed for TA, CON animals had significantly lower ($p < 0.05$) GAST muscle mass and relative muscle mass when compared to both the MDX and TAU groups.

Table 4.2 Comparison of muscle mass between CON, MDX and TAU groups

		Muscle Mass (mg)			
		Day 28	**Day 35**	**Day 45**	**Day 70**
TA	CON	19.1 ± 1.4	19.7 ± 1.0	22.6 ± 2.5	31.7 ± 2.0
	MDX	12.0 ± 1.5 *	22.3 ± 1.1	38.6 ± 2.7 *	52.6 ± 2.1 *
	TAU	21.0 ± 1.4 ^	20.6 ± 1.0	24.1 ± 2.5 ^	47.7 ± 2.0 *
GAST	CON	54.1 ± 3.0	55.5 ± 1.8	59.1 ± 3.2	92.6 ± 3.7
	MDX	38.2 ± 3.2 *	60.4 ± 1.9	98.2 ± 3.4 *	139.8 ± 4.0 *
	TAU	57.1 ± 3.0 ^	55.0 ± 1.8	57.8 ± 3.2 ^	111.6 ± 3.7 ^

* denotes group is significantly different compared to control animals. ^ denotes group is significantly different compared to MDX animals. (n=7-10)

Table 4.3 Normalisation of muscle mass to body mass between CON, MDX and TAU groups

		Relative Muscle Mass (mg/g)			
		Day 28	**Day 35**	**Day 45**	**Day 70**
TA	CON	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
	MDX	1.2 ± 0.1	1.5 ± 0.1	2.1 ± 0.1 *	2.0 ± 0.1*
	TAU	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.2 ^	1.9 ± 0.1*
GAST	CON	3.9 ± 0.2	3.4 ± 0.1	3.4 ± 0.2	4.2 ± 0.2
	MDX	3.7 ± 0.2 *	4.0 ± 0.2	5.7 ± 0.3 *	5.4 ± 0.3 *
	TAU	3.9 ± 0.1 ^	3.9 ± 0.0	4.0 ± 0.2 ^	4.6 ± 0.1 ^

* denotes group is significantly different compared to control animals. ^ denotes group is significantly different compared to MDX animals. (n=7-10)

4.4.3 Hind limb muscle % dw/ww

For TA (Table 4.4), CON mice had a significantly greater % dw/ww than the MDX at day 35 ($p < 0.05$) and day 70 ($p < 0.01$). TAU had a significantly lower dw/ww ratio when compared to CON mice at day 45 ($p < 0.05$) and day 70 ($p < 0.01$). As shown in Table 4.5, there was no significant difference in the % dw/ww for GAST in CON, MDX or TAU groups at any time point examined. There was no significant difference in the percentage of dw/ww between MDX and TAU at any time point for hind limb muscles.

Table 4.4 Percentage of dw/ww for TA across ages in CON, MDX and TAU mice

	TA Freeze Dried Muscle % dw/ww			
	Day 28	**Day 35**	**Day 45**	**Day 70**
CON	24.4 ± 0.7	26.4 ± 0.8	24.8 ± 0.6	25.1 ± 0.2
MDX	22.9 ± 0.7	22.8 ± 0.8 *	23.4 ± 0.6	24.1 ± 0.2 *
TAU	22.9 ± 0.9	24.5 ± 0.9	21.3 ± 0.7 *	23.8 ± 0.2 *

* denotes group is significantly different compared to control animals. (n=7-10)

Table 4.5 Percentage of dw/ww for GAST across ages in CON, MDX and TAU mice

	GAST Freeze Dried Muscle % dw/ww			
	Day 28	**Day 35**	**Day 45**	**Day 70**
CON	23.1 ± 0.8	24.0 ± 0.6	23.9 ± 0.6	23.4 ± 0.5
MDX	23.4 ± 0.8	21.9 ± 0.6	23.3 ± 0.6	22.7 ± 0.5
TAU	22.9 ± 1.0	23.4 ± 0.7	24.9 ± 0.8	22.5 ± 0.5

(n=7-10)

4.4.4 DIA muscle % dw/ww

There was a significant difference in water content between the CON and MDX groups at day 35 ($p < 0.05$), 45 ($p < 0.05$) and day 70 ($p < 0.05$) as shown in Table 4.6. At day 70 both the MDX ($p < 0.05$) and TAU ($p < 0.001$) groups demonstrated significantly lower % dw/ww when compared to CON muscle, as was also the case for MDX mice at day 35 and 45. There was also a significant decrease in % dw/ww in the TAU when compared to MDX at day 70 only.

Table 4.6 Percentage dw/ww for DIA across ages in CON, MDX and TAU mice

	DIA Freeze Dried Muscle % dw/ww			
	Day 28	**Day 35**	**Day 45**	**Day 70**
CON	25.5 ± 0.3	25.9 ± 0.6	26.4 ± 0.6	25.7 ± 0.8
MDX	25.9 ± 0.3	23.1 ± 0.6 *	22.8 ± 0.6 *	24.1 ± 0.9 *
TAU	24.5 ± 0.4	24.1 ± 0.7	25.5 ± 0 .8	20.9 ± 0.6 * ^

* denotes group is significantly different compared to control animals. ^ denotes group is significantly different compared to MDX animals. (n=7-10)

4.4.5 Hind limb muscle taurine content

i) Day 28

Taurine treatment resulted in 31% greater taurine content in the TA when compared to the MDX ($p < 0.001$; Figure 4.1 A) and a significantly higher taurine concentration ($p < 0.01$) when compared to CON mice. The taurine content of the MDX was 17% lower ($p < 0.001$) than that of the CON group. The GAST demonstrated a similar pattern to TA, with taurine treatment significantly increasing ($p < 0.001$; Figure 4.1 B) taurine concentration in the TAU group to 26% higher than that of MDX GAST, although there was no significant difference between the CON and TAU groups for GAST at this time point.

ii) Day 35

Both TA and GAST from TAU mice demonstrated significantly higher taurine content ($p < 0.001$) when compared to both the MDX and CON groups (see Figure 4.1). MDX mice also had significantly less taurine ($p < 0.01$) than the CON group for both TA and GAST.

iii) Day 45

Taurine treatment significantly increased taurine concentration ($p < 0.001$) in TA when compared to both CON and MDX groups (see Figure 4.1A). Interestingly, by day 45, there were no significant difference in TA taurine content between CON and MDX animals as was observed at earlier time points. For GAST, there was a significant increase in taurine content in TAU mice when compared to both the CON ($p < 0.001$) and MDX ($p < 0.05$) groups. MDX mice also demonstrated higher GAST taurine ($p < 0.05$) when compared to CON mice

iv) Day 70

MDX mice demonstrated significantly more (11%) TA taurine content ($p < 0.001$) compared to CON animals of the same age. Taurine treatment further increased TA taurine content in TAU mice by 7% ($p < 0.05$)more than was observed in MDX animals, such that TA taurine content in TAU mice were 19% above that of CON animals (see Figure 4.1A). For GAST, there was no significant difference in taurine content between CON and MDX at this time point. However, taurine content in the TAU treated mice decreased to below that of CON GAST ($p < 0.005$) by day 70, despite the increased intake of taurine in this group.

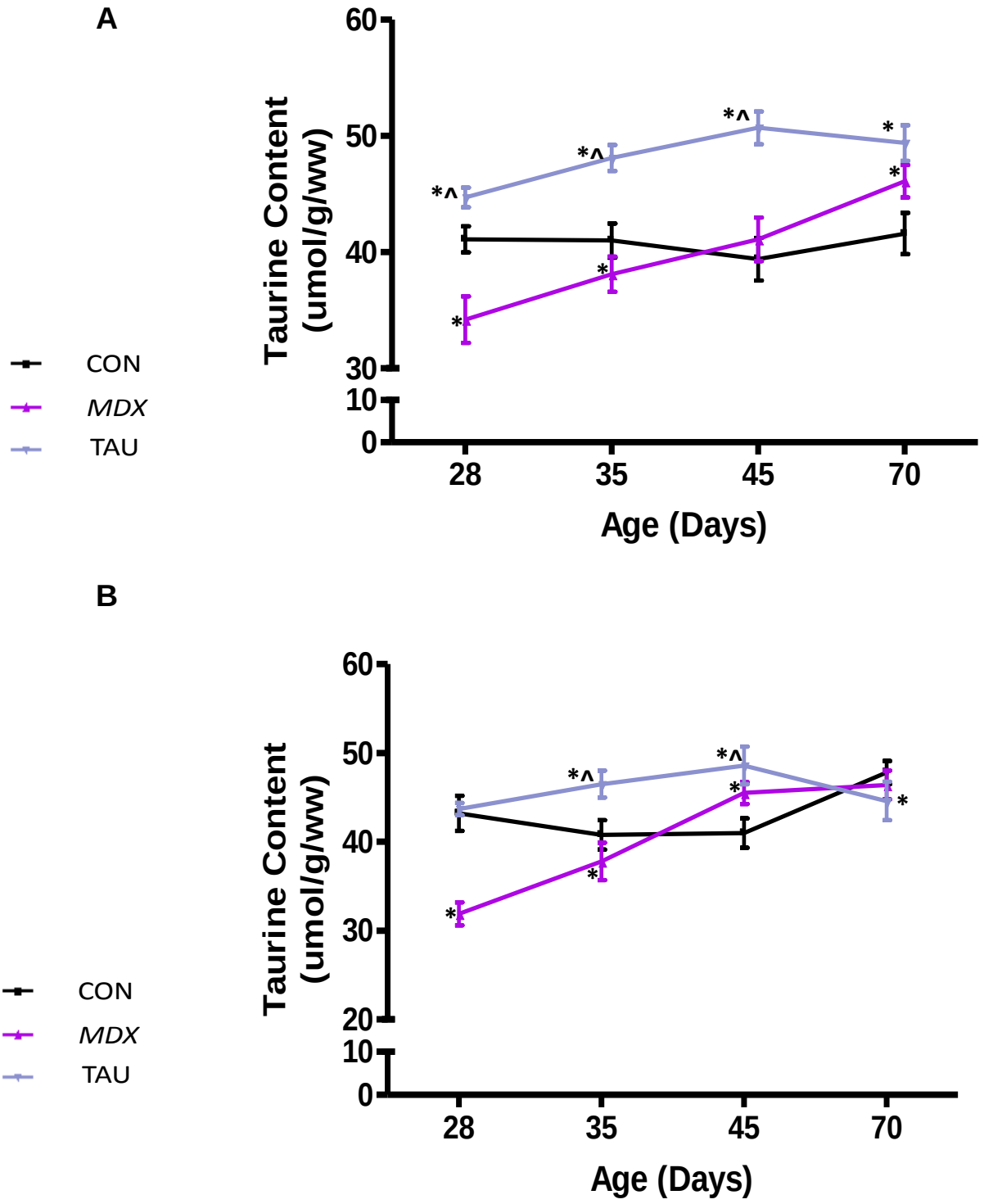


Figure 4.1 Taurine content in TA (A) and GAST (B) from day 28 to day 70 in CON, MDX and TAU mice

All data is presented as mean ± S.D (n=7-10). * group is significantly different compared to control animals ^ group is significantly different compared to MDX animals.

4.4.6 DIA muscle taurine content

i) Day 28

The DIA taurine content of CON mice was significantly greater ($p < 0.001$; Figure 4.2) than MDX mice. TAU mice also had significantly less taurine ($p < 0.001$) compared to CON, although taurine treatment did significantly increase taurine ($p < 0.001$) compared to the MDX group.

ii) Day 35

There was no significant difference in taurine content between CON and MDX animals. Taurine content was significantly increased ($p < 0.05$; Figure 4.2) in the TAU group ($p < 0.01$) when compared to both the CON and MDX mice.

iii) Day 45

No significant difference in taurine content between CON and MDX animals was observed at day 45. Taurine content was significantly increased ($p < 0.001$; Figure 4.2) in the TAU group when compared to both the CON and MDX mice.

iv) Day 70

There was a significant increase in taurine content in both the MDX and TAU groups ($p < 0.05$; Figure 4.2) compared to CON mice, although no difference existed between MDX and TAU groups.

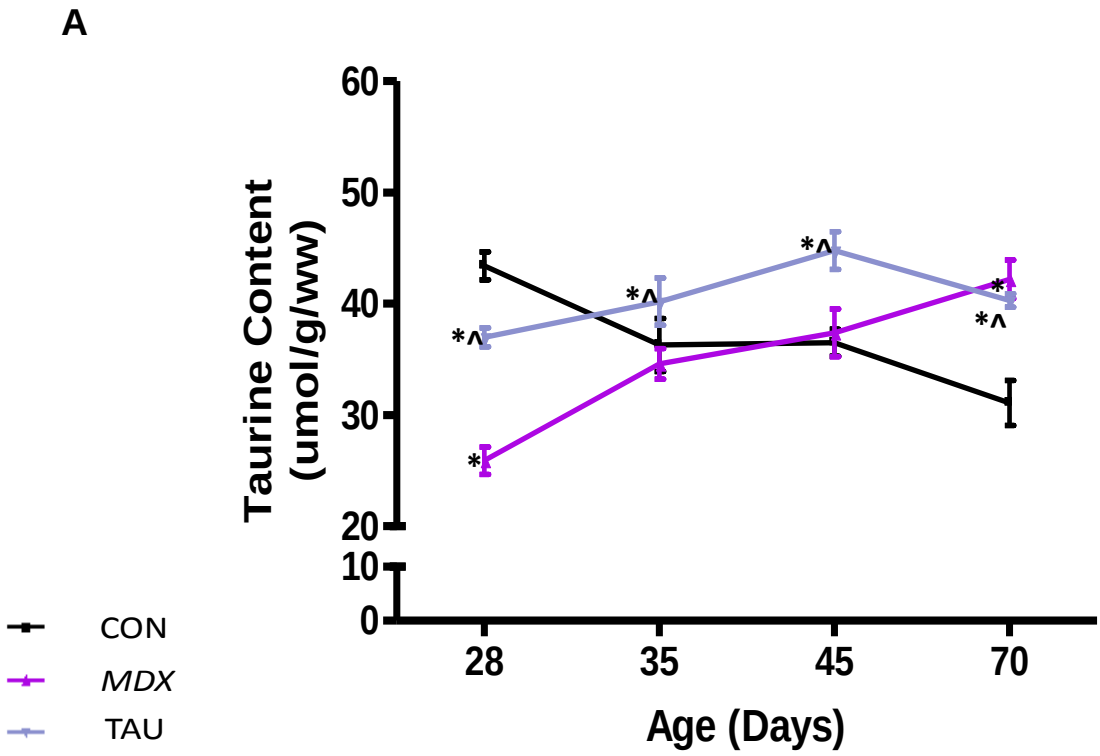


Figure 4.2 Taurine content in DIA (A) from day 28 to day 70 in CON, MDX and TAU mice

All data is presented as mean ± SD (n=7-10) * group is significantly different compared to control animals (n=7-10). ^ group is significantly different compared to MDX animals.

4.4.7 Dystrophin expression

As shown in Figure 4.3, dystrophin was present in CON mice but absent from both the MDX and TAU muscles. This confirms the presence of dystrophy in the *mdx* mice sampled, and that no mutations had occurred within the control litters.

A

B

Figure 4.3 Representative Western blot of dystrophin expression in day 28 (A) and day 70 (B) in CON, MDX and TAU mice

4.4.8 TauT protein expression

As shown in Figure 4.4 A, there was no significant difference between MDX and TAU groups for TauT protein expression at day 28.At day 70 (Figure 4.4 B), MDX mice have significantly less ($p < 0.05$) TauT when compared to CON mice, expressing only 43% of CON. Similarly, TAU expressed only 56% of CON TauT values at day 70, however this did not reach statistical significance. There was no difference in TauT expression between MDX and TAU.

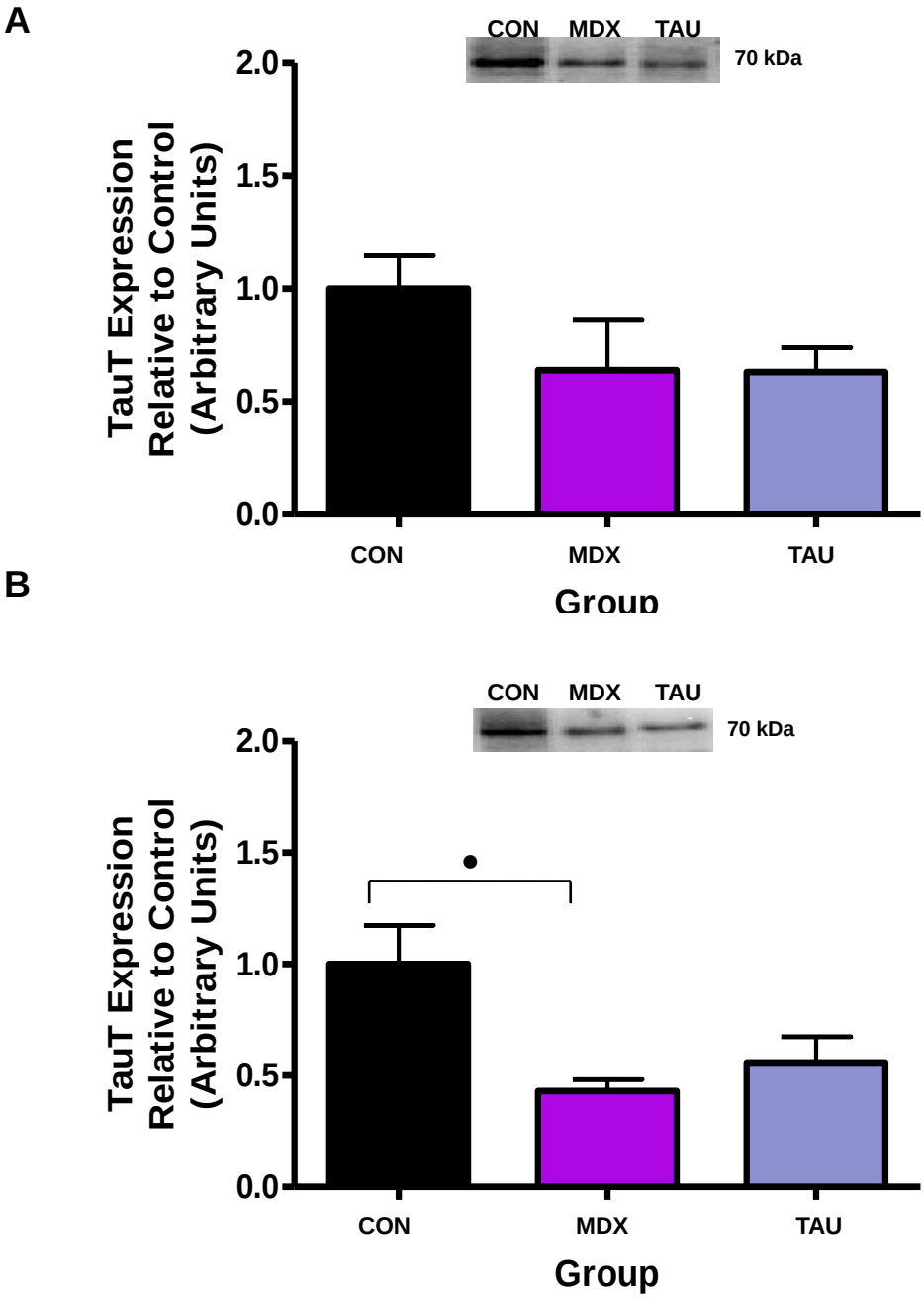


Figure 4.4 TauT expression in day 28 (A) and day 70 (B) CON, MDX and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10). ● denotes a significant difference (● $p < 0.05$).

4.4.9 E-C coupling and Ca^{2+} handling protein expression

i) Contractile protein expression

r, There was no significant difference in myosin protein expression in MDX muscles compared to CON at day 28 for theTAmuscle (see Figure 4.5 A). A (36% decrease in myosin expression observed in MDX TA at day 70 was significantly different from CON muscle ($p < 0.05$; see Figure 4.5 B). Taurine treatment significantly elevated actin expression compared to MDX mice ($p < 0.05$) at day 28 (see Figure 4.6.A), such that it was almost 50% higher than CON expression. This was also observed at day 70, with TAU mice demonstrating significantly increased actin expression compared to the untreated *mdx* group ($p < 0.01$).

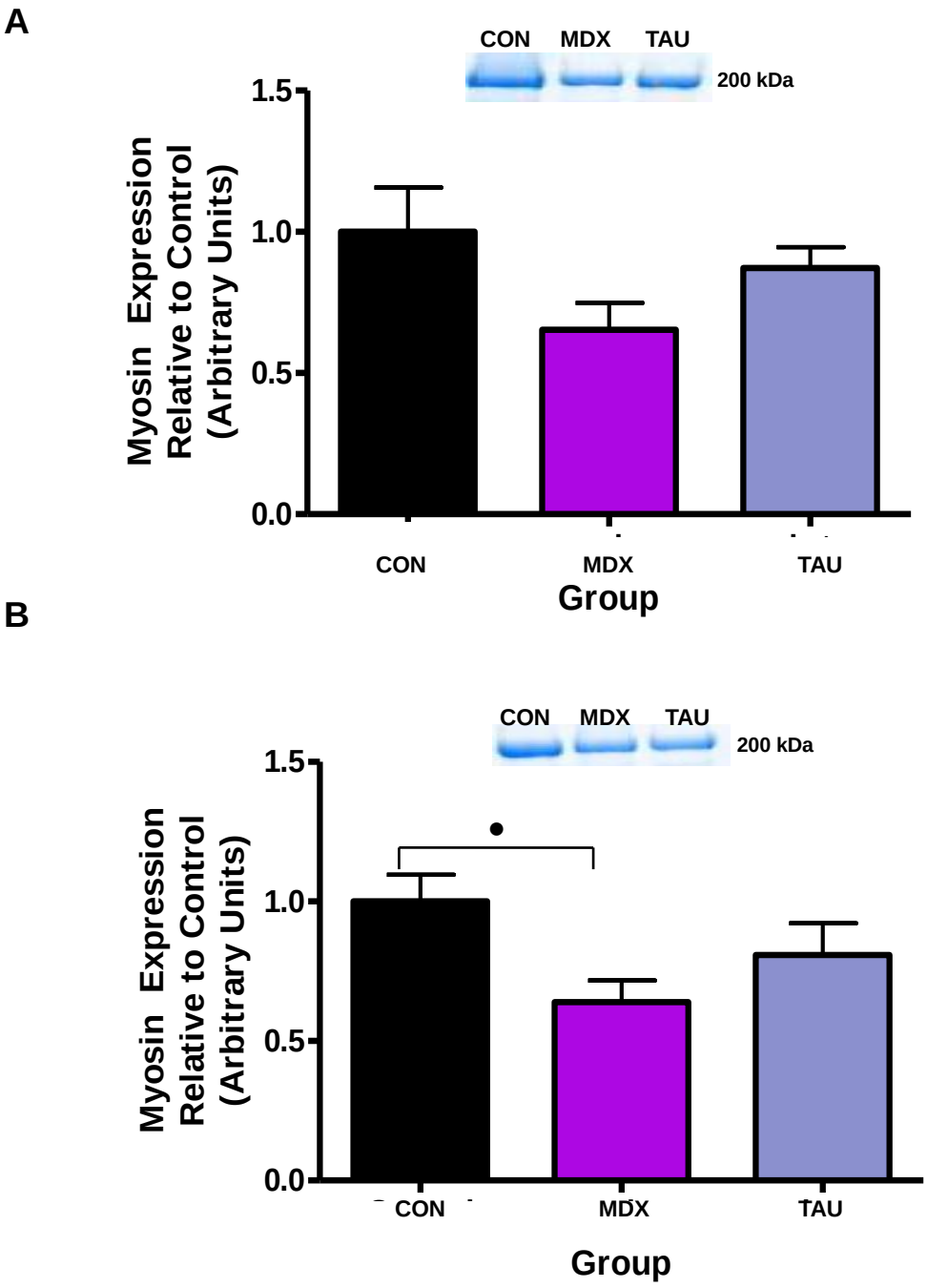


Figure 4.5 Myosin expression in day 28 (A) and day 70 (B) CON MDX, and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10). ● denotes a significant difference (● $p < 0.05$).

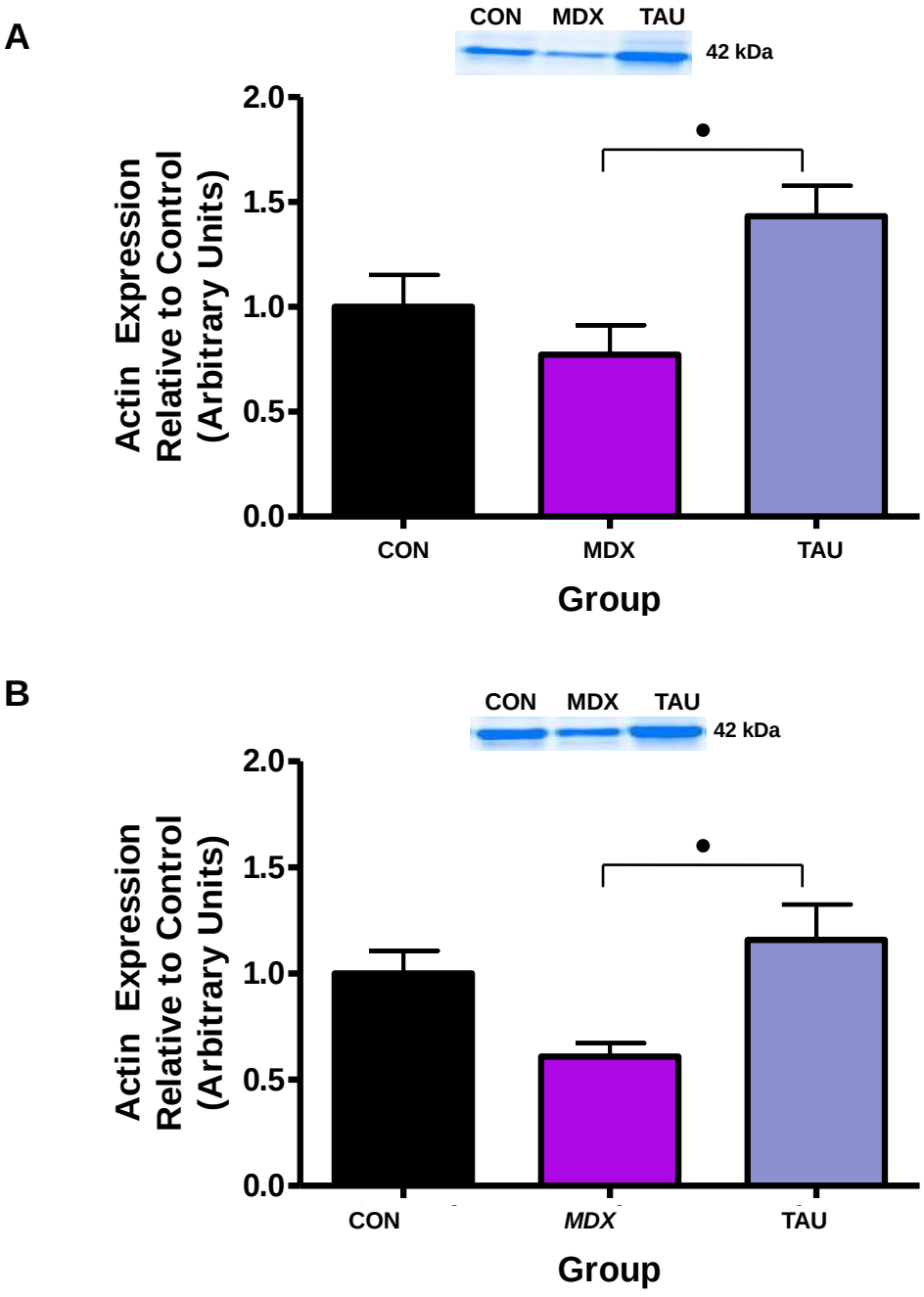


Figure 4.6 Actin expression in day 28 (A) and day 70 (B) CON, MDX and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10). ● denotes a significant difference (● p <0.05).

ii) E-C coupling protein expression

There are no significant differences in DHPR expression between groups (see Figure 4.7 A & BFor RyR expression at day 28, taurine treatment significantly decreased ($p < 0.05$) RyR when compared to the MDX group. There was also a significant difference between CON and MDX for RyR at this time point, with MDX exhibiting 44% lower RyR expression. At day 70 there was no significant difference in RyR expression amongst the groups (see Figure 4.8 B), There was no significant difference in SERCA expression observed between the groups at day 28 or day 70 (see Figure 4.9 A & B)

iii) CSQ protein expression

There were no significant differences in CSQ protein expression at day 28 between groups (see Figure 4.10 A). At day 70 however, there was a significant decrease in CSQ expression ($p < 0.05$) in the MDX group when compared to CON, while treatment with taurine successfully rescued CSQ expression such that no significant difference between CON and TAU groups were apparent. TAU mice had significantly increased CSQ protein expression relative to MDX animals (see Figure 4.10 B).

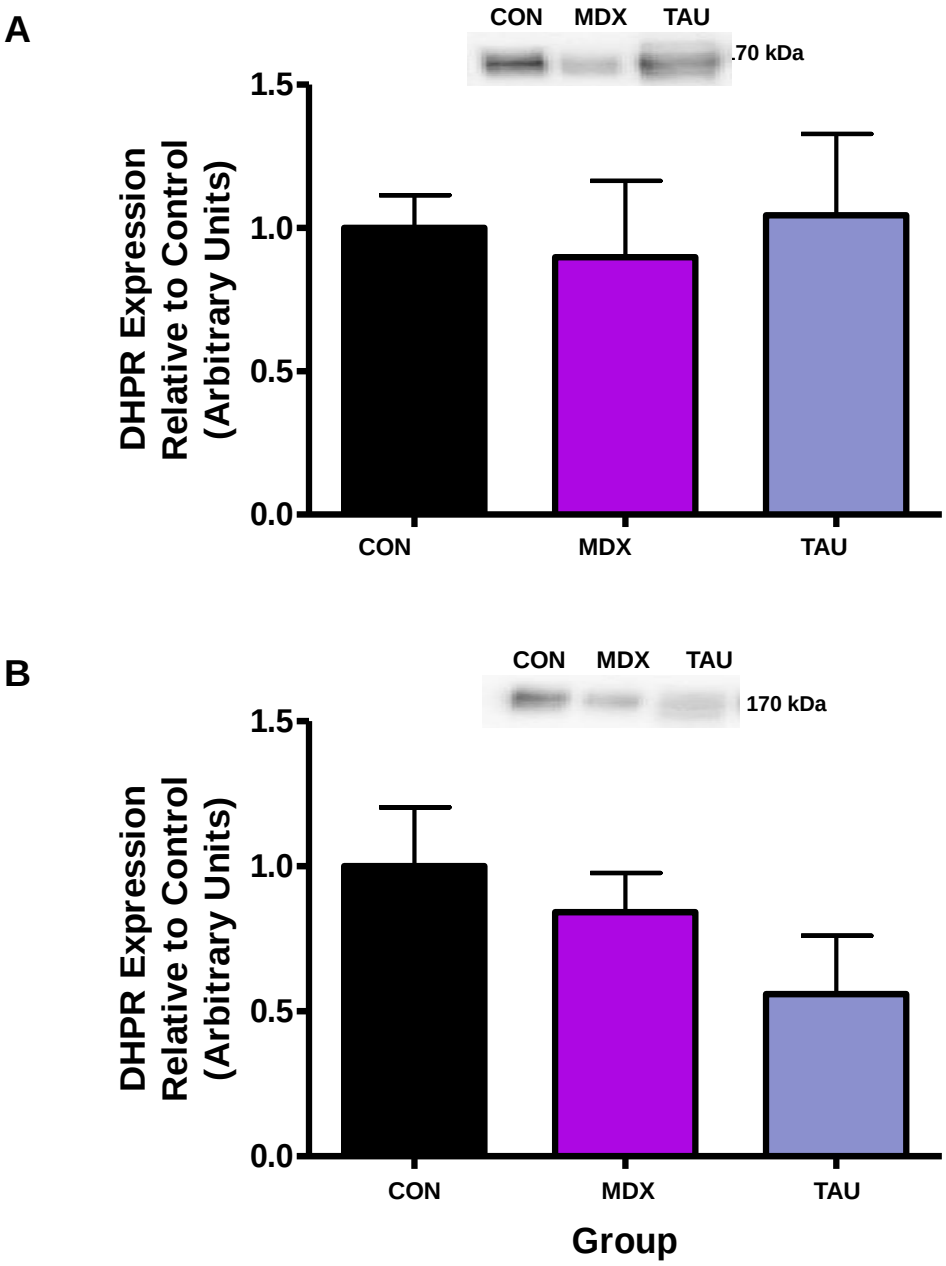


Figure 4.7 Day 28 (A) and day 70 (B) DHPR protein expression in CON, MDX and TAU mice

Representative Western blots and graphed data for DHPR expression. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10).

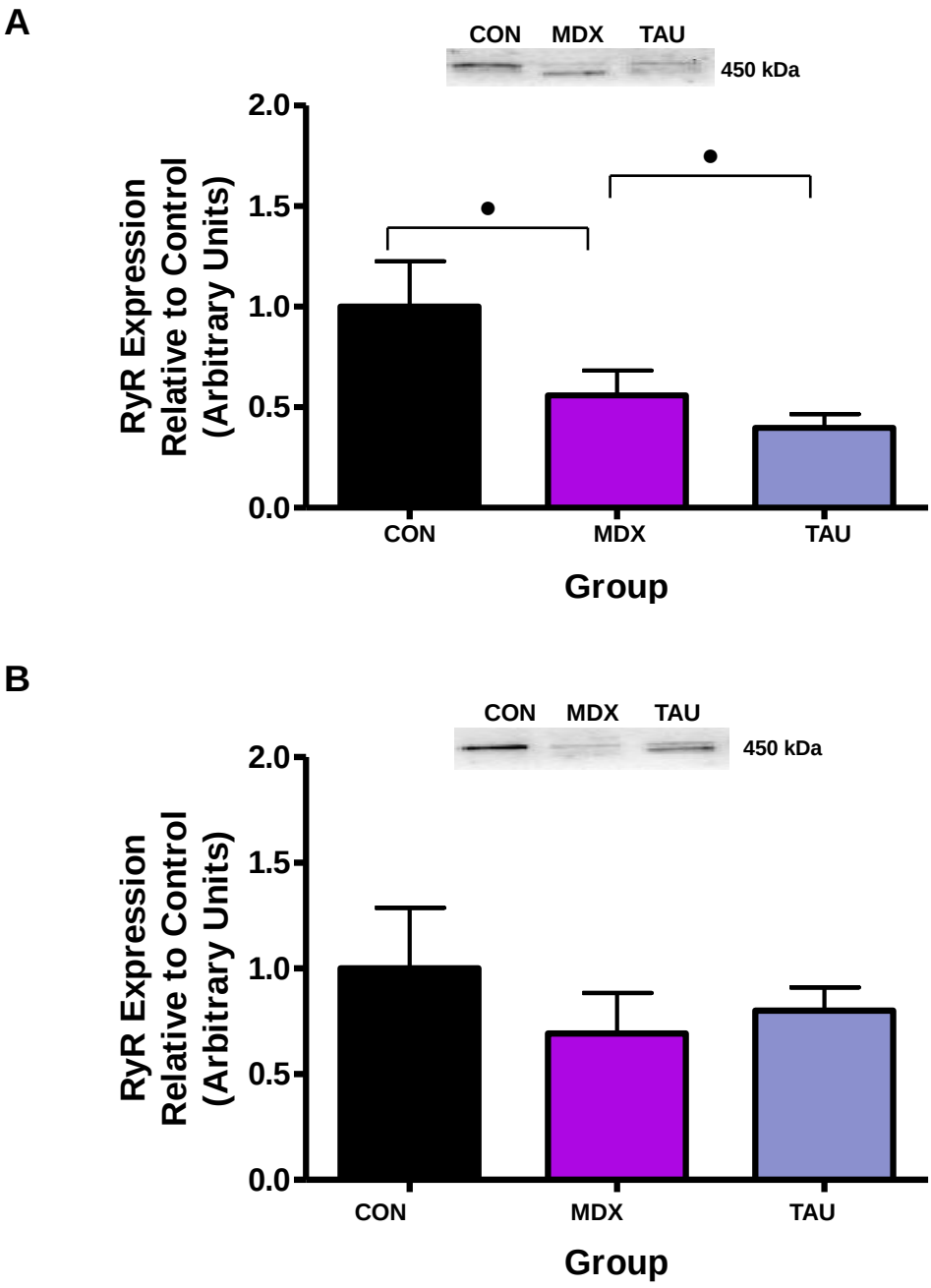


Figure 4.8 Day 28 (A) and day 70 (B) RyR expression in CON, MDX and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10). ● denotes a significant difference (● $p < 0.05$).

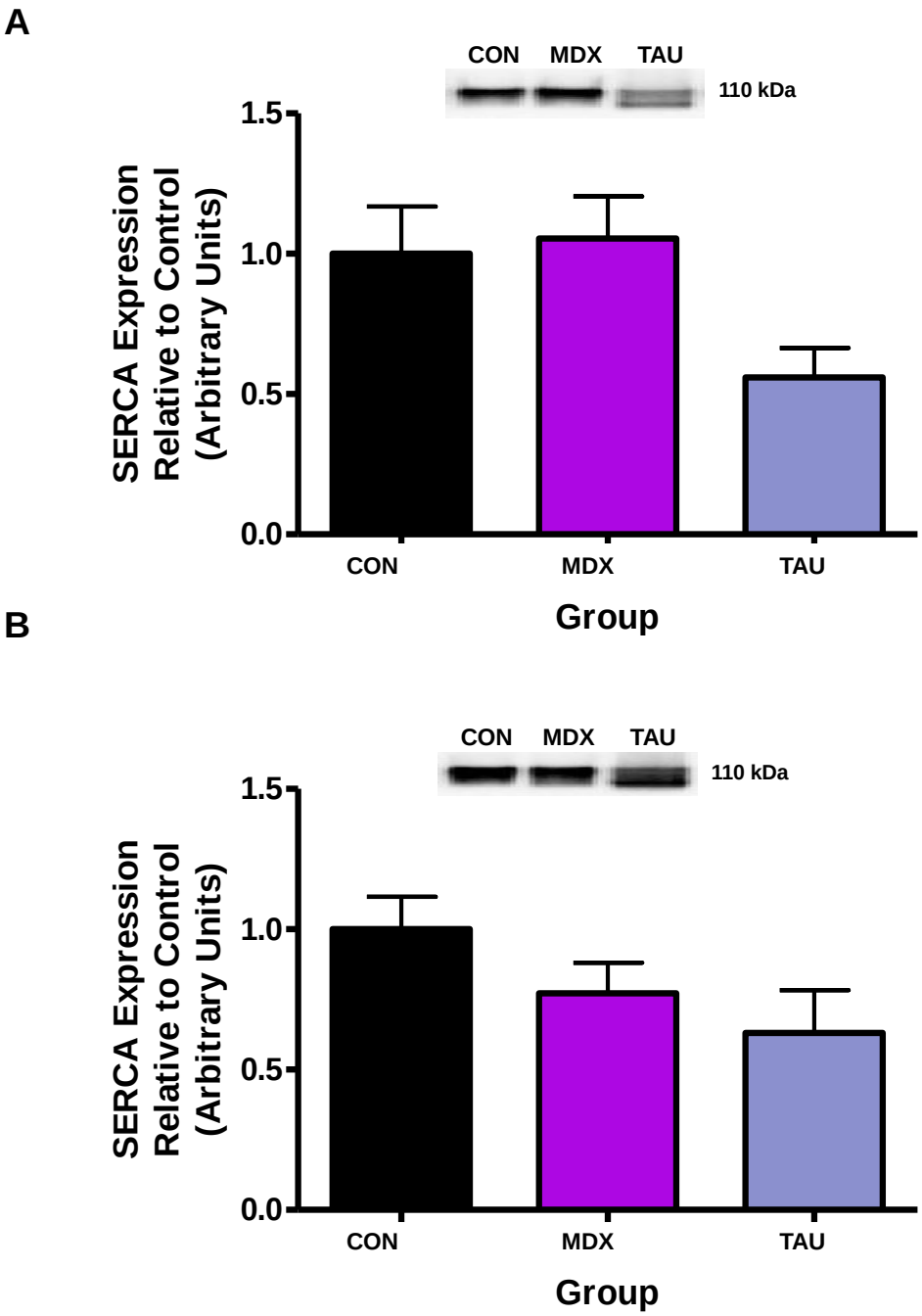
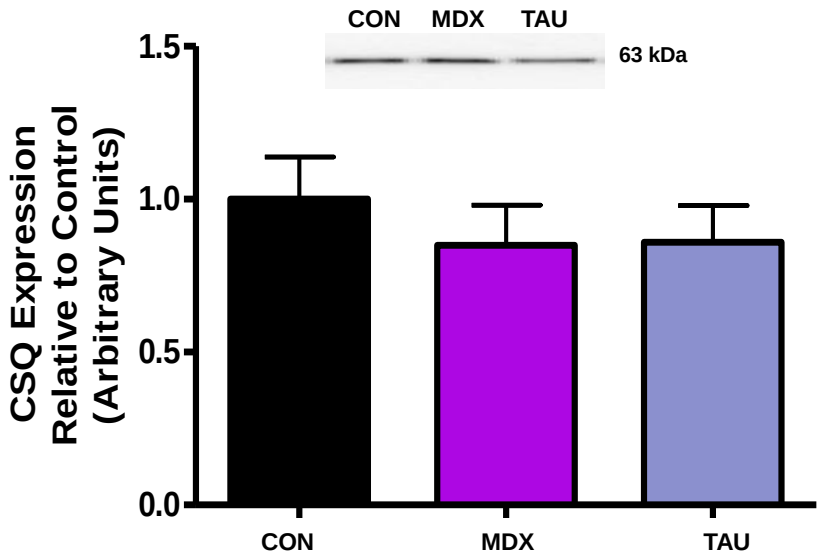


Figure 4.9 Day 28 (A) and day 70 (B) SERCA expression CON, MDX and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10).

A



B

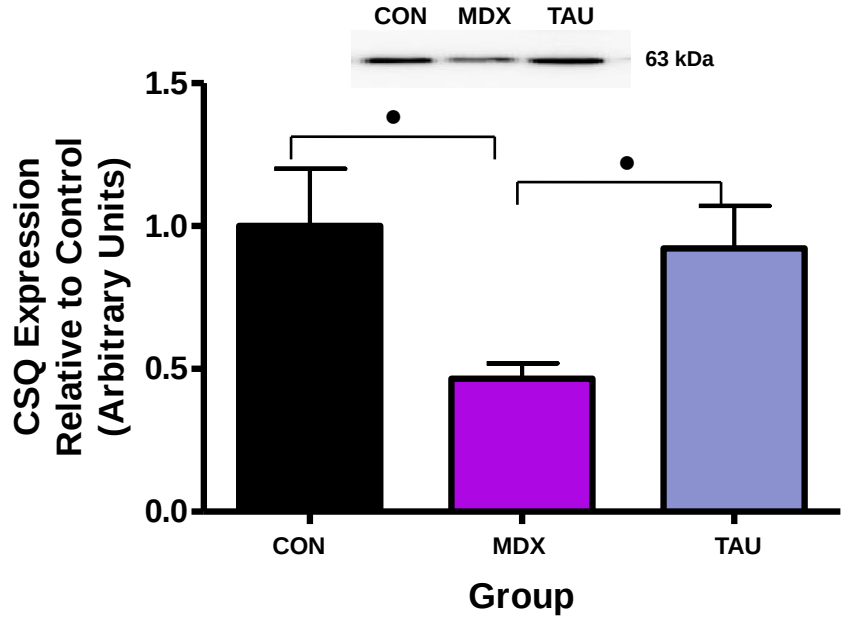


Figure 4.10 Day 28 (A) and day 70 (B) CSQ expression in CON, MDX and TAU mice

Representative Western blot and graph using protein extracted from TA muscle of control, *mdx* and *mdx* taurine supplemented mice. Data is expressed relative to control values ± SEM and has been normalised to tubulin (n=7-10). ● denotes a significant difference (● p <0.05).

4.5 Discussion

The broad aim of this study was to determine if supplementation with taurine could elevate skeletal muscle taurine content in the *mdx* mouse during the acute phase of degeneration, through to a more stabilised phenotype. This study is the first to demonstrate that, despite the suggested impairment in the ability to retain adequate tissue content of taurine in dystrophic skeletal muscle, taurine supplementation is able to significantly increase intramuscular taurine content in the *mdx* mouse, restoring skeletal muscle taurine to above that found in non-dystrophic controls.

Moreover, this is the first study to show that although taurine content in dystrophic *mdx* muscle can be increased, TauT expression is 57% less in *mdx* muscle relative to non-dystrophic controls at 70 days of age. The present study is also the first to provide evidence that taurine treatment effects E-C coupling and Ca^{2+} handling proteins in the *mdx* mouse. Myosin expression is significantly reduced in *mdx* mice at day 70, and taurine supplementation significantly increased actin expression at both day 28 and day 70. RyR expression was significantly lower in both MDX and TAU groups at day 28, while CSQ expression was significantly increased in *mdx* mice with taurine treatment.

4.5.1 Effect of taurine supplementation on body and muscle mass

The effect of taurine supplementation on body and muscle mass within rodents is inconsistent within the literature. Some studies report no change in body or muscle mass with treatment (Goodman et al., 2009, Yatabe et al., 2003), while others report increases in both measures (De Luca et al., 2003, Goodman and Shihabi, 1990, Hultman et al., 2007). In the present study, taurine supplementation was associated with a significant increase in body weight at day 28 only, suggesting that taurine supplementation enhances early growth. Increased TA and GAST muscle mass was also observed at day 28 in TAU mice, such that there was no difference in muscle mass between the TAU and CON mice, while MDX muscles were significantly lighter than both the other groups. These findings are supported by DeLuca et al (2003), who found that at 4 weeks of age, *mdx* mice were significantly lighter than the wild-type control group, while taurine treatment resulted in double the body mass gain in *mdx* mice. Interestingly, while muscle mass is increased with taurine treatment at day 28 in the current study, it appears that

taurine treatment then slows the growth of *mdx* skeletal muscle, as the muscle mass gained between each time point is less than that observed in MDX mice, such that by day 45 and 70 TAU muscle was significantly lighter than MDX. As hypertrophy of skeletal muscle after the acute degenerative bout in the *mdx* mouse is not associated with improvements in specific force production or decreased susceptibility to damage (Consolino and Brooks, 2004, Krahn and Anderson, 1994, Zammit and Partridge, 2002), this unexpected effect of taurine reducing muscle hypertrophy at the later time points may indicate a protective mechanism in *mdx* muscle.

It is well accepted that small diameter muscle fibres are more resistant to necrosis in dystrophic muscle, as larger muscle fibres are preferentially affected in DMD (Karpati et al., 1988, Marques et al., 2007, Massa et al., 1997, Webster et al., 1988). The hypertrophy observed in muscle of the *mdx* mouse is a result of fewer functional fibres being available for contraction, hence the remaining fibres hypertrophy and are at an increased risk of contraction-induced damage (Consolino and Brooks, 2004, Zammit and Partridge, 2002). An example of the detrimental effect of increasing muscle mass in the *mdx* mouse can be seen in the study of Krahn et al. (1994), that found that treatment with anabolic steroids increased muscle hypertrophy and force production in the *mdx* mouse, however, this was coupled with exacerbation of dystrophic pathology. Unfortunately, no functional measures such as muscle force production were made in the current study, however, it is possible that while skeletal muscle of taurine treated *mdx* mice are smaller, they may be functionally superior to the larger muscles of the untreated *mdx* mice. Indeed, functional measurements following manipulation of intramuscular taurine are a key feature of the next two experimental chapters.

4.5.2 Effect of taurine supplementation on skeletal muscle fluid content

An increase in the content of muscle fluid has been observed with some amino acid supplementation protocols (such as creatine), and has been suggested to be a possible mechanism for the positive effects observed in skeletal muscle function in association with supplementation (Santos et al., 2004, van Loon et al., 2003). Increased myoplasmic osmolarity and resultant cell swelling, caused by the elevated amino acid uptake in conjunction with supplementation, has been demonstrated to increase intramuscular protein synthesis and net protein deposition (Demant and Rhodes, 1999, van Loon et al.,

2003), as well as increasing the expression of myogenic growth factors (Hespel et al., 2001). In the present study, there was a significantly decreased % dw/ww in *mdx* TA muscle compared to control at day 35 and day 70, with no significant increases in fluid content associated with taurine supplementation. Interestingly, there was no significant difference in fluid content found in GAST during any time-point examined. It is possible that the difference in water content reported is due to variations in muscular involvement at the time-points examined, as TA demonstrates a far more severe degree of pathology at an earlier stage, with as much as 90% of muscle fibres undergoing significant damage and necrosis during the acute stage dystrophy early in the *mdx* lifespan (De la Porte et al., 1999). Indeed, previous investigations have found smaller % dw/ww in *mdx* skeletal muscle relative to controls, indicating that *mdx* mice have more tissue water within skeletal muscle possibly due to oedema resulting from muscle damage (Dunn and Radda, 1991, Selsby, 2011).

The finding that taurine treatment did not significantly increase muscle water content concurs with results from earlier studies (Goodman et al., 2009, Grindstaff et al., 1997), which suggests that the positive effects of taurine on skeletal muscle function are not associated with cell volume changes, as has been reported with other popular supplements investigated in dystrophy, such as creatine. Similar to the findings for TA, fluid content in the DIA was significantly increased in the *mdx* mouse relative to control tissue, while at day 70 taurine supplementation was associated with a significant increase in water content above that of untreated *mdx* animals. The increase in fluid content in the DIA at day 70 in the TAU group is interesting, as this effect has not been observed in limb muscle. It should be noted however, that at this time point the TAU mice had significantly less DIA taurine content compared to MDX, despite taurine supplementation. Therefore this result is not attributable to elevated taurine content, and could potentially be due to increasing damage and oedema at two months of age, or less muscle repair that is associated with lower taurine content (McIntosh et al., 1998b). Furthermore, as fluid content is increased in DIA, it is possible that taurine efflux is increased in order to maintain the osmolarity of the muscle cells.

4.5.3 Effect of taurine supplementation on skeletal muscle taurine content

Previous studies have shown that young (2-5 week old) *mdx* skeletal muscle contains significantly less taurine relative to non-dystrophic controls, and that taurine content appears to increase with stabilisation of the disease and muscle repair (McIntosh et al., 1998a, McIntosh et al., 1998b). Specifically, McIntosh et al. (1998) examined taurine content in the TA and DIA of young (<3 week old), adolescent (3-6 week old) and adult (> 6 week old) *mdx* mice, finding that lower taurine was characteristic of active degeneration within *mdx* skeletal muscle, but increased with repair until ultimately reaching a greater level in adult *mdx* mice compared to controls. The use of similar time points and the same muscle (TA) in the current study allows direct comparison with those results. This study confirmed that taurine is lower in *mdx* mice during acute degeneration (day 28 & 35) and increases with repair (day 45), until reaching a greater concentration than controls (day 70). The same general pattern was also observed in another hind limb muscle (GAST), with lower taurine at days 28 and 35, yet higher by day 45. The novel finding in the present study was that despite substantial muscle damage and degeneration, taurine supplementation was able to increase hind limb skeletal muscle taurine content to above that of control measures at all time points within the TA, and at most points within GAST. This is significant, as taurine content of skeletal muscle has been shown to be a marker of muscle repair, correlating closely with regeneration and myogenic cell proliferation (McIntosh et al., 1998b).

It should be noted, however, that taurine supplementation appears to become less effective with long-term treatment, as by day 70, in both the TA and GAST, taurine supplemented mice appear to be unable to maintain a steady increase in taurine muscle content, with smaller differences in TA than at earlier time points, while the GAST actually showed significantly less taurine content than controls, despite supplementation. In fact, taurine content of GAST was lower than that of untreated *mdx* mice, although this did not reach statistical significance. This result was unexpected, as while it is assumed that there is a ceiling for intramuscular taurine content, and that eventually despite increasing supplementation the accumulation of taurine in skeletal muscle would plateau, it was not hypothesised that taurine treatment would become ineffective with chronic treatment. This result is unlikely to be due to alterations in TauT expression, as there was no significant difference found between MDX and TAU groups at day 28 or

day 70, and any difference from CON animals appears consistent at both day 28 and day 70. However, it is possible that the activity of the transporter is downregulated with chronic supplementation, or that taurine efflux is increased. Another possible explanation is that taurine supplementation is not ineffective, but rather taurine content becomes normalised within the *mdx* muscle, negating the need to further increase intracellular stores despite the increased availability of taurine. Unfortunately, there are very few examples in the literature of long-term taurine treatment (Di Leo et al., 2002, Yu et al., 2007), with none of the available investigations examining a time course of taurine treatment to determine if there are any changes associated with the duration of supplementation. Pierno et al. (1998) has used chronic (2-3 months) taurine treatment in aged rats, finding that taurine treatment significantly increased muscle taurine content to above non-supplemented aged controls. Yu et al. (2008) showed similar results within the retina of diabetic rats, with 3 months of chronic taurine treatment significantly increasing taurine content back to control values. This, however, is not different from results obtained in the current study, as the decrease in taurine content is not significantly different between the two dystrophic groups, MDX and TAU, but rather between CON and TAU. It is unknown if the observed trend at day 70 for lower taurine content in supplemented *mdx* mice associated with long-term taurine treatment would have continued with increasing age. To the author's knowledge, there is no study that has examined taurine content of skeletal muscle after any longer than 3 months of treatment, and as such the likelihood of this phenomenon of decreased taurine accumulation in muscle with chronic treatment cannot be evaluated at present.

Taurine content of the DIA was also examined in this study, as while this muscle displays a relatively mild dystrophic phenotype at the time points examined, it does undergo significant degeneration and fibrosis at approximately 6 months of age (Louboutin et al., 1993), which could possibly be attenuated if taurine treatment is effective early in the lifespan. In addition, previous investigations have shown that taurine content is significantly lowered in the *mdx* DIA, even at a young age (McIntosh et al., 1998a, McIntosh et al., 1998b). Results from the current study support that MDX DIA taurine content is significantly lower compared to CON muscle, and that supplementation with taurine significantly increases taurine content in the DIA muscle of the *mdx* mouse. As taurine has been shown to be essential for normal skeletal muscle function, and is able to combat many of the key pathological features of dystrophy, such

as impaired Ca^{2+} handling, increased oxidative stress and chronic inflammation, it is possible that this increase in endogenous taurine associated with supplementation in the *mdx* DIA, may decrease pathology later in life.

4.5.4 Effect of taurine supplementation on TauT protein expression

TauT expression in skeletal muscle is known to be upregulated in response to cellular stress as well as during myogenesis (Park et al., 2004, Uozumi et al., 2006a). As such, it was proposed that TauT protein expression would be lower during the beginning of the acute damage phase in day 28 *mdx* mice, but then gradually be upregulated in response to both the cellular stress caused by this damage as well as the increased muscle regeneration occurring by day 70. Unexpectedly, there was no significant difference in TauT expression between CON and the *mdx* group at day 28. Interestingly, a significant 57% decrease in TauT expression in the MDX group was observed at day 70. As taurine accumulation was still observed in *mdx* skeletal muscle with supplementation despite the apparent lower TauT protein expression, it appears that compensatory mechanisms must be activated in response to the downregulation of TauT in dystrophic muscle.

As activity of the TauT was not measured, it is possible that while transporter number is decreased, that the activity of the remaining transporters becomes more efficient in order to compensate for this loss. Indeed, the observed downregulation of TauT protein expression may account for the lower concentration of taurine that is reported in the literature in *mdx* mice, with subsequent upregulation of TauT activity accounting for the improved taurine accumulation observed in adult animals (McIntosh et al., 1998a, McIntosh et al., 1998b). It is possible that supplementation with taurine stimulates the activity of the TauT transporter earlier in the *mdx* lifespan, resulting in increased muscle taurine content. This theory is not supported by previous studies in other tissues and organs, which suggest that supplementation causes a downregulation of TauT activity (Matsell et al., 1997, Tappaz, 2004), however, activity of the TauT in conjunction with supplementation has never been investigated in skeletal muscle. Perhaps adding support to the theory that TauT expression and activity is modulated differently in skeletal muscle is the findings from Goodman et al. (2009), who demonstrated that short-term taurine supplementation resulted in no significant difference in TauT protein expression in rat skeletal muscle. This finding has been confirmed in the present study,

where no significant difference in TauT protein expression in skeletal muscle was found between the MDX and TAU groups. This contradicts the findings of several other studies, which as previously mentioned, have demonstrated a downregulation of TauT mRNA and protein expression in association with taurine treatment in non-skeletal muscle tissues (Matsell et al., 1997, Tappaz, 2004). With such limited research available on TauT expression and regulation in skeletal muscle, and given taurine's apparent importance in this tissue, more research is required before the definitive mechanism allowing increased taurine accumulation in *mdx* muscle despite a significant decrease in TauT protein expression can be elucidated.

4.5.5 Effect of taurine supplementation on E-C coupling and Ca^{2+} handling proteins

The present study is the first to demonstrate that taurine supplementation in the *mdx* mouse alters the expression of contractile and E-C coupling proteins, as well as the SR Ca^{2+} binding protein CSQ. In fact, this is the first study to examine actin and myosin protein expression and E-C coupling proteins in untreated *mdx* mice during the acute phase of degeneration, and then during the stabilised phase of muscle damage/repair. Investigation of contractile protein and E-C coupling expression during these phases is important, as the acute phase of damage in the *mdx* model more closely mimics the human DMD condition, while at day 70 compensatory mechanisms that result in functional recovery in the *mdx* mouse are likely to have occurred, and thus protein expression may be altered as part of this process.

Treatment with taurine appears to be protective of contractile protein expression, as it prevented the decline in myosin expression observed in MDX mice at day 70, while significantly increasing actin expression such that it was significantly increased compared to MDX at day 28 and 70 (see Figures 4.5 and 4.6). Contractile proteins are thought to be damaged as a result of increased proteolysis in dystrophic tissue, and derangement of motor protein structure and activation have been observed in dystrophic muscle (Friedrich et al., 2010). The protection of contractile filaments with taurine supplementation in *mdx* muscles may explain, in part, why taurine has been shown to have beneficial effects on contractile function and force output in the *mdx* mouse (Cozzoli et al., 2011b, De Luca et al., 2003).

While taurine supplementation increases contractile proteins in the *mdx* mouse, E-C coupling proteins were variably affected by taurine supplementation. DHPR expression was unaltered by taurine treatment at both day 28 and day 70The limited studies available in the literature on DHPR protein expression in *mdx* skeletal muscle confirm results from the present study, namely that DHPR expression is unaltered in dystrophic skeletal muscle (Desnuelle et al., 1986, Pereon et al., 1997).

RyR protein expression was altered, with the already significant decline in RyR expression at day 28 in *mdx* muscle being exacerbated by taurine treatment, resulting in significantly less RyR protein expression within the TAU group. The finding that dystrophic muscle displays a decrease in RyR protein expression is as expected, as both structural and functional deficits in RyR have been reported within dystrophic cardiac and skeletal muscle, although there is less information available on protein expression (Bellinger et al., 2009, Fauconnier et al., 2010, Morel et al., 2004). Bellinger et al. (2009) identified alterations to RyR structure and function that contributed to the increased cytosolic Ca^{2+} concentration observed in *mdx* muscle, due to increased leak from RyR channels resulting from nitric oxide-mediated modifications causing increased S-nitrosylation of cystine residues on the RyR. Reductions in RyR protein expression in *mdx* mice may explain the decrease in force production reported during the acute damage phase at day 28, although the effect of taurine treatment causing further reductions to RyR expression is unexpected. It is possible that RyR protein expression is reduced in untreated *mdx* at day 28 as a protective mechanism, as at this time point there is significant muscle degeneration due to a cascade of events such as increased protease activity, ROS production and inflammation, all of which are driven by excessive Ca^{2+} accumulation in skeletal muscle (Bakker et al., 1993, Emery, 2003, Fong et al., 1990, Williams et al., 1990, Yeung et al., 2005). Perhaps decreasing the expression of RyR at day 28 limits Ca^{2+} entry into dystrophic muscle that occurs due to increased Ca^{2+} leak from the RyR, which has been shown to be exacerbated under conditions of increased oxidative stress, as would be the case at this time point (Bellinger et al., 2009, Brookes et al., 2004, Whitehead et al., 2006). While reduced RyR expression, and thus less capacity for Ca^{2+} release from the SR, would also decrease force production, due to the extensive muscle damage and necrosis that occurs in the 3rd to 4th week of life in the *mdx* mouse this reduction in contractile activity may again be protective, limiting

damage to muscle fibres that are known to have increased susceptibility to contraction induced damage (Brussee et al., 1997, Selsby, 2011, Vilquin et al., 1998). Moreover, this loss of contractile function may also be compensated for by the increased expression of contractile proteins. Thus, taurine is potentially increasing this adaptive response during this stage of the *mdx* lifespan to further protect skeletal muscle from damage. The fact that there is no significant difference in RyR expression between CON, MDX or TAU by day 70 where muscle damage and necrosis is decreased, supports this theory.

Previous investigations within the *mdx* mouse have demonstrated no significant difference in SERCA expression (Dowling et al., 2004), although there is some suggestion that the activity of SERCA is reduced in dystrophic muscle causing impaired Ca^{2+} handling and contractile function (Divet et al., 2005, Kargacin and Kargacin, 1996). For example, Divet et al. (2005) found that within fast-twitch muscles of the *mdx* mouse, there was a switch to the slower isoform of SERCA leading to reduced Ca^{2+} uptake. Moreover, a recent investigation by Morine et al. (2010) showed that SERCA overexpression in the *mdx* DIA significantly improved Ca^{2+} handling and decreased muscle damage. The present study found no significant difference inSERCA expression in association with taurine treatment at day 28 or day 70 in the *mdx* mouse, while no difference in SERCA expression was found between CON and the untreated MDX groups. Four weeks of taurine treatment in rats also had no effect on SERCA expression (Goodman et al., 2009). Given that there is no significant muscle degeneration occurring due to Ca^{2+}-dependant processes in the rat, the *mdx* model at day 70 would be more comparable to the rat model employed by Goodman et al. (2009), as skeletal muscle damage is stabilised to a low level at this time, thus yielding similar results.

Decreases in Ca^{2+} binding proteins within dystrophic tissue have previously been reported within the literature, causing drastic reductions in the Ca^{2+} buffering capacity of the SR (Culligan et al., 2002, Dowling et al., 2004). It is proposed that this alteration to Ca^{2+} binding proteins in dystrophic skeletal muscle is a significant contributor to the development of pathogenesis in DMD, as increasing the expression of Ca^{2+} binding proteins reduces muscle necrosis. For example, examination of the effect of two Ca^{2+} channel blockers on skeletal and cardiac muscle damage in the *mdx* mouse found an attenuation of the rise in intracellular Ca^{2+} concentration in *mdx* mice, and increased CSQ expression, which was associated with reduced degree of muscle damage

(Matsumura et al., 2009). Moreover, Ferretti et al. (2009) found that the relatively spared intrinsic laryngeal muscles of the *mdx* mouse naturally contain higher expression of CSQ, in addition to greater expression of SERCA.

In the present study, CSQ expression was not significantly different at day 28 between CON, MDX or TAU groups, however, at day 70, there was a significant decrease in CSQ protein expression in *mdx* mice, while taurine supplementation significantly increased CSQ expression, such that there was no significant difference between the control and taurine-supplemented *mdx*. This increase in CSQ protein expression at day 70 improves the capacity of the SR to store Ca^{2+}, which could improve intracellular Ca^{2+} handling in dystrophic skeletal muscle and limit Ca^{2+} driven damage. Elevated CSQ expression in association with taurine treatment could also result in improved contractile function. Indeed, Goodman et al. (2009) found a 49% increase in CSQ protein content in rats after 2 weeks of taurine supplementation via drinking water. Associated with this reported increase in CSQ was increased peak tetanic and twitch force, as well as improved recovery after a high-frequency continuous stimulation to fatigue (Goodman et al., 2009).

4.6 Conclusions

In summary, this is the first study to demonstrate that taurine supplementation is able to significantly increase skeletal muscle taurine content during peak degeneration, through to recovery in the *mdx* mouse to a normal healthy control level. Taurine has been shown to alter skeletal muscle development in the *mdx* mouse, resulting in less muscle mass when compared to non-dystrophic control mice, although this may result in better protection of skeletal muscle from damage. TauT protein expression was found to be lower in dystrophic *mdx* mice, and as hypothesised expression was unaltered in association with taurine supplementation, suggesting that some of the impairment in retaining normal skeletal muscle taurine concentration may be, in part, due to alterations in TauT expression in dystrophic skeletal muscle.

E-C coupling and contractile proteins are also affected by taurine supplementation, with actin and CSQ all found to be significantly altered in association with taurine treatment. Due to the large increase in CSQ expression, it is possible that taurine may be having positive effects on muscle function via improvements to Ca^{2+} handling. Thus, there is a need for the functional properties of taurine supplemented *mdx* mice to be examined.

CHAPTER FIVE

LONG AND SHORT-TERM TAURINE SUPPLEMENTATION ON CONTRACTILE PROPERTIES AND ENZYME ACTIVITY IN *MDX* SKELETAL MUSCLE

5.1 Introduction

The loss of dystrophin from the skeletal muscle of DMD patients causes myofibres to be inherently more susceptible to damage during contractile activity, which leads to significant muscle necrosis, atrophy and weakness (Beenakker et al., 2005, Emery, 1995). Initially, the damage caused as a consequence of contraction is able to be repaired, however eventually chronic degeneration of muscle fibres exhausts this regenerative capacity (Luz et al., 2002). This leads to significantly impaired muscle function that ultimately results in wheelchair confinement, failure of respiratory muscles and death in the second or third decade of life (Emery, 1995, Kohler et al., 2009).. As DMD results in not only reduced muscle strength, but also increased fatigue, therapeutic interventions that can improve both force output and/or resistance to fatigue would be ideal (Beenakker et al., 2005, Frascarelli et al., 1988, McDonald et al., 2010). Recent research suggests that taurine is essential for normal skeletal muscle function, and is able to modulate contractile function (Dawson et al., 2002, Hamilton et al., 2006, Warskulat et al., 2007). Interestingly, elevating skeletal muscle taurine content has been shown to increase muscle force output, most likely due to alterations in Ca^{2+} handling (Bakker and Berg, 2002, De Luca et al., 2003). Bakker and Berg. (2002) found that the application of taurine to mechanically skinned EDL muscle fibres augmented SR Ca^{2+} accumulation and release and improved maximum force output, while Goodman el al. (2009) found that supplementation with taurine increased rat EDL twitch force by 19% relative to controls, and also increased specific tetanic force. Moreover, while taurine is able to improve muscle strength, it also appears to reduce fatigue (Dawson et al., 2002, Goodman et al., 2009). A study by Matsuzaki et al. (2002) found that the taurine

concentration of fast-twitch skeletal muscle of the rat was significantly decreased after exercise, and this loss was increased with increasing duration of activity. In an extension to this work, Yatabe et al. (2003) later determined that supplementation with taurine prevented the loss of taurine from skeletal muscle with exercise, and also improved physical endurance.

As the concentration of taurine has been found to be decreased in dystrophic skeletal muscle (McIntosh et al., 1998a), it is possible that some of the functional deficits observed in contractile function could be due to, in part, to a lack of taurine, and thus supplementation with taurine may improve muscle force and fatigue. Indeed, the application of taurine to EDL muscle preparations of the *mdx* mouse have been shown to improve E-C coupling and the stability of the sarcolemma (DeLuca et al., 1996). While DeLuca et al. (2003) found that supplementation with taurine significantly improves grip strength in *mdx* mice. A more recent investigation by Cozolli et al. (2011) determined that combined therapy with taurine and corticosteroids significantly improved muscle function and E-C coupling, more than when each treatment was administered in isolation. Despite these positive results, only two studies have supplemented the *mdx* mouse with taurine, and only a limited number of functional measures have been made (Cozzoli et al., 2011b, De Luca et al., 2003), necessitating further investigation into the effect of taurine supplementation on contractile function of dystrophic skeletal muscle. In addition, no study has examined the effect of long and short-term taurine supplementation in the *mdx* mouse, as taurine treatment may have different effects depending on the timing of treatment (i.e. pre or post the acute phase of damage observed at 3-4 weeks of age in the *mdx* model).

As a limited number of studies have examined the effect of taurine supplementation on dystrophic skeletal muscle function, little is known about taurine's possible role(s) in DMD. Taurine has been proposed to play a role in membrane stabilisation, Ca^{2+} handling and mitochondrial function as well as modulating the inflammatory response and the production of ROS (Huxtable, 1992, Schaffer et al., 1995). As muscle contraction and repair of damaged myofibres requires significant energy, and mitochondrial function is known to be impaired in dystrophy, examining the activity of key metabolic enzymes may provide a mechanism for some of taurine's beneficial effects.

While taurine cannot be utilised for energy production directly, it is possible that through minimising Ca^{2+} overload, reducing oxidative stress, muscle damage and inflammation, taurine may decrease energy requirements and prevent mitochondrial pathology in dystrophic muscle tissue (Chang et al., 2004, Schaffer et al., 2009, Sun et al., 2011). In normal muscle, Ca^{2+} is an important regulator of enzymatic activity and stimulates mitochondrial oxidative phosphorylation (ox-phos), however in pathological conditions such as DMD, chronically elevated Ca^{2+} may overload the mitochondria resulting in a cascade of events that leads to down regulation of ox-phos, excessive ROS production, apoptosis and cellular death (Brookes et al., 2004). These detrimental changes to energy metabolism associated with DMD are also of importance due to the increased energy demands associated with Ca^{2+} extrusion and repair of damaged tissues. Several studies in both humans and the *mdx* mouse have shown that dystrophic skeletal muscle has an altered metabolic profile with reductions in the maximal rate of mitochondrial respiration (Kuznetsov et al., 1998), reduced enzyme activity (particularly glycolytic pathway) (Kuznetsov et al., 1998) as well as reductions in creatine and PCr (Passaquin et al., 2002). Despite this, no study has examined the effect of taurine supplementation on metabolic enzyme activity in DMD.

5.2 Aims and hypothesis

The primary aim of this study was to determine if taurine supplementation can improve contractile function in the *mdx* mouse. In an effort to determine the most appropriate timing of taurine treatment, the second aim of this study was to determine if long or short-term taurine supplementation has different effects on contractile function. The activity of key enzymes involved in glucose metabolism and fat oxidation were also examined, to determine if taurine treatment enhanced enzymatic function.

The specific aims of this study were to;

i) Determine if treatment with taurine improved contractile function in *mdx* EDL and SOL.

ii) To determine if long or short-term treatment is more effective in improving contractile function in the *mdx* mouse.

iii) To determine if taurine treatment reduces muscle damage as measured indirectly via plasma CK concentration.

iv) To determine if taurine treatment affects the activity of some key metabolic enzymes in skeletal muscle, specifically, PFK, CK, CS and *β*-HAD.

It was hypothesised that both long and short-term taurine supplementation would significantly improve contractile function in the *mdx* mouse and significantly decrease plasma CK concentration; although it was expected that long-term supplementation would be more effective than the short-term treatment. It was also hypothesised that both taurine treatments would significantly improve the activity of key enzymes involved in glucose and fat metabolism, and that long-term treatment would be more beneficial.

5.3 Methods

5.3.1 Animals

All mice (n = 46) used in this study were male, and with the exception of the short-term taurine supplemented group, were bred at the Melbourne University animal house using stock obtained from the ARC (WA, Australia). The short-term supplement group was ordered in from the ARC prior to supplementation, and housed at the Melbourne University animal house for the duration of the taurine treatment period. Details on the housing conditions of the animals are available in Chapter 3, section 3.1. All control (CON) mice were untreated and served as an indicator of normal contractile function and enzyme activity, and allowed comparison between any improvements observed with taurine treatment to typical values for normal muscle. *Mdx* mice were divided into one of three groups, untreated (MDX), short-term taurine treatment (S-TAU) or long-term taurine treatment (L-TAU).

These two treatment durations were chosen for two reasons, 1) it has been proposed that certain DMD treatments are more beneficial when administered early in disease progression, before significant muscle damage and atrophy have occurred, and are potentially ineffective at decreasing muscle pathology when given at late stages of the disease (Bushby et al., 2004, Manzur et al., 2004) and 2) results from study one (see Chapter 4) suggested that by day 70, there was a decline in muscle taurine content despite supplementation, which if this trend continued, could make long-term supplementation ineffective. The L-TAU group was designed to test if taurine supplementation before the onset (< 3 weeks old) of the acute damage phase though to adulthood in the *mdx* mouse potentially reduced the loss of muscle function due to protection during this period, while the S-TAU group was designed to examine the effect of taurine treatment after the acute damage phase, and thus determine if taurine would still have beneficial effects on contractile function, even if administered after the major bout of degeneration and only for a short time.

The L-TAU group was therefore supplemented throughout the entire lifespan, initially through maternal means and later via drinking water provided *ad libitum.* Maternal supplementation commenced three weeks before being paired with male mice and

continued until offspring were weaned. Once weaned from supplemented mothers, male offspring were separated supplemented with taurine directly via drinking water. The S-TAU group were supplemented for four weeks prior to experimentation. Both groups were given a dosage of 2 % w/v of taurine via drinking water provided *ad libitum,* and remained on the supplement until sacrifice, at six months of age (± 1 week).

5.3.2 Contractile protocol

Contractile testing of both the EDL and SOL muscles were conducted as part of this study to determine if taurine treatment affected both fast and slow-twitch muscles. Briefly, mice were anesthetised with intra-peritoneal injections of 40-50mg/kg of Nembutal (Pentobarbitone Sodium, Rhone Merieux, QLD, Australia) so that they were unresponsive to tactile stimuli. In order to maintain blood supply and nervous innervation until the last possible moment, the EDL muscle was surgically exposed first (taking care not to expose the posterior compartment of the leg) and immediately tested for isometric contractile properties (as described in Chapter 3, section 3.2). During the equilibration time in the muscle bath, the contralateral EDL was dissected and placed in an identical muscle bath for the duration of the contractile experiments, but was not exposed to stimulation. Muscle baths were maintained at 24 °C for the duration of the experiment, as this temperature allowed the maintenance of optimal isometric force *in vitro*. While contractile properties of the EDL were being tested, the mouse remained under anaesthesia with a saline soaked gauze pad covering the surgery sites to prevent drying of the tissues. Once testing of the EDL was completed, both SOL muscles were dissected and placed in the baths as previously described for EDL. Blood was then collected for plasma CK analysis.

The force frequency relationship was determined using stimulation frequencies of 30, 60, 80, 100, 120, 140 and 160 Hz for EDL and 30, 60, 80, 100, 120, 140 Hz for SOL. A three minute rest was given between stimulations. Muscles were then subjected to a fatigue protocol with continuous stimulation at 120 Hz for 10 seconds for EDL, and 100 Hz for 1 minute for SOL. This protocol was chosen as Goodman et al. (2009) demonstrated significant differences in fatigue in taurine-supplemented rats with a similar high-frequency continuous stimulation protocol. Once the contractile protocol was complete,

muscles were quickly weighed, snap frozen in liquid nitrogen and then stored at -80 °C until further analysis.

5.3.3 Plasma CK measurement

Chest cavity blood was collected for analysis of plasma CK after removal of the heart. Plasma concentration of CK was measured for all groups as previously described in Chapter 3, section 3.4.2.

5.3.4 Enzyme activity analysis in muscle

The activity of key metabolic enzymes for glycolysis, as well as fat and carbohydrate metabolism, were measured, including PFK, CK, CS, *β*-HAD for EDL and SOL. As analysis showed no significant difference in enzyme activity between the rested and contracted muscles, only the data for contracted muscles has been presented. Details on the procedure are available in Chapter 3, section 3.4.

5.3.5 Statistical Analysis

All data are expressed as mean ± standard error (SEM). Contractile and enzyme activity data were analysed using a one-way analysis of variance (1-way ANOVA) and any significant differences examined using a Bonferroni Post Hoc Test on GraphPad Prism 4.1 (GraphPad Software). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

5.4 Results

5.4.1 Body mass

As shown in Table 5.1, CON mice had a significantly lower body mass ($p < 0.001$) when compared to all MDX groups. There was no significant difference in body mass between the MDX and L-TAU group, however, there was a significant increase ($p < 0.01$) in body mass in the S-TAU treatment group relative to both the MDX and L-TAU groups.

Table 5.1 Body mass with taurine treatment

	CON	MDX	L-TAU	S-TAU
Body Mass (mg)	28.3 ± 3.7	33.3 ± 3.8 *	33.6 ± 1.6 * •	38.4 ± 1.4 * ^

* significantly different from CON group. ^ significantly different from MDX group.

• significantly different to S-TAU group. (n=7-10)

5.4.2 Muscle mass and relative muscle mass

i) EDL

Consistent with results for body mass, the EDL of CON mice had a significantly lower muscle mass and relative mass ($p < 0.001$; Table 5.2) when compared to all groups of *mdx* mice. S-TAU EDL muscle and relative muscle mass was significantly increased ($p < 0.001$) relative to both the MDX and L-TAU groups. No significant difference in EDL muscle mass was observed between the untreated *mdx* and long-term taurine treated group.

ii) SOL

As expected, SOL muscle mass and relative muscle mass was significantly higher ($p < 0.001$; Table 5.2) in all *mdx* groups relative to the CON mice. There was also a significant increase in muscle and relative muscle mass in the S-TAU compared to MDX ($p < 0.05$) and L-TAU ($p < 0.001$) groups, as was the case for body mass and EDL muscle mass.

Table 5.2 Muscle mass and relative muscle mass with taurine treatment

		CON	MDX	L-TAU	S-TAU
EDL	Muscle mass (mg)	9.6 ± 1.4	16.9 ± 2.2 *	16.6 ± 1.6 * •	21.1 ± 1.2 * ^
	Relative muscle mass (mg/g)	0.34 ± 0.01	0.51 ± 0.01*	0.50 ± 0.01*	0.54 ± 0.01*^
SOL	Muscle mass (mg)	7.5 ± 0.2	12.9 ± 0.6 *	11.7 ± 0.3 * •	14.5 ± 0.3 * ^
	Relative muscle mass (mg/g)	0.26 ± 0.01	0.38 ± 0.01*	0.35 ± 0.01*	0.38 ± 0.01*^

* significantly different from CON group. ^ significant difference relative to MDX group.

● significant difference relative to S-TAU group. (n=7-10)

5.4.3 Plasma CK

As shown in Figure 5.1, significantly lower plasma CK was observed in the CON group relative to both the MDX ($p < 0.001$) and S-TAU ($p < 0.001$) groups. Interestingly, long-term taurine treatment appears to reduce CK leakage from muscle, such that no significant difference in plasma CK concentration between the CON and L-TAU group was observed. This indicates that L-TAU treatment significantly decreases permeability of skeletal muscle fibres to CK, suggesting that there is possibly lower damage in this group of *mdx* mice. Due to this improvement, there was also a significant difference ($p < 0.001$) observed between the MDX and S-TAU groups relative to the L-TAU group, with the L-TAU group having significantly lower plasma CK relative to the MDX ($p < 0.05$) and S-TAU ($p < 0.001$) groups.

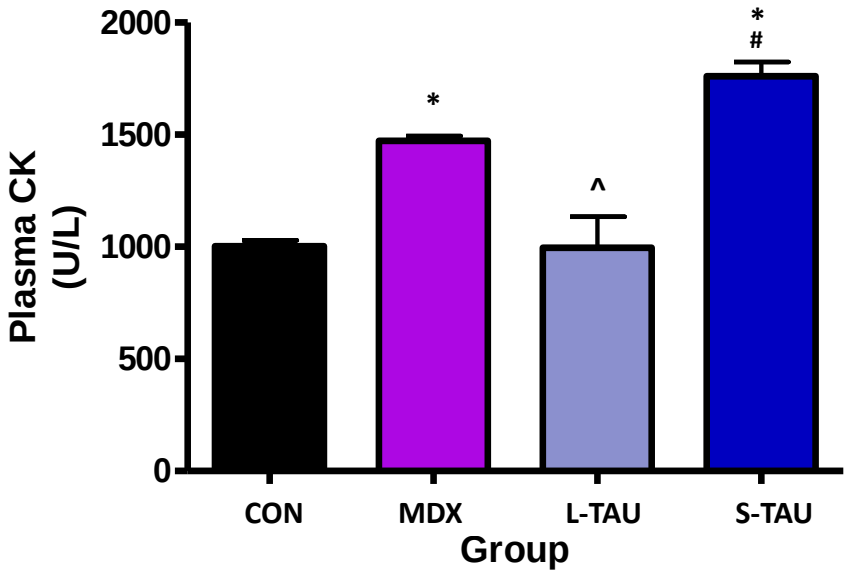


Figure 5.1 Plasma CK concentration in CON, MDX and taurine supplemented groups

***** significant difference relative to CON group. ^ significant difference relative to MDX group. # significant difference relative to L-TAU group. (n=7-10)

5.4.4 EDL isometric contractile properties

i) Twitch characteristics, CSA and L_0

As shown in Table 5.3, CON mice demonstrated significantly greater ($p < 0.001$) normalised twitch force relative to all the *mdx* groups. Significantly decreased twitch force was observed in the L-TAU group, compared to both the MDX ($p < 0.001$) and S-TAU ($p < 0.01$) mice. Despite this, there was no significant difference observed in TTP, although a significant increase in ½ RT was observed in the L-TAU ($p < 0.001$) when compared to S-TAU mice. As expected due to the increase in muscle mass in *mdx* mice, there was a significant increase in EDL muscle CSA in all *mdx* groups relative to the CON group. The S-TAU group also demonstrated a significantly increased CSA when compared to MDX. S-TAU group had significantly increased L_0 when compared to CON, and L-TAU mice had significantly less L_0 relative to S-TAU. No other significant differences in L_0 were observed.

Table 5.3 Isometric contractile properties of EDL with taurine treatment

	CON	MDX	L-TAU	S-TAU
Lo (mm)	12.9 ± 1.0	13.4 ± 1.3	12.8 ± 1.2 •	14.7 ± 0.6 *
CSA (mm^2)	1.6 ± 0.1	2.6 ± 0.1 *	2.7 ± 0.1 *	3.1 ± 0.0 * ^
Pt (N/cm^2)	12.5 ± 0.7	8.0 ± 0.7 *	4.3 ± 0.5 * ^ •	7.9 ± 0.5 *
TTP (msec)	34.5 ± 0.6	34.9 ± 0.8	35.8 ± 0.5	34.0 ± 0.8
½ RT (msec)	27.0 ± 1.3	26.4 ± 1.4	30.5 ± 1.3 •	22.5 ± 1.0

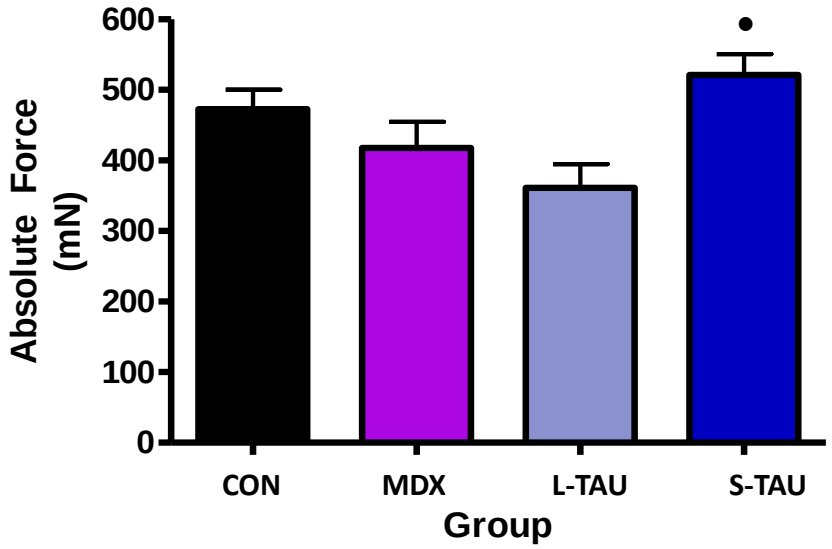
* significantly different from CON group. ^ significant difference relative to MDX group.

● significant difference relative to S-TAU group. (n=7-10)

ii) Peak tetanic force

There was no significant difference in absolute force between CON mice and all *mdx* groups (see Figure 5.2 A). There was, however, a significant increase ($p < 0.01$) in absolute force between the S-TAU and L-TAU groups. However, when normalised to CSA, there was no significant differences observed between any of the *mdx* groups tested, although all *mdx* groups demonstrated significantly lower ($p < 0.001$) specific force relative to the CON group.

A



B

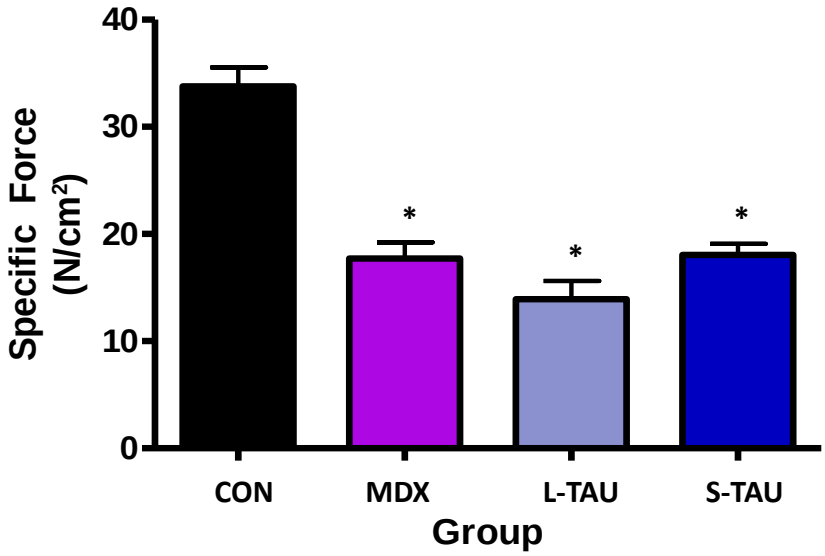


Figure 5.2 Absolute (A) and specific (B) force production for the EDL muscles from CON, MDX and taurine treated *mdx* mice

***** significant difference relative to CON group. ● significant difference relative to L-TAU group. (n=7-10)

iii) Force-Frequency Relationship

There was no significant difference in the relative force-frequency relationship between CON and MDX (see Figure 5.3), or between any of the treatment groups at stimulation frequencies of 10, 30, 50, 80 and 120 Hz. At higher stimulation frequencies of 140 and 160 Hz, there was a significant ($p < 0.001$) decline in force production in all *mdx* groups, with no significant differences observed with taurine treatment, demonstrating that *mdx* muscle is unable to maintain force production at high stimulation frequencies, relative to CON.

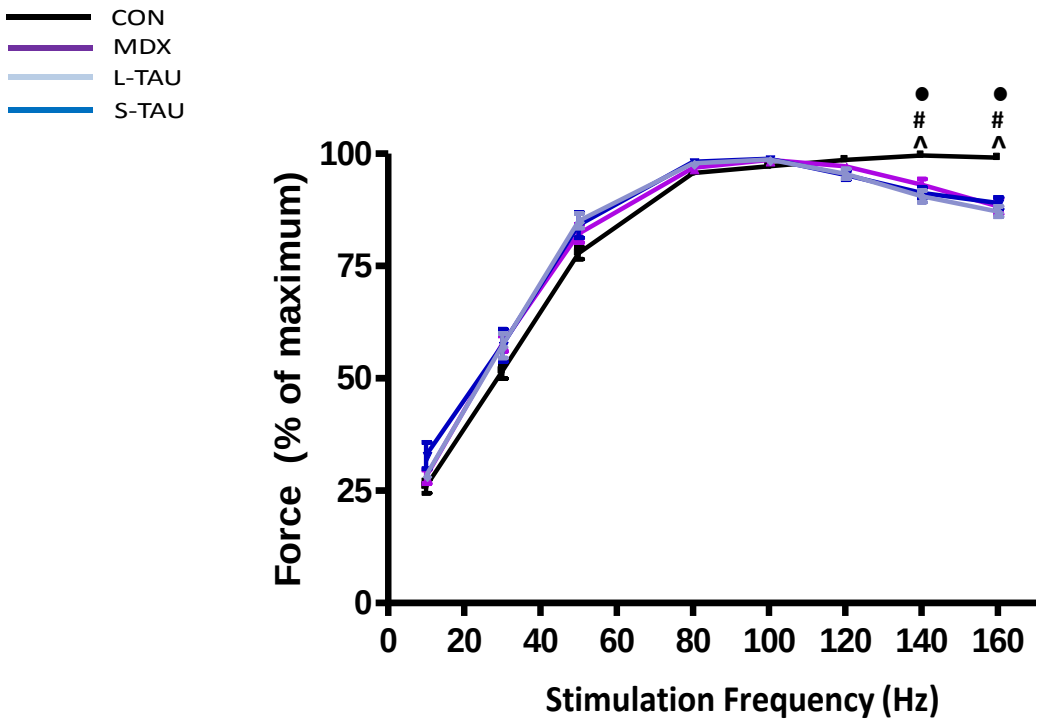


Figure 5.3 Relationship between stimulation frequency and force production for the EDL muscles from CON, MDX taurine treated *mdx* mice

^ significant difference relative to MDX group. # significant difference relative to L-TAU group.

● significant difference relative to S-TAU group. (n=7-10)

iv) Fatigue

As shown in Figure 5.4 A, there was no significant difference in the time taken to reach 70% of original force during a continuous, high-frequency fatiguing stimulation between CON and MDX, or between any of the treatment groups. Force generated by MDX muscles, however, reached 50% of original force faster ($p < 0.01$) than CON, with a similar result ($p < 0.001$) observed S-TAU group, such that no significant difference in time to fatigue was seen between MDX and S-TAU mice. Interestingly, L-TAU mice demonstrated increased resistance to fatigue, taking significantly longer to reach 50% of original force when compared to both the MDX ($p < 0.05$) and the S-TAU ($p < 0.001$) groups. This increased resistance to fatigue with long-term taurine supplementation decreased muscle fatigue in the L-TAU, such that no significant differences within the group relative to CON values were observed. Relative to CON and L-TAU, both the MDX ($p < 0.001$) and S-TAU ($p < 0.01$) groups took less time to reach 30% of original force.

v) Percentage of original force post-fatigue

As shown in Figure 5.4 B, at the end of 10 seconds of stimulation, all of the *mdx* groups MDX ($p < 0.01$), L-TAU ($p < 0.05$) and S-TAU ($p < 0.001$)) demonstrated a significantly greater decline in force relative to CON values. Taurine supplementation in the long- or short- term was unable to decrease fatigability, although there was a tendency for the L-TAU group to retain slightly more force than the MDX and S-TAU groups.

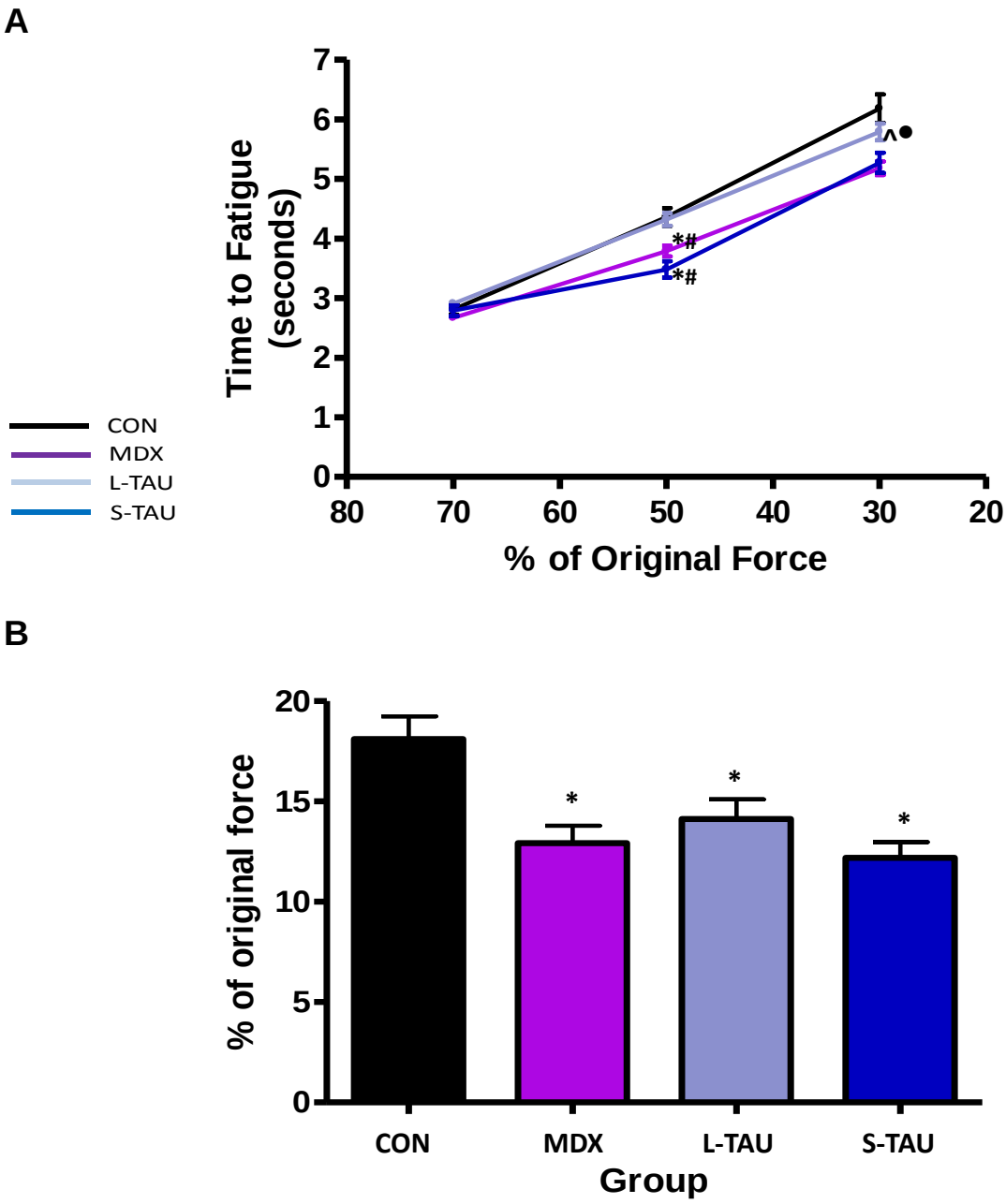


Figure 5.4 Time taken for EDL force to fatigue to 70, 50 and 30% of original force (A) and the percentage of original force attained at the end of the fatiguing stimulation (B)

***** significant difference relative to CON group. ^ significant difference relative to MDX group. # significant difference relative to L-TAU group. ● significant difference relative to S-TAU group. (n=7-10)

5.4.5 Enzyme activity for EDL

i) PFK

As shown in Figure 5.5, there was a significant difference between CON and MDX groups, with MDX demonstrating significantly lower ($p < 0.05$) PFK activity. Interestingly, long-term taurine supplementation resulted in a further decrease in PFK activity, such that L-TAU was significantly lower than both the CON ($p < 0.001$) and MDX ($p < 0.05$) groups. The S-TAU group were not significantly different to the MDX mice, however short-term taurine supplementation did not result in further decreases to PFK activity as was observed for L-TAU supplementation, thus making the S-TAU significantly higher ($p < 0.05$) than L-TAU mice.

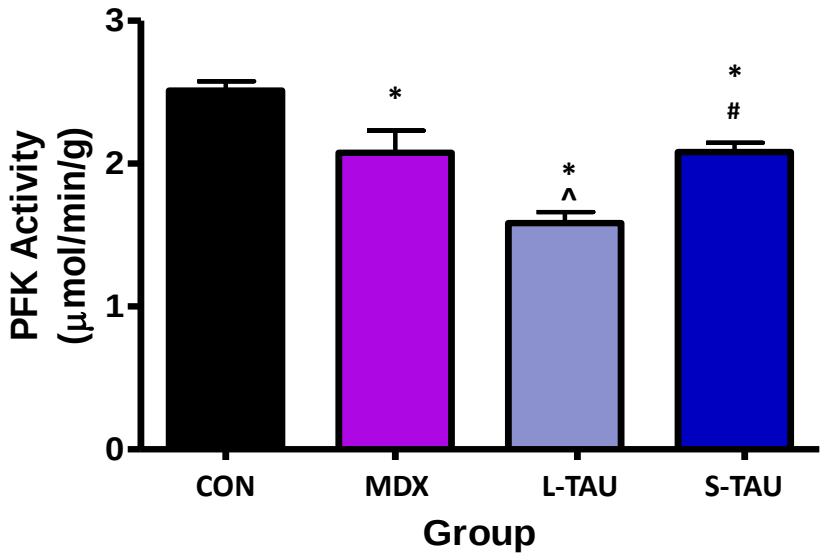


Figure 5.5 EDL PFK activity in CON, MDX and taurine supplemented groups

***** significant difference relative to CON group. ^ significant difference relative to MDX group.
# significant difference relative to L-TAU group. (n=7-10)

ii) CK

No significant difference in CK activity was observed in association with the supplementation protocols or between CON and *mdx* mice (Figure 5.6).

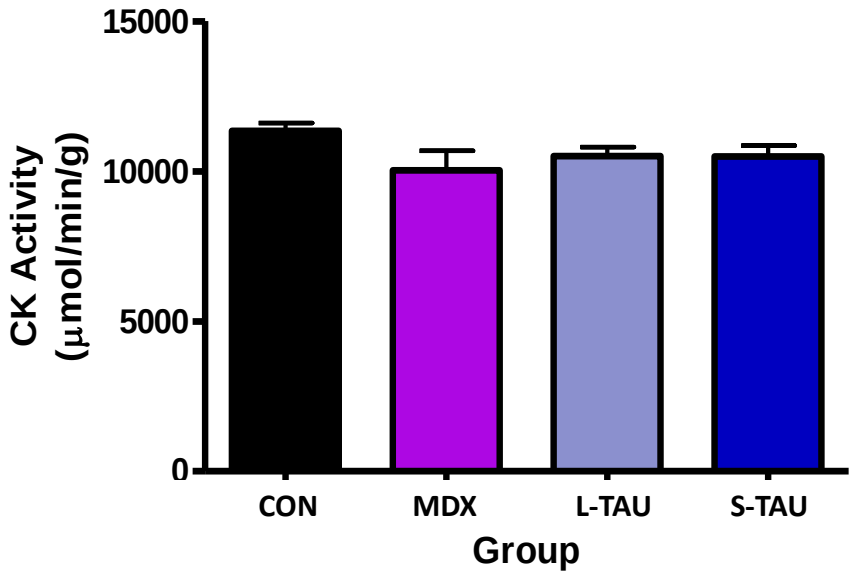


Figure 5.6 EDL CK activity in CON, MDX and taurine supplemented groups
(n=7-10)

iii) *β*-HAD

The activity of *β*-HAD was more than doubled (52% higher) in MDX mice relative to CON values. L-TAU treatment blunted this increase, such that no significant difference between CON and L-TAU mice was observed, but a significant decrease was noted ($p < 0.05$; Figure 5.7) compared to MDX. There was no significant difference between the S-TAU and either the MDX or CON groups.

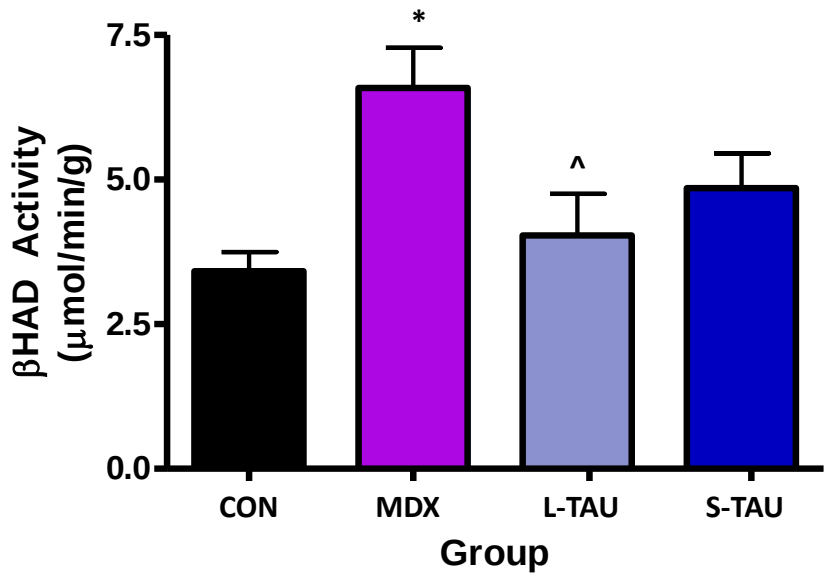


Figure 5.7 EDL β-HAD activity in CON, MDX and taurine supplemented groups

***** significant difference relative to CON group. ^ significant difference relative to MDX group. (n=7-10)

iv) CS

No significant differences in CS activity were observed between CON or any of the *mdx* groups for EDL (see Figure 5.8).

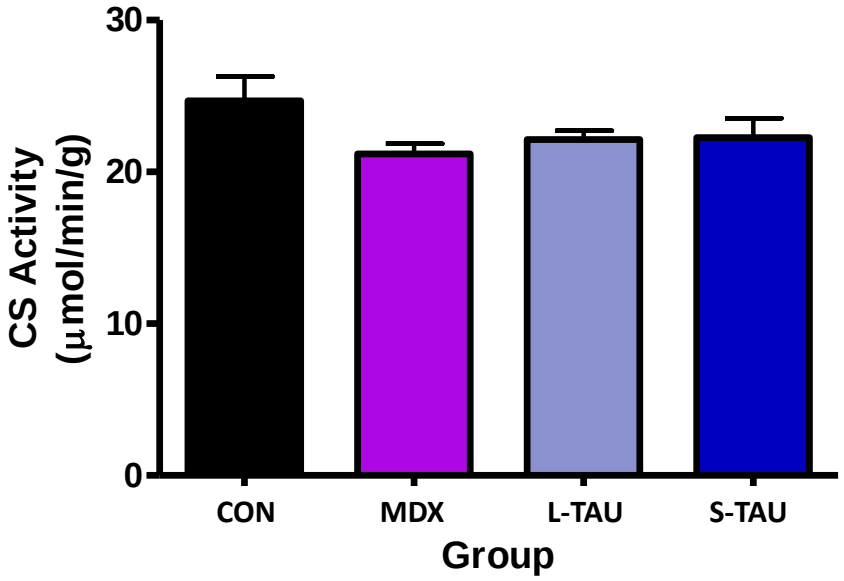


Figure 5.8 EDL CS activity in CON, MDX and taurine supplemented groups (n=7-10)

5.4.6 SOL isometric contractile properties

i) Twitch characteristics, CSA and L_0

As shown in Table 5.4, all *mdx* groups demonstrated significantly less ($p < 0.001$) specific twitch force when compared the CON group, while no significant difference in twitch force was observed in association with either taurine treatment. No significant difference in TTP or ½ RT was observed between any of the groups. As expected due to the larger muscle mass in *mdx* mice, all *mdx* groups demonstrated significantly greater CSA ($p < 0.001$) when compared to the CON mice. A significant increase in L_0 was also observed in the S-TAU group relative to L-TAU only.

Table 5.4 Isometric contractile properties of SOL with taurine treatment

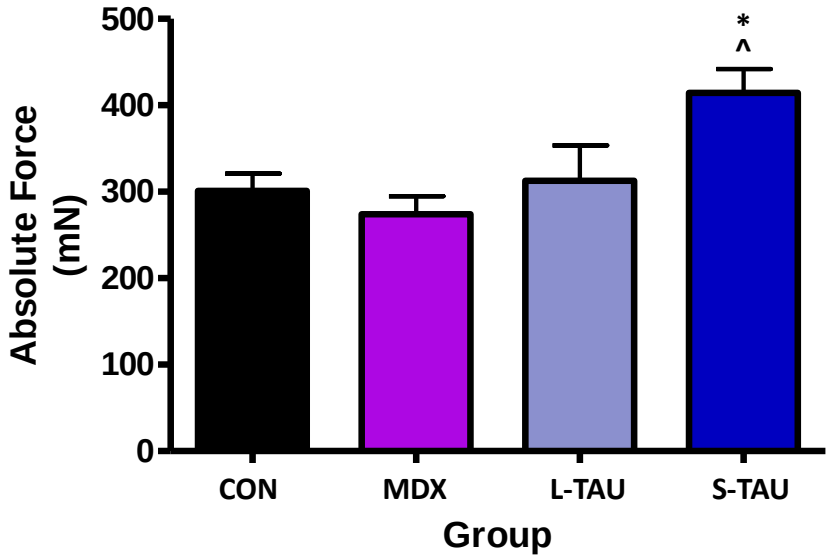
	CON	MDX	L-TAU	S-TAU
Lo (mm)	12.6 ± 0.8	12.5 ± 0.4	12.0 ± 0.3	13.6 ± 0.2 #
CSA (mm^2)	0.8 ± 0.0	1.4 ± 0.1 *	1.3 ± 0.1 *	1.3 ± 0.0 *
Pt (N/cm^2)	13.6 ± 0.5	8.0 ± 0.6 *	8.5 ± 0.9 *	9.4 ± 0.5 *
TTP (msec)	56.1 ± 0.6	55.0 ± 1.3	54.1 ± 1.2	54.7 ± 1.2
½ RT (msec)	66.0 ± 2.0	65.7 ± 2.8	62.7 ± 2.7	67.7 ± 2.3

***** significant difference relative to CON group. # significant difference relative to L-TAU group. (n=7-10)

ii) Peak tetanic force

As shown in Figure 5.9 A, the S-TAU group demonstrated significantly higher absolute force when compared to the CON ($p < 0.05$) and MDX ($p < 0.05$) groups. When normalised to CSA (Figure 5.9 B), all *mdx* groups demonstrated significantly less force than CON mice, however no significant differences in specific force were observed in association with either of the taurine supplementation groups.

A



B

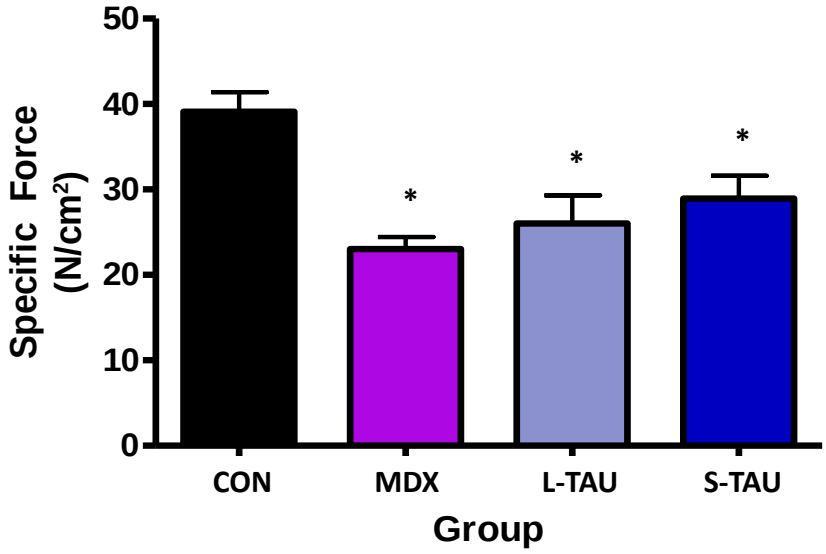


Figure 5.9 Absolute (A) and specific (B) force production for the SOL muscle between CON and MDX mice, as well as in association with taurine treatment

***** significant difference relative to CON group. ^ significant difference relative to MDX group. (n=7-10)

iii) Force-Frequency Relationship

There was no significant difference in the relative force-frequency relationship between CON and *mdx* mice (see Figure 5.10), or between any of the treatment groups at any of the stimulation frequencies

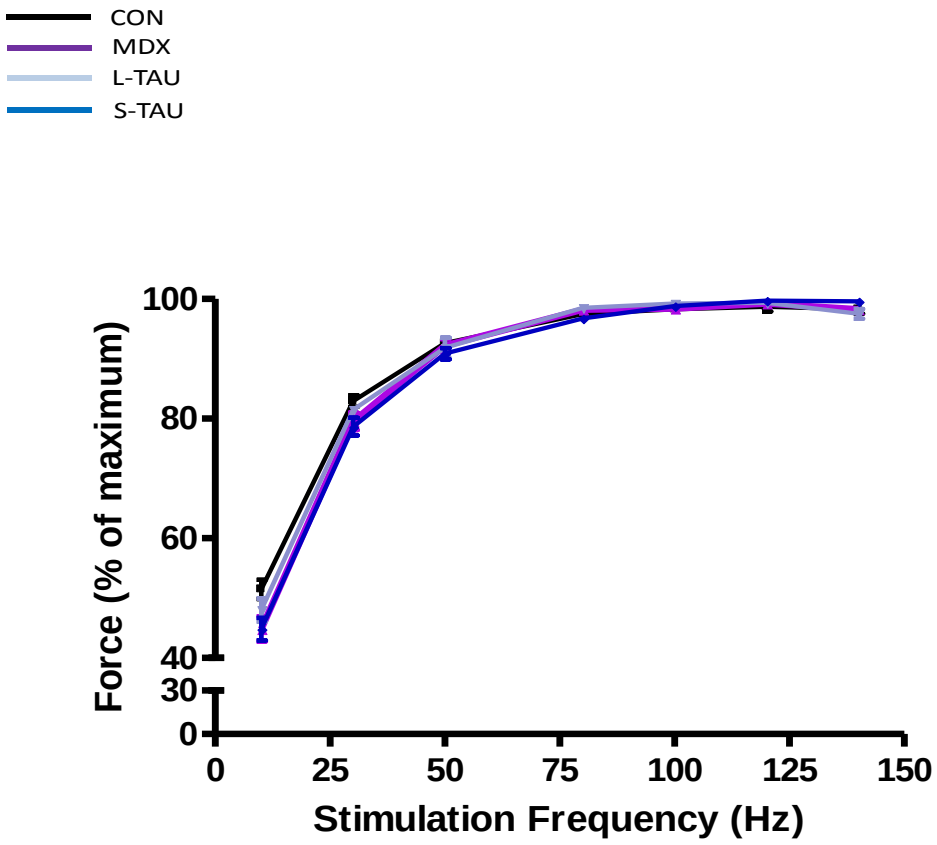


Figure 5.10 Relationship between stimulation frequency and relative force production for the SOL between CON and MDX mice, as well as in association with taurine treatment. (n-7-10)

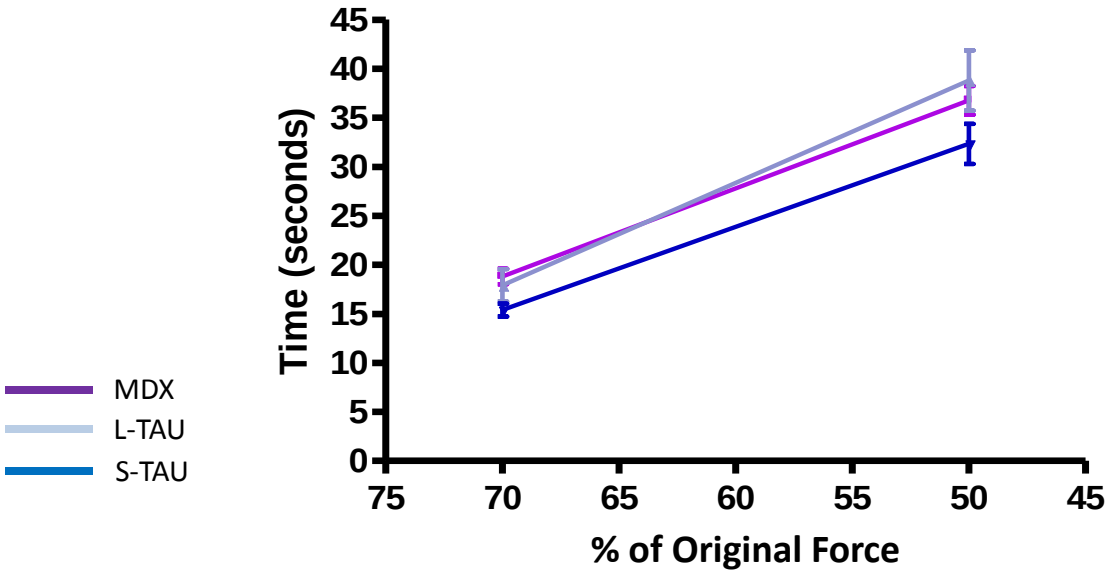
iv) Fatigue

At the continuous stimulation frequency of 100 Hz for 1 minute for SOL, CON muscles were significantly more resistant to fatigue ($p < 0.001$) than *mdx* SOL at both fatigue points examined. In fact, many of the CON muscles did not reach 50% of fatigue by the end of the stimulation, and thus accurate comparisons between CON and *mdx* groups cannot be made. As such, the CON group has been excluded from Figure 5.11 A, to allow comparison only between the *mdx* groups tested. There was no significant difference between the *mdx* groups in the time taken to reach 70% or 50% of original force during the fatigue bout.

v) Percentage of original force post-fatigue

As shown in Figure 5.11 B, all of the *mdx* groups demonstrated significantly less force MDX ($p < 0.01$), L-TAU ($p < 0.001$), S-TAU ($p < 0.001$)) at the end of the fatiguing stimulation when compared to CON mice. There was no significant difference in the percentage of force remaining at the end of fatigue between the *mdx* groups.

A



B

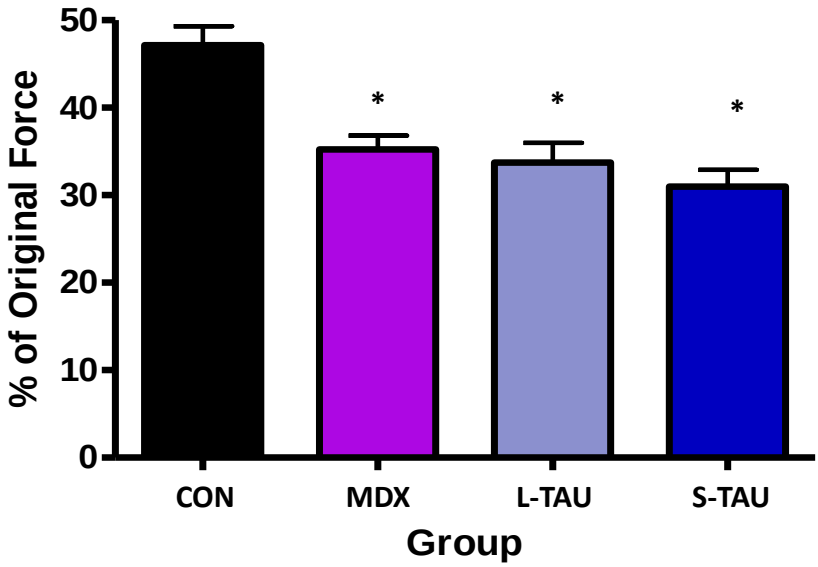


Figure 5.11 Time taken for SOL force to fatigue to 70, 50 and 30% of original force (A) and the percentage of original force attained at the end of the fatiguing stimulation (B)

***** significant difference relative to CON group. (n=7-10)

5.4.7 Enzyme activity for SOL

i) PFK

As shown in Figure 5.12, there was no significant difference in PFK activity observed between CON and MDX mice, or in association with taurine supplementation.

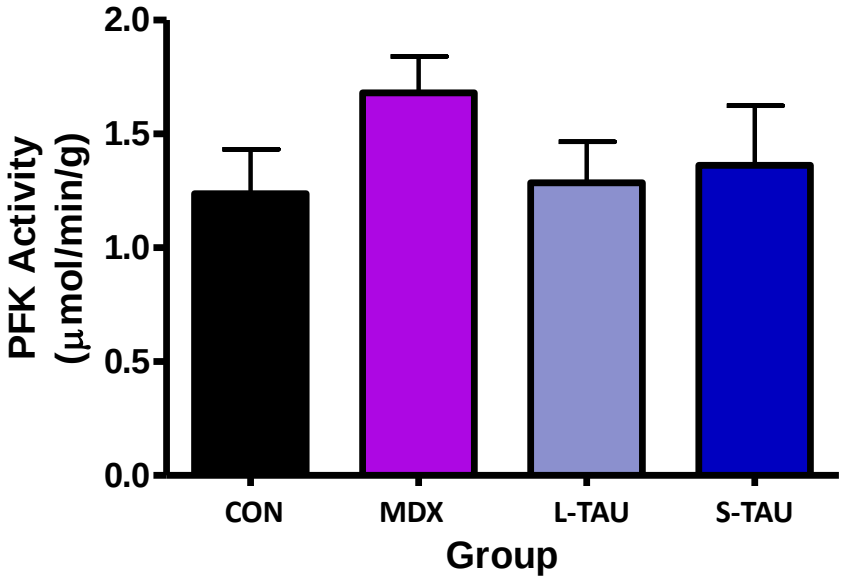


Figure 5.12 SOL PFK activity in CON, MDX and taurine supplemented groups (n=7-10)

ii) CK

CK activity was significantly ($p < 0.001$; Figure 5.13) lower in all *mdx* groups compared to CON. No significant differences in CK activity were observed between the MDX and L-TAU group, however, the S-TAU mice demonstrated significantly increased CK activity ($p < 0.001$) relative to MDX.

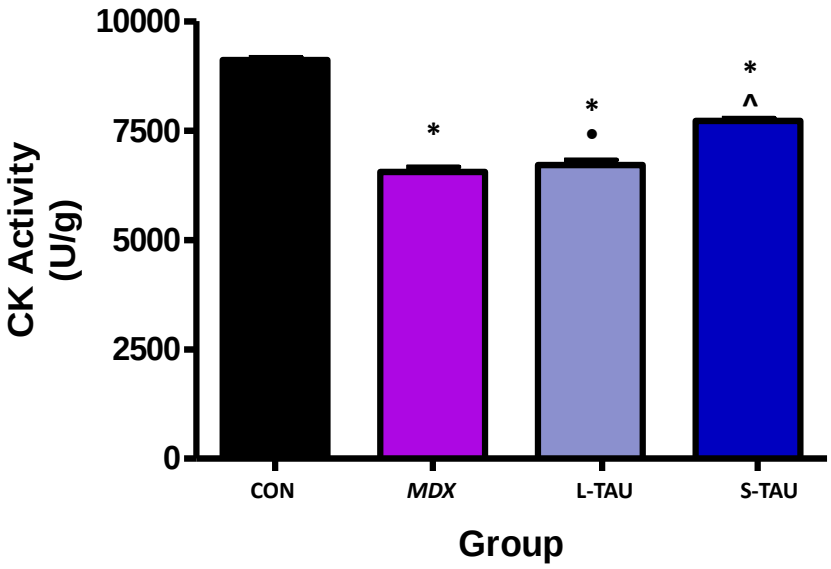


Figure 5.13 SOL CK activity in CON, MDX and taurine supplemented groups

***** significant difference relative to CON group. ^ significant difference relative to MDX group. ● significant difference relative to S-TAU group. (n=7-10)

iii) *β*-HAD

There was no significant difference in *β*-HAD activity observed between any of the experimental groups for SOL (See Figure 5.14).

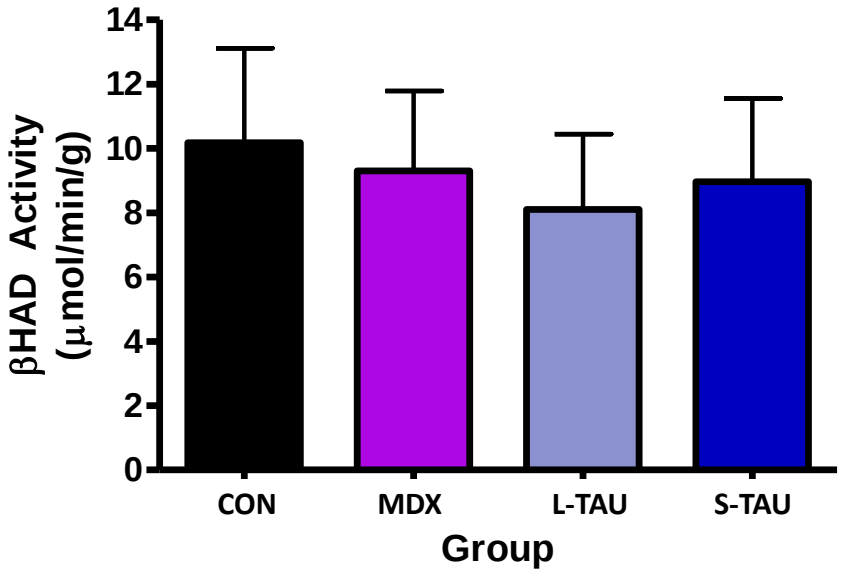


Figure 5.14 SOL βHAD activity in CON, MDX and taurine supplemented groups (n=7-10)

iv) CS

As shown in Figure 5.15, there was a significant difference between the CON and MDX groups, with MDX mice demonstrating lower ($p < 0.05$) CS activity relative to the CON mice. Short-term taurine supplementation prevented some of this decline, with the S-TAU group being significantly higher than MDX.

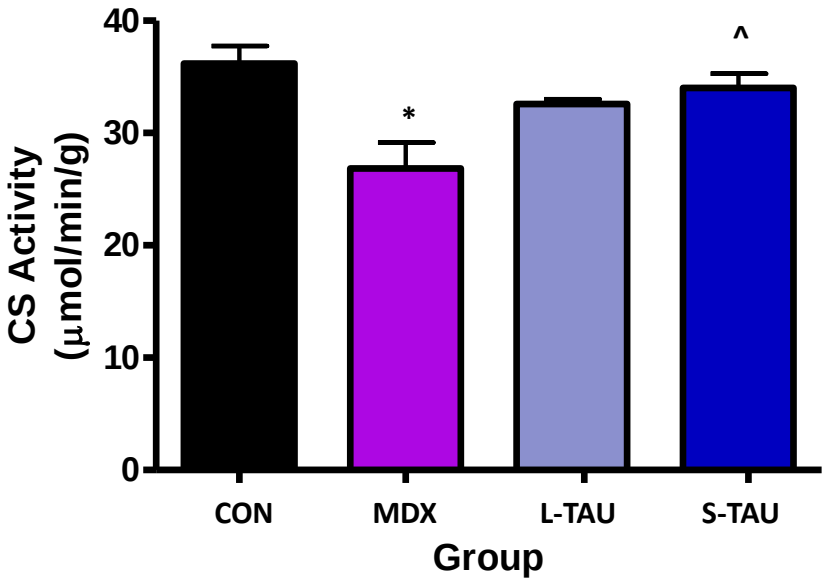


Figure 5.15 SOL CS activity in CON, MDX and taurine supplemented groups

***** significant difference relative to CON group. ^ significant difference relative to MDX group. (n=7-10)

5.5 Discussion

The main aim of this study was to determine if taurine supplementation improved contractile function in *mdx* mouse EDL and SOL, and if short or long-term taurine treatment was equally as effective in improving contractile function. This is the first study to supplement the *mdx* mouse with taurine for over three months duration. Results demonstrated that long-term taurine supplementation was unable to improve peak tetanic and twitch force in the *mdx* EDL muscle, as was also observed with short-term supplementation. However, long-term taurine supplementation did significantly increase the time taken to reach fatigue to 50% of pre-fatigue force, with a tendency towards improved resistance to fatigue at 30%. This suggests that the duration of taurine supplementation could be important, and that supplementation before the onset of dystrophic pathology could result in additional protection from skeletal muscle fatigue, but be ineffective in improving peak tetanic force in dystrophic skeletal muscle. This is also the first study to examine the effect of taurine supplementation on SOL contractile function, finding that as was the case with the fast-twitch EDL, both long- and short-term taurine supplementation were unable to improve the contractile properties of slow-twitch muscle. Interestingly, despite no change in contractile function with long-term taurine treatment, this group did demonstrate significantly decreased plasma CK, indicating reduced plasma membrane permeability, or suggesting reduced muscle damage. However, short-term taurine treatment had no effect on plasma CK .

The secondary aim of this study was to determine if taurine supplementation could alter the activity of some of the key metabolic enzymes within *mdx* skeletal muscle, with results showing that PFK activity is significantly decreased in *mdx* EDL, while long-term taurine supplementation further depresses PFK activity, while short-term taurine supplementation had no effect on this rate limiting enzyme for glycolysis. CK and CS were unchanged, however *β*-HAD activity was more than doubled in the *mdx* EDL relative to CON, suggesting and increased reliance on fat metabolism, possibly due to the reduction in PFK activity decreasing glycolytic flux into the CAC. Interestingly, long-term taurine supplementation appears to attenuate this increase, returning *β*-HAD activity to levels consistent with CON mice. In SOL, CK activity was significantly lower in all *mdx* groups relative to CON.. CS was also decreased in MDX mice, with the increase in CS activity in the SOL of the S-TAU group reaching statistical significance. These

results suggest that taurine, although unable to be directly used for energy production, may be modulating energy production and mitochondrial function through indirect actions.

5.5.1 Effect of taurine supplementation on body and muscle mass

After the acute degenerative phase that occurs in the *mdx* mouse at 3-4 weeks of age, the remaining functional myofibres in the hind limb muscle hypertrophy, which then contract synergistically with damaged skeletal muscle fibres in order to compensate for the impaired muscle function (Consolino and Brooks, 2004, Zammit and Partridge, 2002). As such, *mdx* mice are frequently reported to have increased body and muscle mass relative to non-dystrophic C57BL/10 mice of the same age (Dellorusso et al., 2001a, Hayes et al., 1993, Lynch et al., 2001b). In the present study, all of the *mdx* mouse groups demonstrated significantly increased body and muscle mass relative to the CON mice (see Table 5.1 and 5.2), as is typical for 6 month-old animals. Long-term taurine treatment was not associated with any change to body or muscle mass relative to the MDX group which was expected, as results from Chapter 4 demonstrated that although taurine treatment early in the *mdx* lifespan appears to slow growth from day 28 to day 70, at day 70 there was no significant difference in muscle mass between the MDX and TAU group. However, short-term taurine supplementation significantly increased body and muscle mass relative to both the MDX and L-TAU groups. While there are examples in the literature showing increased body and muscle mass with taurine supplementation (De Luca et al., 2003, Goodman and Shihabi, 1990, Hultman et al., 2007), in the case of the present study, the increase in body mass may not be attributable to the taurine supplementation protocol.

The S-TAU group were not bred within the Melbourne University animal house, as was the case for the CON, MDX and L-MDX groups, but rather were ordered in from ARC at ~4 months of age, allowed to adjust to the new housing conditions and then placed on taurine water. The conditions under which mice are housed (such as cage design, noise, interaction with housing staff and contact with microorganisms) can significantly impact the phenotype of laboratory mice (Gartner, 1990, Grounds et al., 2008b), leading to increased biological variation. Since the completion of this study, it has been noted from other experiments conducted within our laboratory that animals ordered from ARC are

larger than those that have been bred at the Melbourne University animal housing facility, despite no difference in age. This is likely due to differences in the housing conditions between the two facilities, as the original stock used for breeding in the CON, MDX and L-MDX groups were all obtained from ARC originally. Unfortunately, no measures of body mass were made prior to placing the S-TAU animals on taurine water, so an effect of short-term taurine supplementation on body mass cannot be completely excluded. In the two previous studies that have supplemented the *mdx* mouse, DeLuca et al. (2003) found that 4 weeks of taurine supplementation beginning at 3-4 weeks of age in exercised *mdx* mice resulted in doubling of the body mass gained relative to untreated *mdx*, while Cozzoli et al. (2011) showed no significant increase in body weight in association with 4 weeks of taurine supplementation in exercised *mdx* mice. Since the exercise performed in these two studies would have increased muscle turnover, while the current study commenced supplementation at five months of age, when there is little turnover in *mdx* dystrophic hind limb muscles, this highlights that the effect of taurine supplementation on body and muscle mass of *mdx* mice appears to be variable. It is likely influenced by factors such as the age of the mouse, exercise, dose of taurine supplementation and the method of administration.

5.5.2 Effect of taurine supplementation on plasma CK concentration

In the present study, long-term taurine supplementation significantly decreased plasma CK in the *mdx* mouse, such that no significant difference between CON and L-TAU mice was observed. The CK enzyme is typically found in high concentrations within cardiac and skeletal muscle, and is commonly used as a marker of skeletal muscle injury as damaged myofibres leak CK into plasma (Cozzoli et al., 2011b, Dawson et al., 2002, Emery, 1995, Shiny et al., 2005). Indeed, one of the earliest pathological findings in DMD patients, and the dystrophic *mdx* mouse, was elevated plasma CK, indicating considerable muscle damage, which is evident even before the onset of significant functional impairment (Emery, 1995). The decrease in plasma CK seen in the group of long-term taurine supplemented *mdx* mice suggests that although not manifesting in increased peak force production, there was an improved resistance to fatigue, perhaps due to less susceptibility to damage.

Interestingly, short-term taurine supplementation had no effect on plasma CK , which is surprising, as taurine has been proposed to protect skeletal muscles from damage (Dawson et al., 2002, Huxtable, 1992, Uozumi et al., 2006b). Within the literature the few studies that have examined the effect on taurine supplementation on plasma CK have shown no effect on plasma CK concentration with short-term taurine treatment (Cozzoli et al., 2011b, Dawson et al., 2002)although one study using an isoprenaline-induced model of cardiomyopathy has reported reductions in plasma CK with taurine supplementation (Shiny et al., 2005).

The results from the present study in addition to that obtained by Cozzoli et al (2011), suggests that short-term taurine treatment in the *mdx* mouse may be ineffective in protecting against sarcolemmal weakness and dystrophic degeneration. Indeed, Cozzoli et al. (2011) found no significant beneficial effects on skeletal muscle morphology with combined therapy of PDN + Taurine, above that which was observed with PDN treatment alone. Unfortunately, this study did not examine the effect of taurine treatment in isolation on muscle morphology of dystrophic muscle (Cozzoli et al., 2011b), and thus, detailed studies examining the effect of taurine treatment on muscle histology are needed to assess the therapeutic implications of taurine treatment on muscle damage. It should also be noted that despite no apparent reduction in muscle damage with short-term taurine treatment, this duration of taurine supplementation has been shown to have several beneficial effects on measures of muscle function such as grip strength, Ca^{2+} handling and E-C coupling in the *mdx* mouse (Cozzoli et al., 2011b, De Luca et al., 2003), demonstrating that while taurine is able to improve dystrophic muscle function, although it is unlikely that this is through membrane protection (Cozzoli et al., 2011b).

5.5.3 Effect of taurine supplementation on isometric contractile properties of EDL

Taurine is essential for normal skeletal muscle function and has previously been shown to modulate E-C coupling, improve both tetanic and twitch force output as well as increase resistance to fatigue (Bakker and Berg, 2002, Conte-Camerino et al., 1987, Dawson et al., 2002, Goodman et al., 2009, Pierno et al., 1996, Schaffer et al., 2010). In the current study, both long and short-term taurine supplementation was ineffective in increasing peak tetanic and twitch force in the *mdx* EDL. This was unexpected, as both DeLuca et al. (2003) and Cozzoli et al. (2011) have previously shown that ~4 weeks of taurine supplementation significantly improves grip strength in the *mdx* mouse, while other studies comparing normal skeletal muscle taurine content to taurine depleted skeletal muscle in non-dystrophic rodents have also shown improvements in muscle force output with higher taurine content (Bakker and Berg, 2002).

The variable results obtained with short-term taurine supplementation in the present study, relative to these two previous investigations examining taurine supplementation in *mdx* mice (Cozzoli et al., 2011b, De Luca et al., 2003), may be due differences in the experimental protocol used to assess muscle force, and also the age of the mice at the time of experimentation. In order to enhance the dystrophic phenotype of experimental animals, both DeLuca et al. (2003) and Cozzoli et al. (2011) employed a chronic exercise protocol on a treadmill during the supplementation period prior to muscle testing. As the *mdx* mice in the current study were six months of age, where chronic but only low degree of skeletal muscle damage persist, it is possible that the beneficial effects of taurine treatment are not evident due to only minor functional deficits that are observed in unexercised *mdx* mice of this age. In addition, it is possible that the positive effects of short-term taurine supplementation on muscle strength in the *mdx* mouse are best evaluated using *in vivo* methods (such as grip strength), or at the very least using stimulation frequencies and temperatures that are more physiological (Hamilton et al., 2006, Wineinger et al., 1998). This latter aspect is explored further in the experiments described in the next chapter. It should also be noted that as taurine is also a key amino acid within the nervous system, which also lacks dystrophin, discrepancy between our *in vitro* and others *in vivo* assessment of muscle strength could also be due taurine supplementation atering motor neuron function within the brain.

In addition to the findings on tetanic force, long-term taurine supplementation was also shown to significantly increase the time taken to reach 50% of original force during a high-frequency continuous fatiguing stimulation of the EDL, while no effect on fatigue was observed with short-term taurine treatment. As this is the first study to examine the effect of taurine supplementation on measures of fatigue in the *mdx* mouse, no comparison with current literature can be made with dystrophic tissue specifically, although several studies conducted in other animal models have shown that taurine treatment is able to significantly decrease muscle fatigue (Dawson et al., 2002, Yatabe et al., 2009c).

For example, taurine supplementation in rats has been shown to significantly increase the running time to exhaustion in treadmill experiments (Dawson et al., 2002, Yatabe et al., 2009c). Moreover, it appears that the typical exercise-induced loss of taurine from fast-twitch skeletal muscle can be attenuated with taurine treatment, which may be a factor in taurine's ability to improve performance. Furthermore, Goodman et al. (2009), using a comparable fatigue protocol to the current study in taurine-supplemented rats, found that taurine treatment significantly increased the relative force maintained during a high-frequency continuous stimulation in fast-twitch muscle. Interestingly, all of the studies examining the effect of taurine supplementation on muscle fatigue have only used short-term supplementation protocols (Dawson et al., 2002, Goodman et al., 2009, Yatabe et al., 2009c), yet in the *mdx* mouse only long-term supplementation was able to significantly decrease muscle fatigue.

This suggests that the timing and duration of taurine supplementation may be important in dystrophy, as has been shown for other therapeutic treatments (Bushby et al., 2004, Manzur et al., 2004). Indeed, initiation of corticosteroid treatment before physical performance declines and well before the onset of wheelchair use in dystrophic patients is current best practice for DMD therapy (Bushby et al., 2004, Manzur et al., 2004). Moreover, to date there is no clinical evidence that beginning steroid use after the loss of ambulation in steroid naive patients has any beneficial effects on patient outcome (Bushby et al., 2004). Similarly, it has been suggested that the discrepancies observed in the effect of antioxidant therapy between animal and human clinical trials may be due to the timing of treatment, as human trials have utilised patients already experiencing

skeletal muscle degeneration and impaired function, and thus may limit the beneficial effects observed with this treatment (Selsby, 2011). Results from the current study suggest that supplementation early in the lifespan, before the onset of the acute degenerative bout in the *mdx* mouse, may be essential for rescuing muscle function in dystrophic skeletal muscle and maximising the therapeutic potential of taurine. It is possible that short-term taurine supplementation beginning at 3 weeks of age in *mdx* mice would have demonstrated significant effects. Indeed, exacerbating dystrophic pathology prior to supplementation resulted in significant improvements in skeletal muscle function, despite only a short-term supplementation protocol being utilised (Cozzoli et al., 2011b, De Luca et al., 2003). Thus the ability of taurine to improve muscle function may be largely dependent on the phenotype of the muscle being investigated, as well as being influenced by the type of muscle being investigated, as the slow-twitch SOL exhibited different responses to taurine.

5.5.4 Effect of taurine supplementation on isometric contractile properties of SOL

Both the long and short-term taurine supplementation protocols in this study were unable to improve contractile function of the slow-twitch SOL muscle in the *mdx* mouse. This is despite all groups of *mdx* mice demonstrating significantly reduced specific force and an increased susceptibility to fatigue. As taurine content is significantly higher in slow-twitch relative to fast-twitch skeletal muscle (Iwata et al., 1986), it is possible that stores of taurine were unable to be significantly increased by supplementation, and thus have limited effects on contractile measures. Dawson et al. (2002) found no significant increase in the taurine content of rat SOL following four weeks of supplementation with 3% taurine in drinking water, while Cozzoli et al. (2011) found a small, but non-significant increase in taurine content of the *mdx* SOL after treatment with taurine. As the taurine content of SOL following supplementation was not evaluated in this study, the ability for taurine stores to be increased through either long- or short-term taurine supplementation in the *mdx* mouse cannot be elucidated from the present study. However, as changes to enzymatic activity were observed in both the L-TAU and S-TAU groups within SOL, it could be assumed that some alterations to muscle taurine content have occurred. However, it is possible that these alterations were not of a great enough magnitude to elicit improvements in contractile function

5.5.5 Effect of taurine supplementation on enzyme activity

Energy metabolism in dystrophic skeletal muscle has been shown to be impaired, causing a compromised cellular energy status that could significantly limit skeletal muscle's ability not only for contraction, but also regeneration and repair (Jongpiputvanich et al., 2005, Tseng et al., 2002). Glycolytic metabolism in particular has been shown to be affected, possibly due to lowered nNOS that results from dislocation of the DAP complex in DMD (Zhou et al., 2006). NO has been shown to regulate the rate-limiting glycolytic enzyme PFK, while nNOS itself can directly bind to PFK and thus modulate its activity (Wehling-Henricks et al., 2009a). Markedly depressed PFK activity have previously been observed within dystrophic skeletal muscle, possibly due to inhibition of PFK activity by increased intracellular Ca^{2+} (Lilling and Beitner, 1991, Wehling-Henricks et al., 2009a). Consistent with this literature, the present study showed a significant decrease in the activity of PFK in the EDL muscle of all the *mdx* groups. Interestingly, long-term taurine supplementation caused a further decrease in PFK activity in EDL of *mdx* mice, suggesting the capacity for glycolytic metabolism is further impaired with long-term taurine treatment, although short-term taurine supplementation had no significant effect on PFK activity. Despite these changes in EDL, no significant differences between CON and MDX mice were observed for PFK activity in SOL, nor in association with either of the taurine treatment protocols. This is most likely due to the fibre type differences, where EDL is more reliant on glycolytic metabolism than the SOL, which has a greater mitochondrial density and therefore enhanced capacity for oxidative metabolism. Moreover, it is not unusual for differences in metabolism to be reported between fibre types in dystrophy (Chi et al., 1987).

To date, there is only one example available in the literature examining glycolytic metabolism in association with taurine supplementation in skeletal muscle specifically. Takekura et al. (1986) investigated glycolytic and oxidative enzyme activity in EDL and SOL muscles of rats after sprint and endurance running activity, finding that taurine supplementation significantly increased PFK activity after both activities in the EDL, but not the SOL. This conflicts with the findings of the present study with regard to EDL only, where taurine supplementation further decreased PFK activity in dystrophic skeletal muscle. This result is interesting, as taurine has been proposed to have insulin-like effects, increasing glucose uptake into tissues and thus improving substrate supply for

glycolytic metabolism (Chang et al., 2004, Haber et al., 2003, Parvez et al., 2008). It may be that exercise provided a stimulus for taurine to exert these effects which would not have been present in the current study. Indeed, studies of glycolytic metabolism in association with taurine supplementation in tissues other than skeletal muscle have shown similar results to the present study. For example, Obrosova and Stevens (1999) found that taurine supplementation decreased G-6-P in the lens of the eye in diabetic rats. Reduced lactate were also observed in this study, although no alterations to other important glycolytic metabolytes such as F-6-P were found (Obrosova and Stevens, 1999). Moreover, a study by Kim and Kim (2009) showed that taurine treatment prevented the typical increase in glucose uptake and upregulation in GLUT1 expression that occurs in activated macrophages. Thus it appears that the effect of taurine on glucose handling and glycolytic metabolism is regulated differently depending on the tissue being examined, and as only one investigation of glycolytic metabolism in skeletal muscle is currently available further investigations into taurine supplementation and glycolytic metabolism in both healthy and dystrophic skeletal muscle are necessary. Furthermore, it should be noted that the study by Takekura et al. (1986) showed no significant difference in PFK enzyme activity between EDL and SOL which is unexpected, as it is generally well accepted that PFK activity is higher in fast-twitch skeletal muscle due to its preferential reliance on glycolytic metabolism (Chi et al., 1987b, Takekura and Yoshioka, 1987, Zonderland et al., 1999) suggesting some methodological concerns. Indeed, PFK on CON EDL were approximately double those in CON SOL in the current study, consistent with this expectation. The reduction in PFK activity in *mdx* EDL with taurine supplementation could also be due to differences associated with muscle fibre type, as a transition to a slower phenotype (remembering that fast-twitch fibres are preferentially affected in dystrophic muscles) would decrease PFK activity in the EDL. However, fibre-type differences were not examined in the present study and thus cannot be confirmed.

As expected, all groups of *mdx* mice demonstrated significantly decreased muscle CK activity in SOL relative to CON mice. In skeletal muscle, CK plays a central role in skeletal muscle metabolism, acting as a fast energy buffer and shuttle between the sites of energy production (mitochondria) and energy consumption (cross bridges and ion pumps) (Bruton et al., 2003). Creatine metabolism has been shown to be impaired in DMD patients, which have a higher urinary excretion of creatinine coupled with

significant reductions in total creatine within muscle tissue (Fitch and Moody, 1969, Sharma et al., 2003). Interestingly, long-term taurine supplementation had no effect on the activity of CK in *mdx* muscle, while short-term taurine supplementation increased CK activity within the SOL. It is interesting that this increase in CK activity did not result in improved contractile function, as PCr is a key energy source for SERCA and the contractile filaments and thus impacts muscle function (Volek et al., 1999). Given that there is cytosolic CK as well as mitochondrial CK, further work would be required to clarify any functional effects of this increase.

While no significant difference is CS activity was observed in the fast-twitch EDL, slow-twitch dystrophic muscle demonstrated significantly reduced CS activity, which appears to be partially rescued with taurine supplementation. CS is the enzyme which catalyses the reaction that ultimately converts pyruvate into citrate, which then enters the CAC to be oxidised, producing energy via the electron transport chain (Davidson et al., 2006). As this reaction is a rate-limiting step in this process, this change in CS activity indicates that *mdx* mice have a lower capacity for oxidative metabolism, and that taurine supplementation (especially in the short-term) improves this capacity, bringing oxidative metabolism closer to CON values. Indeed, it was recently shown that a lack of taurine can result in impaired translation of mitochondrial encoded proteins (MEP), which are involved in the assembly of respiratory chain complexes and thus reduce ATP production (Ricci et al., 2008, Schaffer et al., 2009). Therefore, it is possible that the elevation in skeletal muscle taurine content in the present study may have increased electron transport flux, and increased the rate of ATP production. Furthermore, taurine may be having positive effects on oxidative metabolism by protecting mitochondria from damage (CS is also used as a measure of mitochondrial number), as taurine has been shown protect against mitochondrial-mediated cell death, and preserve mitochondrial function when tissues are exposed to stress (Das et al., 2010, Sun et al., 2011).

The activity of *β*-HAD in EDL was nearly doubled in MDX relative to CON mice, suggesting that the capacity for fatty acid oxidation is significantly increased in six month old *mdx* mice. This may be due to increased availability of fatty acids, as serum lipids have been shown to be elevated in dystrophy (Srivastava et al., 2010). Moreover, increased lipid oxidation in the *mdx* mouse has also been reported following feeding (Mokhtarian et al., 1996), which may be a necessary adaption due to the reductions in

glucose metabolism that are frequently reported in the literature (Lilling and Beitner, 1991, Wehling-Henricks et al., 2009a), and which were also shown in the present study. Interestingly, long-term taurine supplementation significantly attenuated this increase in *β*-HAD activity, such that no difference was observed between CON and L-TAU mice. Short-term taurine supplementation also appears to attenuate this increase, as 29% less *β*-HAD activity was observed in S-TAU relative to MDX, although this did not reach statistical significance. This result was unexpected, as taurine supplementation has previously been shown to significantly increase fat oxidation during a submaximal cycling bout in trained athletes (Rutherford et al., 2010). In addition, if the increase in fatty acid metabolism is a compensatory effected for the reductions in glucose metabolism, the results from this study showing significantly decreased PFK activity with long-term taurine treatment would suggest that taurine potentially impairs metabolism in dystrophic skeletal muscle. However, as the enzyme assays used in this study are examining maximal activity of the enzymes, under optimal conditions, exactly what effect, if any, these changes would have on metabolism *in vivo* requires further investigation.

5.6 Conclusions

In summary, the present study is the first to demonstrate that while both long and short-term taurine supplementation are unable to improve peak tetanic and twitch force in EDL or SOL, long-term taurine supplementation does enhance fatigue resistance in the *mdx* EDL. This may be due to the ability of long-term taurine supplementation to attenuate skeletal muscle damage, as indicated by the decrease in plasma CK, to a level that is comparable with non-dystrophic control mice. The results from the present study conflict with results from the two previous investigations of taurine supplementation on muscle strength in the *mdx* mouse, which have shown an increase in grip strength following taurine treatment (Cozzoli et al., 2011b, De Luca et al., 2003). It is likely that this is due to differences in the degreeof muscle damage prior to supplementation, but could also be due to the non-physiological stimulation frequencies and muscle bath temperature used in the present study. The use of high stimulation frequencies could be considered to be unrepresentative of normal exercise-induced muscle fatigue and thus is a limitation to the present study, indicating that additional examination on the effect of taurine supplementation on contractile function under more physiological conditions in warranted. This is also the first to examine the effect of taurine supplementation on metabolic enzyme function in dystrophic muscle, demonstrating that taurine may be involved in modulating substrate selection and enzyme activity in the *mdx* mouse in both slow- and fast-twitch skeletal muscle. Specifically, PFK and β-HAD metabolism are altered in the EDL, while CK and CS activity is improved in the SOL.

CHAPTER SIX

THE EFFECT OF TAURINE AND *β*-ALANINE SUPPLEMENTATION ON CONTRACTILE PROPERTIES, TAURINE TRANSPORTER AND E-C COUPLING PROTEIN EXPRESSION IN C57BL/10 AND *MDX* MICE

6.1 Introduction

Taurine is essential for normal skeletal muscle function and performance (Dawson et al., 2002, Hamilton et al., 2006, Warskulat et al., 2007). The most convincing evidence for this comes from investigations into the TauT knock-out mouse, which displays reduced muscular taurine content of up to 96% (Ito et al., 2010, Warskulat et al., 2007) coupled with significant functional deficits, including an 80% decrease in physical endurance (Warskulat et al., 2007), loss of muscle mass, development of necrosis as well as destabilisation of myofilaments and other cytosolic organelles (Ito et al., 2010). Depletion of taurine though the use of competitive transport inhibitors, such as GES or *β*-alanine, depletes skeletal muscle taurine content to 30-50% of control values (Dawson et al., 2002, Hamilton et al., 2006), which, although significantly less depletion than is achieved with TauT knockout mice, still results in decreased force production (Hamilton et al., 2006), altered membrane excitability, reduced chloride conductance (DeLuca et al., 1996), reduced exercise tolerance, increased ROS production (Dawson et al., 2002) and impaired Ca^{2+}ATPase pump activity (in cardiac tissue).

To date, however, there are only a limited number of investigations using either of these compounds to deplete taurine in skeletal muscle (Dawson et al., 2002, DeLuca et al., 1996, Hamilton et al., 2006). Conversely, increasing taurine content has been shown to significantly improve skeletal muscle function (Yatabe et al., 2009a). For example, taurine supplementation has been shown to improve running endurance in rats (Dawson

et al., 2002, Yatabe et al., 2003) and that a loss of taurine from skeletal muscle during exercise may be a limiting factor in performance (Yatabe et al., 2009a). At the single muscle fibre level, taurine has been shown to increase force production by increasing SR Ca^{2+} release (Bakker and Berg, 2002).

Interestingly, the *mdx* mouse exhibits many of the pathological features that are associated with taurine depletion, and have been shown to have reduced skeletal muscle taurine content (De Luca et al., 2003, McIntosh et al., 1998a). Given the importance of taurine in skeletal muscle function, it is possible that dysregualtion of taurine accumulation and storage may contribute to some of the pathology observed in *mdx* mice. Moreover, maintaining a high concentration of taurine in skeletal muscle may protect against some of the functional impairments in DMD, while depletion of taurine content may worsen the dystrophic pathology in skeletal muscle. Only two examples are available in the literature that have examined the effect of taurine supplementation on *mdx* muscle function, with both investigations demonstrating improved grip force, Cl^- conductance and stabilised MT (Cozzoli et al., 2011b, De Luca et al., 2003). Moreover, the study by Cozzoli et al. (2011) found that the application of corticosteroid + taurine therapy resulted in further improvements to MT, than that which was observed with glucocorticoid treatment in isolation. Furthermore, taurine treatment was also able to counteract the over-activity of the subset of Ca^{2+} channels that have previously been found to contribute to the altered Ca^{2+} homeostasis observed in dystrophic myofibres (Cozzoli et al., 2011b).

The findings from these two studies contrast with the findings in Chapter 5 of this thesis, where no significant improvements in muscle force were observed with long or short-term taurine treatment. This is possibly due to differences in the protocol used, as both Cozzoli et al. (2011) and DeLuca et al. (2003) used grip strength to measure force, which is a functional *in vivo* measure versus the *in vitro* measure used in Chapter 5. Moreover, as the contractile protocol in Chapter 5 was not particularly physiological (high-frequency stimulations with the muscle bath at 24 °C) it is possible that no significant difference in function was being observed because of the contractile protocol selected, and thus the present study utilises more physiological stimulation frequencies and a muscle bath heated to 30 °C, which is the approximate temperature that the muscles examined would experience *in vivo* (Hamilton et al., 2006). The dose of taurine

supplementation was also increased by 50% in the present study, as 3% w/v appeared to be well tolerated by *mdx* mice, despite this dose being shown to cause experimental complications in rats (Dawson et al., 2002).

While two studies have examined the effect of taurine supplementation on the *mdx* mouse muscle, no study has investigated the effect of taurine depletion on dystrophic skeletal muscle function. As the *mdx* mouse demonstrates increased muscle taurine content after six weeks of age, which actually exceeds the taurine content found in control muscle, it may be difficult to assess the importance of taurine in *mdx* mice after six weeks of age and potentially even earlier in the lifespan as taurine concentration is steadily increasing. Despite the promising results from studies that have supplemented the *mdx* mouse with taurine, and the suggestion that taurine depletion adversely affects skeletal muscle function, the extent to which taurine content of skeletal muscle impacts on dystrophic skeletal muscle function is still largely unknown. It is possible that manipulating taurine content through supplementation and depletion may help to explain the relative importance of taurine in *mdx* skeletal muscle function. However, to date no study has examined the effect of taurine supplementation and depletion on contractile function in the *mdx*.

Despite reports of decreased intramuscular taurine content and a 140% increase in plasma concentration of the amino acid in *mdx* mice (De Luca et al., 2001b), no study has examined TauT expression in *mdx* mice. Results from Chapter 4 of this thesis showed that TauT expression is significantly decreased however, the expression of TauT in older *mdx* mice was not evaluated. Results from Chapter 4 also showed that TauT expression was not altered with taurine supplementation, however, it is also unknown if depleting skeletal muscle taurine content alters the expression of the TauT in *mdx* tissue. Moreover, while taurine and GES treatment have been shown to affect E-C coupling in non-dystrophic skeletal muscle, and E-C coupling is impaired in *mdx* mice, no study has investigated the role of taurine in E-C protein expression in the *mdx* model. As results from Chapter 4 demonstrated alterations to E-C coupling proteins with taurine treatment, further investigations into the effect of taurine depletion on E-C coupling proteins are indicated.

6.2 Specific aims and hypothesis

The purpose of this study was to determine if 3% w/v taurine supplementation would improve contractile function when measured with a physiological contractile protocol, and if this effect was the same in both non-dystrophic control and *mdx* mice. In addition, to determine if depletion of skeletal muscle taurine, using the competitive transport inhibitor *β*-alanine, would significantly impair contractile function and again, if the degree of this impairment is the same for both control and *mdx* mice. Protein expression of the TauT and several key E-C coupling and Ca^{2+} handling proteins were also examined to determine if taurine or *β*-alanine supplementation alter their expression.

The specific aims of this study were;

i) To determine if 4 weeks of 3% [w/v] taurine or *β*-alanine treatment can significantly increase and decrease taurine content, respectively, in fast and slow-twitch skeletal muscle of 6 month old control and *mdx* mice.

ii) To determine if taurine or *β*-alanine treatment alter contractile function when measured with a physiological contractile protocol, including fatigue and recovery characteristics in the EDL and SOL muscles of control and *mdx* mice.

iii) To determine if 4 weeks of taurine or *β*-alanine treatment alter the expression of the TauT in EDL, and several key E-C coupling and Ca^{2+} handling proteins such as CSQ, RyR, DHPR and SERCA in EDL.

It was hypothesised that taurine supplementation would significantly increase skeletal muscle taurine content and improve contractile function while *β*-alanine supplementation would significantly decrease tissue taurine content and reduce contractile function in both control and *mdx* mice. It was hypothesised that TauT expression would be unaltered with taurine supplementation but would increase with *β*-alanine supplementation, while contractile proteins CSQ and RyR would be altered with taurine and *β*-alanine treatment.

6.3 Methods

6.3.1 Animals

Male *mdx* and C57BL/10 (control) mice (n=60) were obtained from the ARC (WA, Australia) at 4 ½ months of age and housed at the Melbourne University animal housing facility for the duration of the treatment period. Details on the housing conditions of the animals are available in Chapter 3, section 3.1. After a two-week adjustment period to the new housing conditions control (CON) and *mdx (*MDX*)* animals were assigned to one of three groups; 1. Untreated (UNT), 2. taurine (TAU) treated and 3. *β*-alanine (BAL) treated. Treatments were administered via drinking water containing a 3% [w/v] solution of taurine (Sigma, Australia) or *β*-alanine (Sigma, Australia) provided *ad libitum.* This supplementation protocol was chosen as Dawson et al. (2002) has previously shown this to be effective in increasing (taurine) and depleting (*β*-alanine) skeletal muscle taurine content in the rat. It was also chosen as this was a higher dose of taurine supplementation than has been used in previous chapters, which may result in detectable improvements to muscle function. Water intake was monitored during the treatment period to determine if there were any changes to water consumption associated with the supplements. Experiments were conducted on the animals at six months of age (± 7 days).

6.3.2 Dissection and contractile protocol

Mice were anesthetised with an IP injection of 40-50mg/kg of Nembutal (Pentobarbitone Sodium, Rhone Merieux, QLD, Australia) so that they were unresponsive to tactile stimuli. In order to maintain blood supply and nervous innervation until the last possible moment, the EDL muscle was surgically exposed first (taking care not to expose the posterior compartment of the leg) and immediately tested for isometric contractile properties (as described in Chapter 3, section 3.2). During the equilibration time in the muscle bath, the contralateral EDL was dissected out and placed in an identical muscle bath for the duration of the contractile experiments, but was not exposed to stimulation. Muscle baths were maintained at 30 °C for the duration of the experiment as this temperature is within the limits of maintaining optimal isometric force *in vitro*, but approaches the normal *in vivo* temperatures for limb muscle (Hamilton et al., 2006).

While contractile properties of the EDL were being tested, the mouse remained under aesthesia with a saline soaked gauze pad covering the surgery sites to prevent drying of the tissues. Once testing of the EDL was completed both SOL muscles were dissected and placed in the baths as previously described for EDL. Post-removal of SOL the PLANT muscles from both limbs were also removed and snap frozen (for taurine content analysis) before blood was collected via cardiac puncture for plasma CK analysis and the diaphragm also removed.

Stimulation frequencies from 10 to160 Hz at 10 Hz intervals were used for both EDL and SOL to determine the force frequency relationship, with three minutes rest between stimulation bouts, to allow the muscle to adequately recover from fatigue. Muscles were then subjected to a fatigue protocol with stimulation at 70 Hz for 250 ms every 1 s for 1 minute for EDL, and 30 Hz for 500 ms every 2 s for a total of 3 minutes for SOL. This fatigue protocol was chosen, as results from Chapter 5 indicated some conflict between results from the present work with the literature (Cozzoli et al., 2011b, De Luca et al., 2003). It was concluded that this could be due to the degree of damage occurring within the muscle during testing (exercised versus non-exercised *mdx* mice) or due to the use of functional measures such as grip strength providing a better indication of muscle function. Thus, as no access was available to equipment that could effectively exercise the *mdx* mice to worsen the phenotype of skeletal muscle, it was decided to test the effect of short-term supplementation on contractile measures using a more physiological protocol than was previously tested. As such, the contractile protocol utilised in this study was modelled on the protocol previously used by Hamilton et al. (2006), as these frequencies of stimulation correspond to the mean motor unit output frequency that would be experience by these muscles in vivo and was successful in demonstrating differences between taurine-depleted and normal mice.

Post fatigue, both EDL and SOL were followed for a total of 1 hour at various time intervals (1, 2, 5, 10, 20, 30, 45 and 60 min) to determine recovery, as was previously used by Goodman et al. (2009). Recovery was assessed at stimulation frequencies of 100 Hz for EDL and 80 Hz for SOL. Once the contractile protocol was complete muscles were quickly weighed, snap frozen in liquid nitrogen and then stored at -80°C until further analysis.

6.3.3 Measurement of skeletal muscle taurine content

Taurine content was measured in the PLANT and DIA muscles for each of the experimental groups as detailed in Chapter 3, section 3.3. PLANT was utilised as an indicator of fast-twitch hind limb muscle taurine content, as insufficient muscle mass was available in the EDL for all the planned analysis. The DIA muscle was examined at six months of age as the phenotypic profile of this muscle more closely mirrors the human condition. Unfortunately, due to the small muscle mass available in the SOL, and no other available hind limb muscles with a similar muscle fibre profile, taurine content was unable to be measured for SOL or any other slow-twitch muscle in this study. Separating the red and white GAST was considered as an option to attain slow-twitch muscle, however, reliably separating red and white GAST is difficult. In addition to this, as the contractile protocol for SOL needed to be completed, limited time was available to dissect out remaining muscles and thus, the use of red GAST for analysis of slow-twitch muscle was not completed.

6.3.4 Measurement of plasma CK

Plasma concentration of CK were measured for all groups as previously described in Chapter 3, section 3.1.2.

6.3.5 Western blot analysis of dystrophin expression

Western blot analysis in rested EDL muscles was used to confirm the presence or absence of dystrophin in the experimental groups. As no significant differences in contractile function were observed in SOL, only the EDL was used for the following Western blot analysis of TauT, E-C coupling and Ca^{2+} handling protein expression. Details on the procedure are available in Chapter 3, section 3.5.

6.3.6 Western blot analysis of TauT protein expression

Taurine transporter expression were measured via Western blot in CON and MDX mice across all treatment groups in the rested EDL only to determine if there were any changes between the strains, or in association with treatment. Details on the procedure are available in Chapter 3, section 3.5.

6.3.7 Western blot analysis of E-C coupling and Ca^{2+} handling protein expression

DHPR, RyR, SERCA, CSQ and contractile protein expression were analysed using Western blot in CON and *mdx* mice across all treatment groups in the rested EDL only, to determine if there were any changes between the strains or in association with treatments. Details on the procedure are available in Chapter 3, section 3.5.

6.3.8 Statistical analysis

All data are expressed as mean ± error of the mean (SEM) unless otherwise stated. Data obtained were analysed using a two-way analysis of variance (2-way ANOVA), with strain and supplement group as the two between group factors, and any significant differences examined using a Bonferroni Post Hoc Test on GraphPad Prism 4.1 (GraphPad Software). If significant differences were observed with treatments, a t-test was used to compare CON and *mdx* mice for each condition. A probability level of <0.05 was adopted throughout to determine statistical significance.

6.4 Results

6.4.1 Fluid consumption

i) CON

There were no significant differences in fluid consumption for CON mice in association with the treatments during the 1st week. By week 2, CON-UNT consumed significantly more water ($p < 0.01$) than both the CON-BAL and CON-TAU groups, while there was no significant difference in fluid consumption when comparing the BAL and TAU treatments in CON mice. As shown in Table 6.1, by week 3 CON-BAL mice consumed significantly less fluid ($p < 0.01$) when compared to both the CON-UNT and CON-TAU mice. *β*-alanine treated mice continued to drink significantly less ($p < 0.05$) water than the CON-UNT group, while no other significant differences within the CON group were observed at this time point.

ii) *Mdx*

In both week 1 and 2, MDX-BAL mice drank significantly less water ($p < 0.001$; Table 6.1) when compared to both the MDX-UNT and MDX-TAU groups while no difference in fluid consumption was found between the MDX-UNT and MDX-TAU. By week 3, MDX-UNT mice drank significantly more ($p < 0.01$; Table 6.1) when compared to MDX-BAL but significantly less ($p < 0.001$) when compared to MDX-TAU. Taurine treatment also significantly increased fluid intake ($p < 0.001$; Table 6.1) when compared to the MDX-BAL group in week 3. As shown in Table 6.1, by week 4 both the MDX-UNT and MDX-TAU mice consumed significantly more water ($p < 0.001$; Table 6.1) when compared to the MDX-BAL group however; there were no differences between MDX-UNT and MDX-TAU mice.

iii) Comparison of fluid consumption between CON and *mdx* mice

Overall, the effect of *β*-alanine on fluid consumption between CON and *mdx* mice was the same, with *β*-alanine treated mice tending to consume significantly less water than the untreated and taurine treated mice irrespective of strain. It should be noted that at almost all time points (except week 3), *mdx* mice drank significantly ($p < 0.0001$) more water than CON, however, this is likely to be due to the increased body mass of the *mdx* mice relative to CON.

Table 6.1 Fluid consumption with taurine and *β*-alanine treatment

		Fluid Consumed (ml/mouse/week)			
		Week 1	**Week 2**	**Week 3**	**Week 4**
CON	**UNT**	18 ± 1.7	23 ± 4.6	25 ± 3.1	24 ± 1.5
	BAL	16 ± 2.9	13 ± 5.3 *	17 ± 3.2 *	18 ± 3.7 *
	TAU	20 ± 3.0	15 ± 1.3 *	22 ± 2.9 ∞	23 ± 2.0
MDX	**UNT**	33 ± 5.2	30 ± 4.0 ^	26 ± 3.8	30 ± 5.1 ^
	BAL	15 ± 2.9 *	22 ± 3.3 * ^	18 ± 2.9 *	21 ± 3.7 *
	TAU	31 ± 2.9 ∞ ^	32 ± 2.9 ∞ ^	34 ± 2.1 ∞ ^	34 ± 1.8 ∞ ^

* Significantly different when compared to UNT group of the same strain. ∞ Significantly different when compared to the BAL group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment. (n=7-12)

6.4.2 Body mass

There was no significant difference in body mass associated with taurine or *β*-alanine treatment in CON mice. A significant decrease in body weight ($p < 0.05$; Table 6.2) was associated with taurine treatment in MDX mice when compared to MDX-UNT while there was no significant difference between MDX-BAL and the MDX-UNT or MDX-TAU groups. CON body mass was significantly lower than that of *mdx* mice across all groups.

Table 6.2 Body mass with taurine and *β*-alanine treatment

	Body Mass (g)		
	UNT	**BAL**	**TAU**
CON	33.8 ± 0.5	33.6 ± 0.9	33.7 ± 0.8
MDX	38.6 ± 0.4 ^	37.1 ± 0.7 ^	35.7 ± 0.7 * ^

* Significantly different when compared to UNT group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment. (n=7-12)

6.4.3 Muscle mass and relative muscle mass

i) EDL

Treatment with BAL and TAU had no effect on EDL muscle mass, or relative muscle mass in CON mice. A significant decrease in EDL muscle mass and relative muscle mass ($p < 0.001$; Table 6.3) was observed in MDX-TAU when compared to both the MDX-UNT and MDX-BAL groups. No significant difference between MDX-UNT and MDX-BAL groups were observed. EDL muscle mass was significantly higher in MDX compared to CON animals.

Table 6.3 EDL muscle mass and relative muscle mass with taurine and *β*-alanine treatment

		EDL		
		UNT	**BAL**	**TAU**
CON	Muscle Mass (mg)	12.2 ± 0.3	12.6 ± 0.3	12.7 ± 0.2
	Relative Muscle mass (mg/g)	0.37 ± 0.01	0.38 ± 0.01	0.36 ± 0.01
MDX	Muscle Mass (mg)	22.7 ± 0.9 ^	22.3 ± 0.8 ^	19.1 ± 0.8 * ∞ ^
	Relative Muscle mass (mg/g)	0.60 ± 0.01 ^	0.60 ± 0.01^	0.54 ± 0.01 * ∞ ^

* significantly different when compared to UNT group of the same strain. ∞ significantly different when compared to the BAL group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment. (n=7-12)

ii) SOL

As shown in Table 6.4, there was a significant increase ($p < 0.05$) in SOL muscle mass and relative muscle mass associated with BAL treatment when compared to CON-UNT mice. No significant differences were observed between TAU and BAL treatment. There were no significant differences in SOL muscle mass in association with TAU or BAL treatment in MDX mice. CON SOL was significantly smaller ($p < 0.0001$) when compared to MDX SOL.

Table 6.4 SOL Muscle mass and relative muscle mass with taurine and *β*-alanine treatment

		SOL		
		UNT	**BAL**	**TAU**
CON	Muscle Mass (mg)	8.7 ± 0.3	10.0 ± 0.2 *	9.5 ± 0.4
	Relative Muscle mass (mg/g)	0.26 ± 0.01	0.30 ± 0.01*	0.30 ± 0.01
MDX	Muscle Mass (mg)	14.9 ± 0.2 ^	14.5 ± 0.5 ^	14.5 ± 0.3 ^
	Relative Muscle mass (mg/g)	0.40 ± 0.01	0.40 ± 0.01 ^	0.41 ± 0.01^

* significantly different when compared to UNT group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment. (n=7-10)

6.4.4 Freeze dried muscle % dw/ww

i) PLANT

There was no significant difference in the dw/ww percentage in association with TAU or BAL treatment in CON or MDX mice. However, there was a significant difference in this percentage ($p < 0.0001$) between the CON and MDX groups, with CON mice having a higher percentage.

Table 6.5 PLANT % dw/ww

	PLANT % dw/ww		
	UNT	**BAL**	**TAU**
CON	26.9 ± 0.5	26.40 ± 0.4	26.2 ± 0.3
MDX	24.0 ± 0.4 ^	24.5 ± 0.8 ^	24.0 ± 0.4 ^

^ significant difference between CON and *mdx* mice for the same treatment. (n=7-10)

ii) DIA

Results for DIA percentage dw/ww are shown in Table 6.6. There was no significant difference in muscle fluid content between the treatment groups however; consistently with PLANT data, CON mice had a significantly higher percentage ($p < 0.0001$) when compared to MDX mice.

Table 6.6 DIA % dw/ww

	DIA % dw/ww		
	UNT	**BAL**	**TAU**
CON	26.1 ± 0.3	26.3 ± 0.5	26.3 ± 0.7
MDX	24.2 ± 0.6 ^	22.9 ± 0.8 ^	24.0 ± 0.5 ^

^ significant difference between CON and *mdx* mice for the same treatment. (n=7-10)

6.4.5 Muscle taurine content

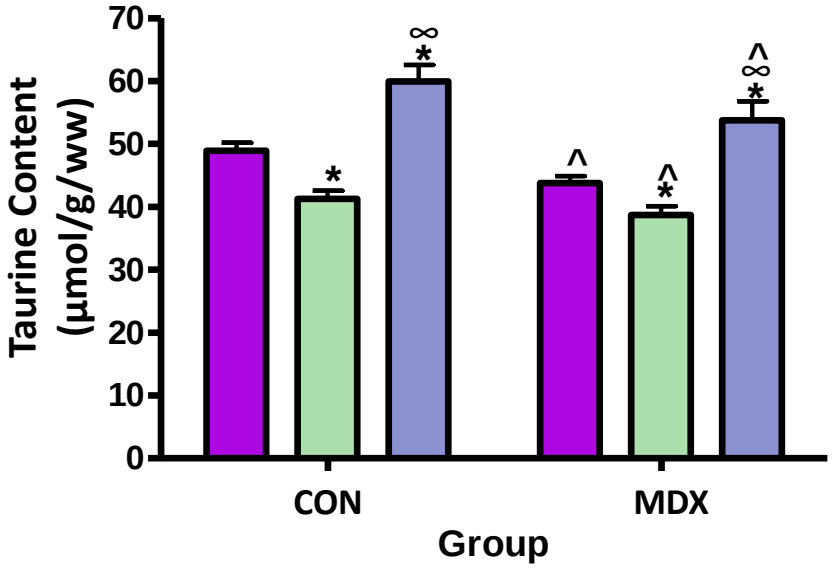
i) PLANT

As seen in Figure 6.1 A, taurine content was 18% and 31% ($p < 0.001$) higher in CON-TAU when compared to CON-UNT and CON-BAL groups, respectively. BAL treatment significantly lowered taurine content ($p < 0.001$) compared to CON-UNT (16%) and CON-TAU (31%) muscles. Similarly for the *mdx* groups, there was a significant (18%) increase in taurine content associated with taurine treatment ($p < 0.001$) when compared to MDX-UNT mice, with taurine content also significantly higher compared to MDX-BAL. Treatment with BAL depleted taurine content by 12% ($p < 0.001$) when compared to MDX-UNT. Interestingly, MDX-UNT and MDX-TAU demonstrated significantly less ($p < 0.0001$) skeletal muscle taurine content when compared to CON-UNT and CON-TAU, respectively. There was also significantly less ($p < 0.001$) taurine content in the MDX-BAL relative to CON-BAL. This is likely due to the lower taurine content in the mdx group to begin with, as the percentage changes with taurine and *β*-alanine treatment are similar.

ii) DIA

Taurine treatment increased DIA taurine content in both CON and MDX mice ($p < 0.001$; Figure 6.1 B, while treatment with BAL decreased taurine content ($p < 0.001$) in both strains. When comparing CON and *mdx* mice in association with each treatment, there was a significantly less taurine content for both taurine and *β*-alanine treatment in *mdx* mice relative to CON for the same treatment.

A



B

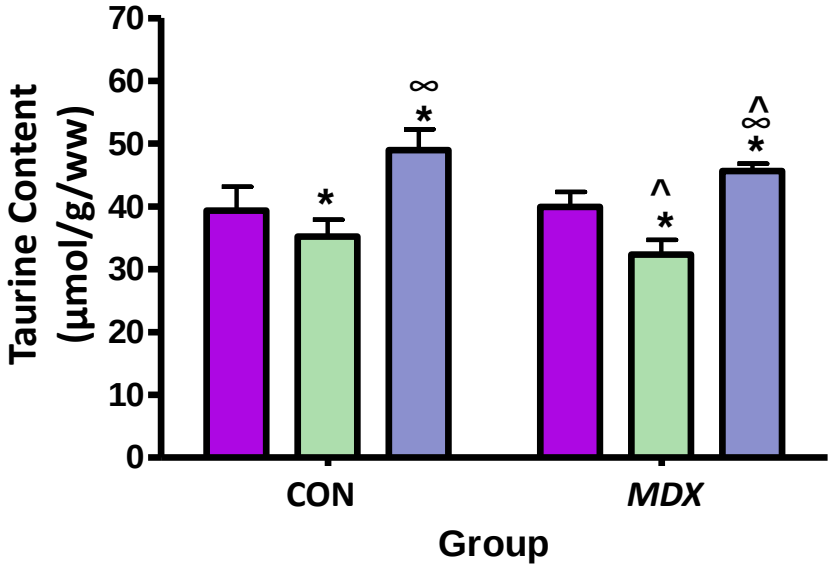


Figure 6.1 Taurine content for (A) PLANT and (B) DIA for CON and *mdx* mice in association with treatments

* denotes a significant difference when compared to UNT group of the same strain. ∞ significantly different when compared to the BAL group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment (n=7-10). Purple bars ■ represent untreated groups, green bars ■ represent *β*-alanine treated groups and blue ■ represents taurine treated groups.

6.4.6 Plasma CK concentration

As shown in Figure 6.2, plasma CK was unchanged with treatment in CON mice. The MDX-TAU treated group had significantly increased ($p < 0.05$) plasma f CK when compared to both the MDX-UNT and MDX-BAL groups, while no change occurred due to *β*-alanine treatment. As expected, plasma CK was significantly higher ($p < 0.0001$) in *mdx* mice relative to CON animals, irrespective of treatment.

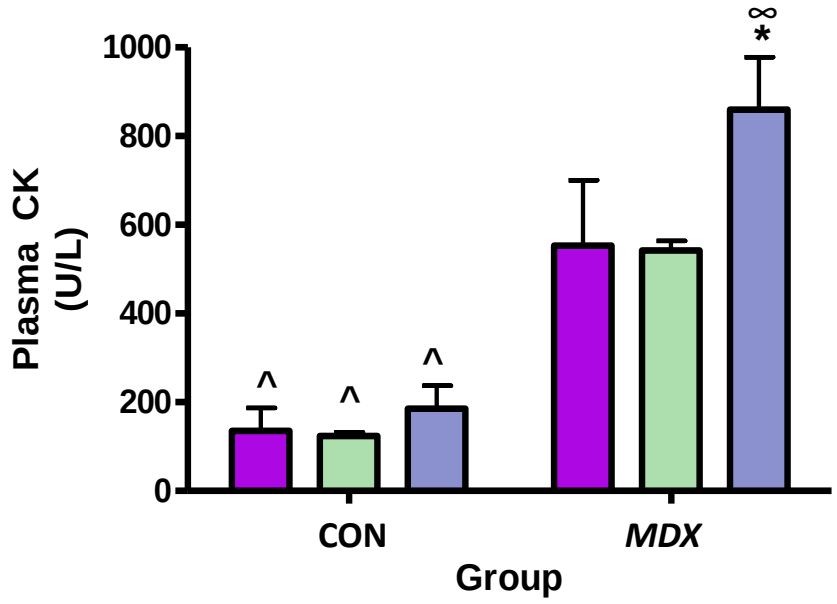


Figure 6.2 Plasma CK concentration for CON and *MDX* mice in association with treatments

* significant difference when compared to UNT group of the same strain. ∞ Significantly different when compared to the BAL group of the same strain. ^ significant difference between CON and *mdx* mice for the same treatment (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

6.4.7 The effect of treatments on the isometric contractile properties of EDL

i) Twitch characteristics, CSA and L_0

As shown in Table 6.7, there were no significant differences in P_t, TTP or ½ RT associated with either of the treatment protocols for CON or *mdx* mice. No significant differences in CSA were found in association with treatment in CON mice. However, CON CSA was significantly lower when compared to *mdx* groups. As such, CON mice had significantly higher ($p < 0.0001$) normalised twitch force when compared to *mdx* mice. No significant difference in L_0 was found between groups for either strain.

Table 6.7 Isometric contractile properties of EDL with taurine and *β*-alanine treatment

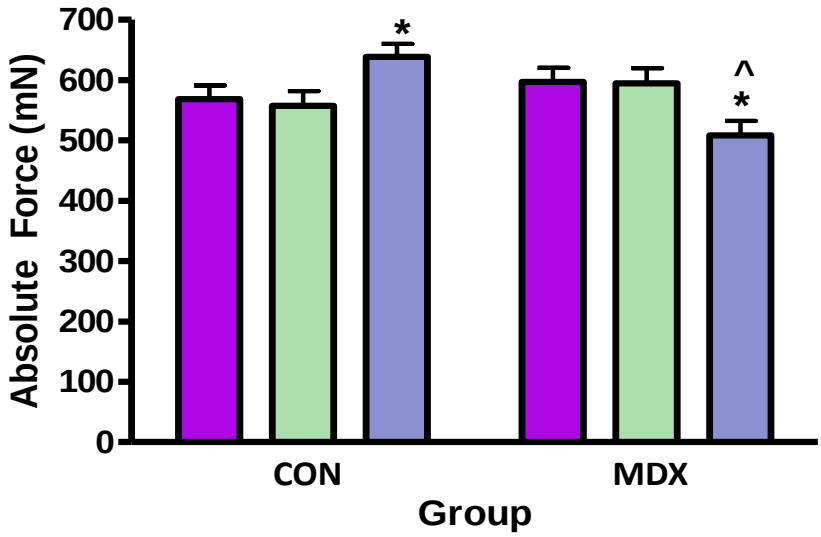
		UNT	BAL	TAU
CON	Lo (mm)	13.3 ± 0.2	13.8 ± 0.2	13.3 ± 0.2
	CSA (mm^2)	1.9 ± 0.0	1.9 ± 0.1	2.0 ± 0.1
	Pt (N/cm^2)	5.4 ± 0.2	6.0 ± 0.3	5.4 ± 0.2
	TTP (msec)	29.8 ± 0.2	30.2 ± 0.3	29.6 ± 0.3
	½ RT (msec)	19.5 ± 0.7	20.0 ± 0.4	19.0 ± 0.5
MDX	Lo (mm)	14.2 ± 0.2	14.2 ± 0.2	13.8 ± 0.2
	CSA (mm^2)	3.4 ± 0.1 ^	3.2 ± 0.1 ^	2.9 ± 0.1 ^
	Pt (N/cm^2)	4.0 ± 0.2 ^	3.7 ± 0.2 ^	4.0 ± 0.3 ^
	TTP (msec)	29.0 ± 0.2	28.3 ± 0.2	29.7 ± 0.1
	½ RT (msec)	18.7 ± 0.6	18.7 ± 0.3	19.3 ± 0.3

^ significant difference between CON and *mdx* mice (n=7-10).

ii) Peak tetanic force

There was a significant increase ($p < 0.05$; Figure 6.3 A) in absolute force for the CONT-TAU when compared to CON-BAL. MDX-TAU displayed significantly less ($p < 0.05$) absolute force when compared to both MDX-UNT and MDX-BAL. However, as shown in Figure 6.3 B, when this data was normalised to CSA there were no significant differences associated with treatments for CON or *mdx* mice. CON mice, however, demonstrated significantly greater ($p < 0.0001$) specific force when compared to the *mdx* groups.

A



B

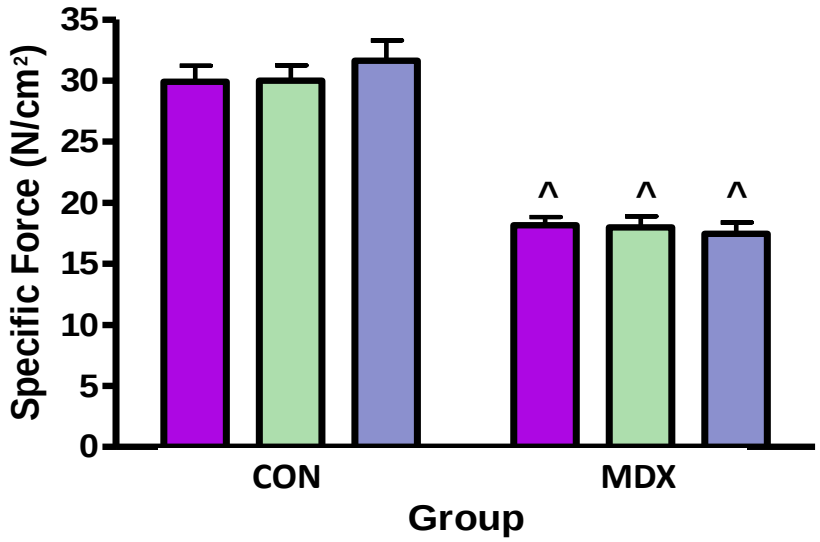


Figure 6.3 Absolute (A) and specific (B) force for EDL in CON and *mdx* mice in association with treatments

* significant difference when compared to UNT group of the same strain. ^ significant difference between CON and *mdx* mice across all groups (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

iii) Force-Frequency Relationship

Force produced by CON-BAL group was significantly lower ($p < 0.05$) than both the CON-UNT and CON-TAU over the range of 90-120 Hz. No significant differences in the force frequency relationship were observed in association with TAU or BAL treatment in *mdx* mice. Stimulation frequencies between 10-110 Hz resulted in significantly higher percentage of maximum force ($p < 0.01$) being produced by *mdx* muscles until 120 Hz, where there was no longer a significant difference between the groups. From 130-160 Hz, MDX EDL produced a significantly lower percentage of maximum force ($p < 0.0001$) than CON muscle.

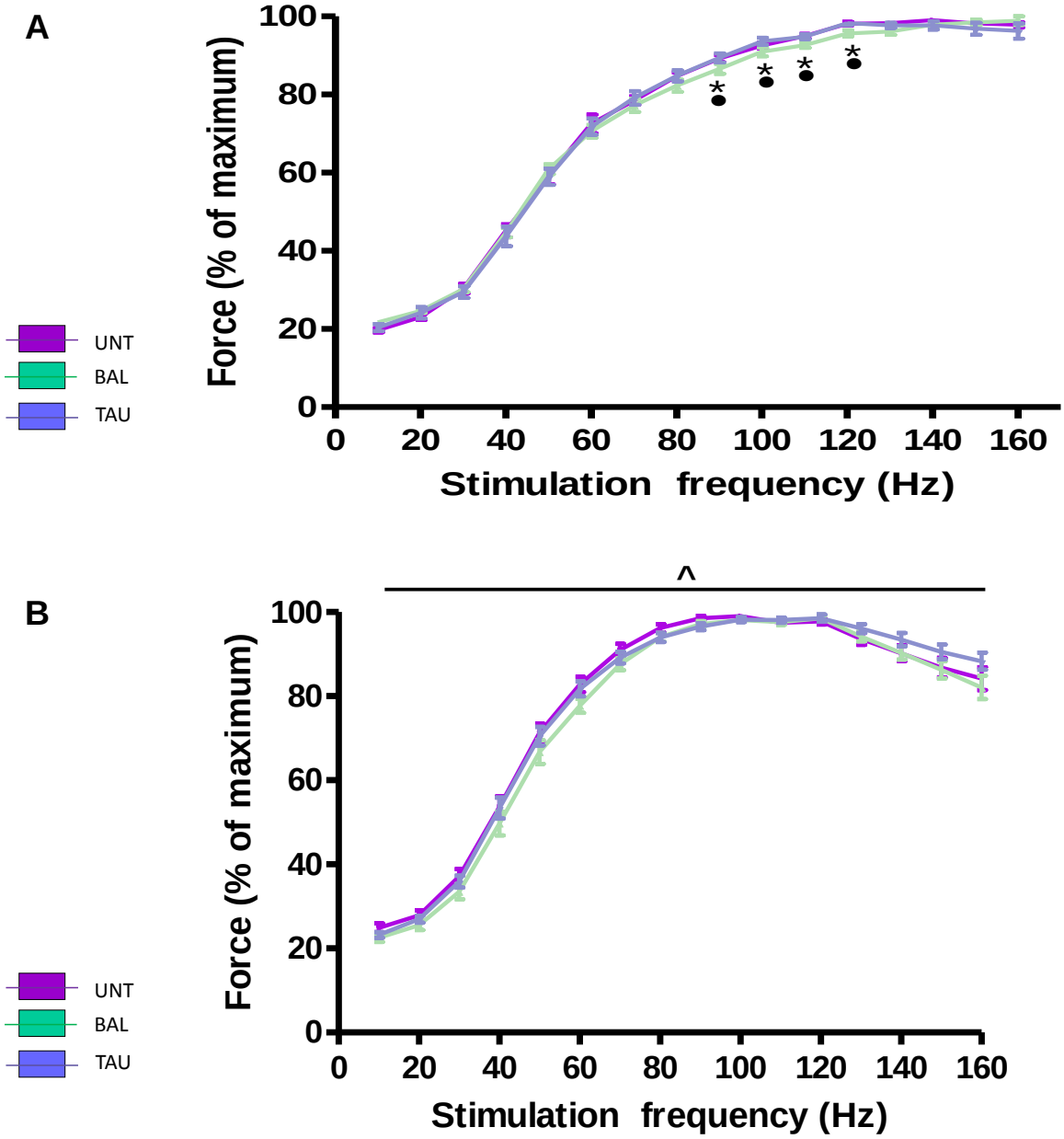


Figure 6.4 The relationship between relative peak force (% of maximum) and stimulation frequency for EDL in CON (A) and *mdx* (B) mice in association with treatments.

* denotes a significant difference when compared to UNT group of the same strain. ● significantly different when compared to the TAU group of the same strain. ^ denotes a significant difference between CON and *mdx* mice (n=7-10).

iv) Fatigue

As shown in Figure 6.5, there was a significant decrease ($p < 0.001$) in fatigue associated with BAL treatment in CON mice when compared to both the CON-UNT and CON-TAU groups. No changes in fatigue were associated with TAU treatment in CON mice. For *mdx* mice, there was a significant decrease in fatigue for both the TAU ($p < 001$) and BAL ($p < 0.05$) treated groups when compared to MDX-UNT, such that there was no significant difference observed in fatigue between TAU or BAL treatment. There was no significant difference in fatigue between CON-UNT and MDX-UNT groups. MDX-BAL demonstrated a significantly less force ($p < 0.01$) when compared to CON-BAL, while MDX-TAU had significantly greater force ($p < 0.01$) relative to the CON-TAU group.

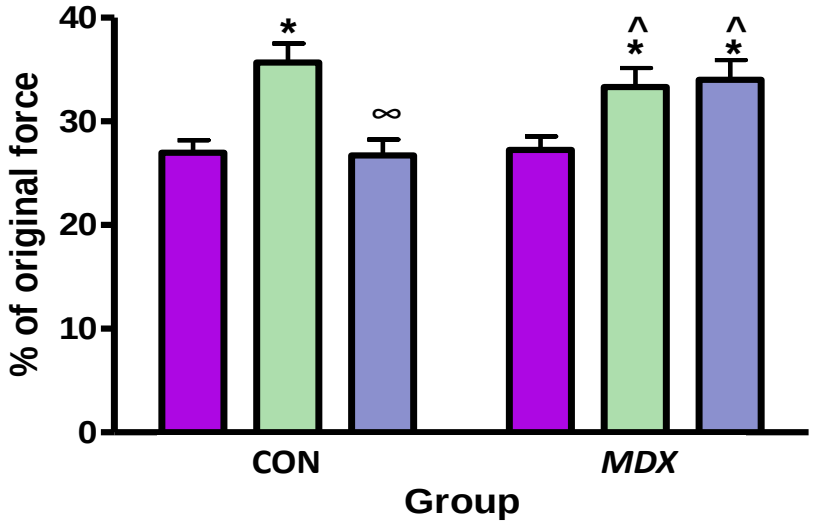


Figure 6.5 Percentage of the original force at the end of a 1 minute of fatiguing stimulation at 70Hz for 250 ms every 1s in CON and *mdx* mice in association with treatments.

* denotes a significant difference when compared to UNT group of the same strain. ∞ Significantly different when compared to the BAL group of the same strain. ^ denotes a significant difference between CON and *mdx* mice (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

v) Recovery of EDL

a) CON

As shown in Figure 6.6 A, CON-BAL mice maintained a significantly higher percentage of original force after 1 ($p < 0.001$), 2 ($p < 0.001$) and, minutes 5 ($p < 0.01$)) of recovery relative to both the CON-UNT and CON-TAU groups. At 10 minutes, CON-BAL still produced higher ($p < 0.05$) percentage of original force compared to the CON-UNT group. After 20 minutes of recovery, however, no significant differences in force was observed between any of the CON groups.

b) Mdx

There was no significant difference in the percentage of original force attained between any of the *mdx* groups in the first two minutes of recovery (see Figure 6.6 B). By 5 minutes, however, there was a significant difference ($p < 0.05$) in recovery when comparing the taurine and *β*-alanine treatment groups. From 10 to 60 minutes of recovery, MDX-TAU demonstrate significantly increased force (10 and 20 minute ($p < 0.05$), 30 minutes UNT ($p < 0.05$) BAL ($p < 0.01$), 45 minutes ($p < 0.05$), 60 minutes UNT ($p < 0.05$) and BAL ($p < 0.001$)) relative to both the MDX-UNT and MDX-BAL groups.

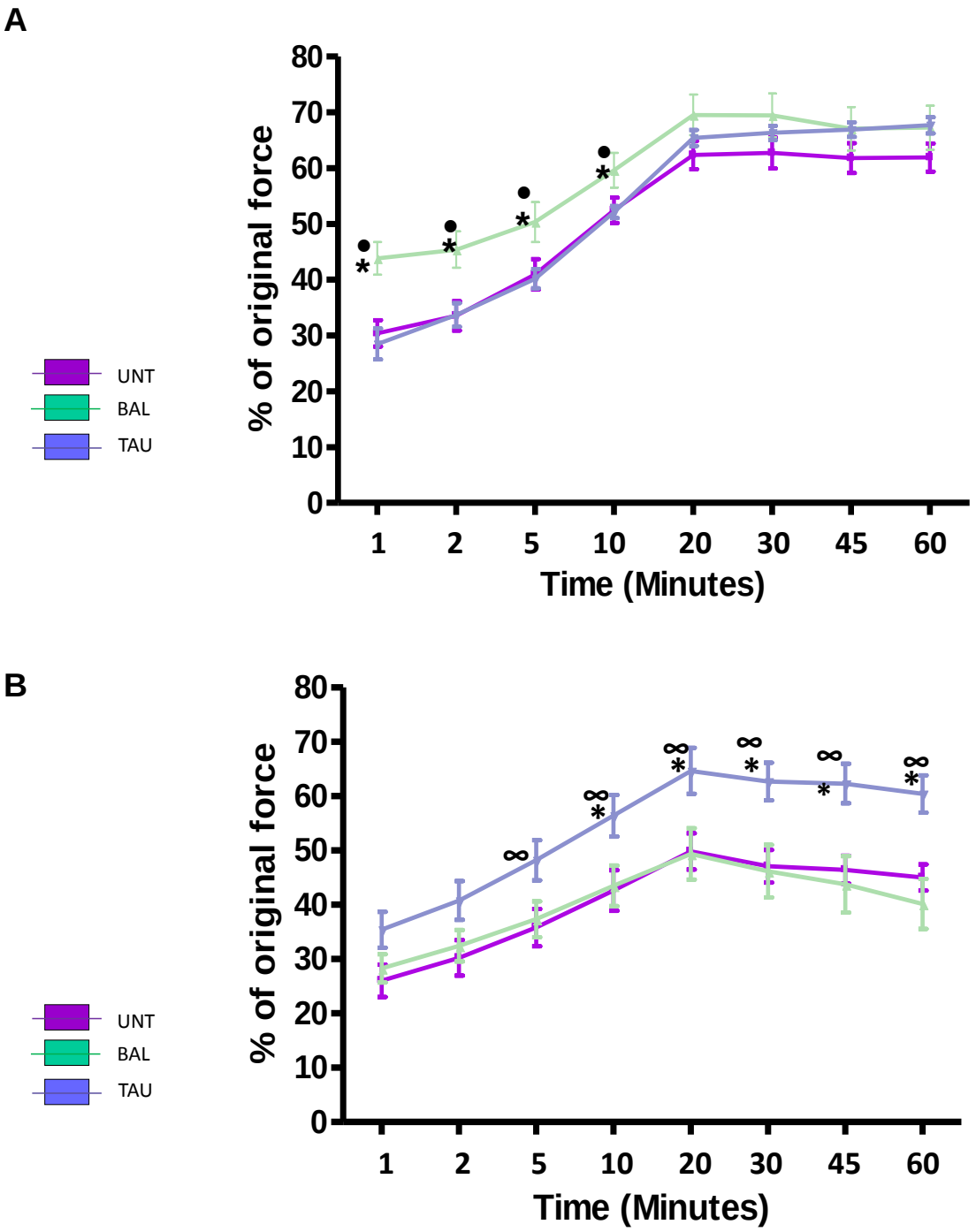


Figure 6.6 Recovery of CON (A) and *mdx* (B) EDL muscles after a fatiguing stimulation measured using a stimulation frequency of 100 Hz

* significantly different when compared to UNT group of the same strain. ∞ significantly different when compared to the BAL group of the same strain. ● significantly different when compared to the TAU group of the same strain. (n=7-10).

6.4.8 The effect of treatments on the isometric contractile properties of SOL

i) Twitch characteristics

As shown in Table 6.8, while no significant differences in L_0, CSA or Pt were observed with taurine or *β*-alanine supplementation in CON mice, *β*-alanine treatment was associated with a significant decrease in TTP relative to CON-UNT ($p < 0.001$) and CON-TAU ($p < 0.05$) groups. A significant decrease ($p < 0.05$) in ½ RT was also observed in CON-BAL relative to CON-UNT. No significant differences in L_0, CSA, Pt, TTP or ½ RT were observed between any of the *mdx* groups examined. Relative to CON mice, all groups of *mdx* mice demonstrated significantly greater CSA ($p < 0.0001$) and significantly less normalised twitch force ($p < 0.0001$). There was also a significant difference ($p < 0.05$) in ½ RT between UNT-CON and UNT-MDX mice with *mdx* SOL exhibiting faster relaxation.

Table 6.8 Isometric contractile properties of SOL with taurine and *β*-alanine treatment

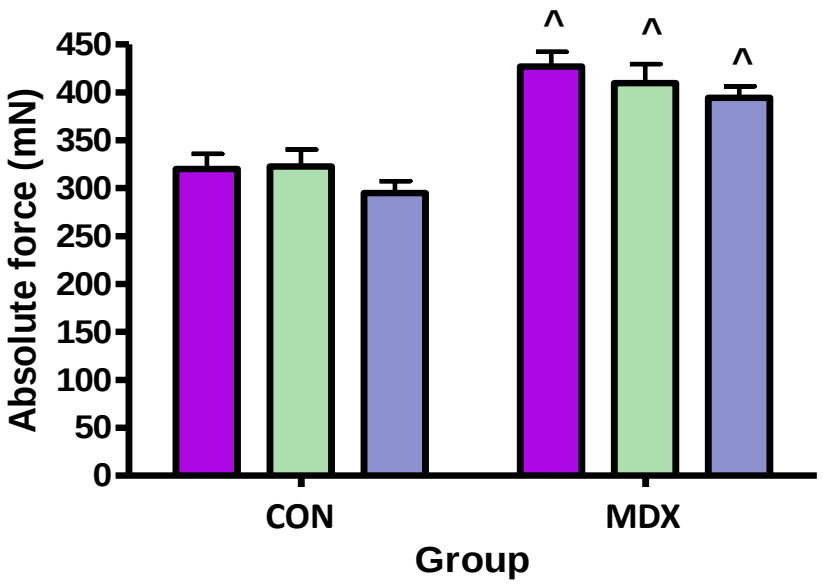
		UNT	*B*AL	TAU
CON	Lo (mm)	12.5 ± 0.2	12.7 ± 0.2	13.2 ± 0.3
	CSA (mm^2)	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
	Pt (N/cm^2)	4.5 ± 0.2	4.0 ± 0.2	4.3 ± 0.2
	TTP (msec)	42.2 ± 0.5	40.0 ± 0.4 *	41.8 ± 0.3 ∞
	½ RT (msec)	42.7 ± 1.7	37.2 ± 1.3 *	41.5 ± 1.5
MDX	Lo (mm)	12.7 ± 0.1	12.8 ± 0.3	12.6 ± 0.2
	CSA (mm^2)	2.4 ± 0.0 ^	2.4 ± 0.1 ^	2.5 ± 0.1 ^
	Pt (N/cm^2)	3.0 ± 0.1 ^	3.2 ± 0.2 ^	2.9 ± 0.1 ^
	TTP (msec)	40.3 ± 0.2	40.2 ± 0.3	40.7 ± 0.5
	½ RT (msec)	37.7 ± 1.4 ^	34.6 ± 0.9	38.2 ± 1.7

* denotes a significant difference when compared to UNT group of the same strain. ^ denotes a significant difference between CON and *mdx* mice (n=7-10).

ii) Peak tetanic force

There was no significant difference in absolute or specific force for CON and *mdx* mice in association with taurine or *β*-alanine treatment. CON mice demonstrated significantly less absolute force ($p < 0.001$; Figure 6.7), but significantly more specific force when compared to *mdx* mice.

A



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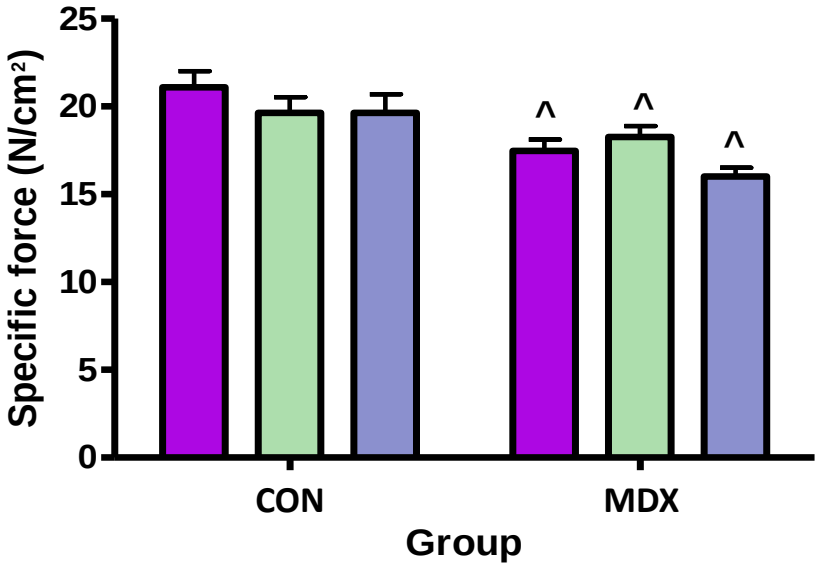


Figure 6.7: Absolute (A) and specific (B) force for SOL in CON and *mdx* mice in association with treatments

.^ significant difference between CON and *mdx* mice across all groups (n=7-10). Purple bars (represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

iii) Force-Frequency Relationship

As shown in Figure 6.8, there were no significant differences in the force-frequency relationship associated with treatment for CON or *mdx* mice. There was a significant difference between the strains from 10 to 40 Hz, with CON mice demonstrating significantly higher ($p < 0.05$) percentage of maximum force at these stimulation frequencies (see Figure 6.8).

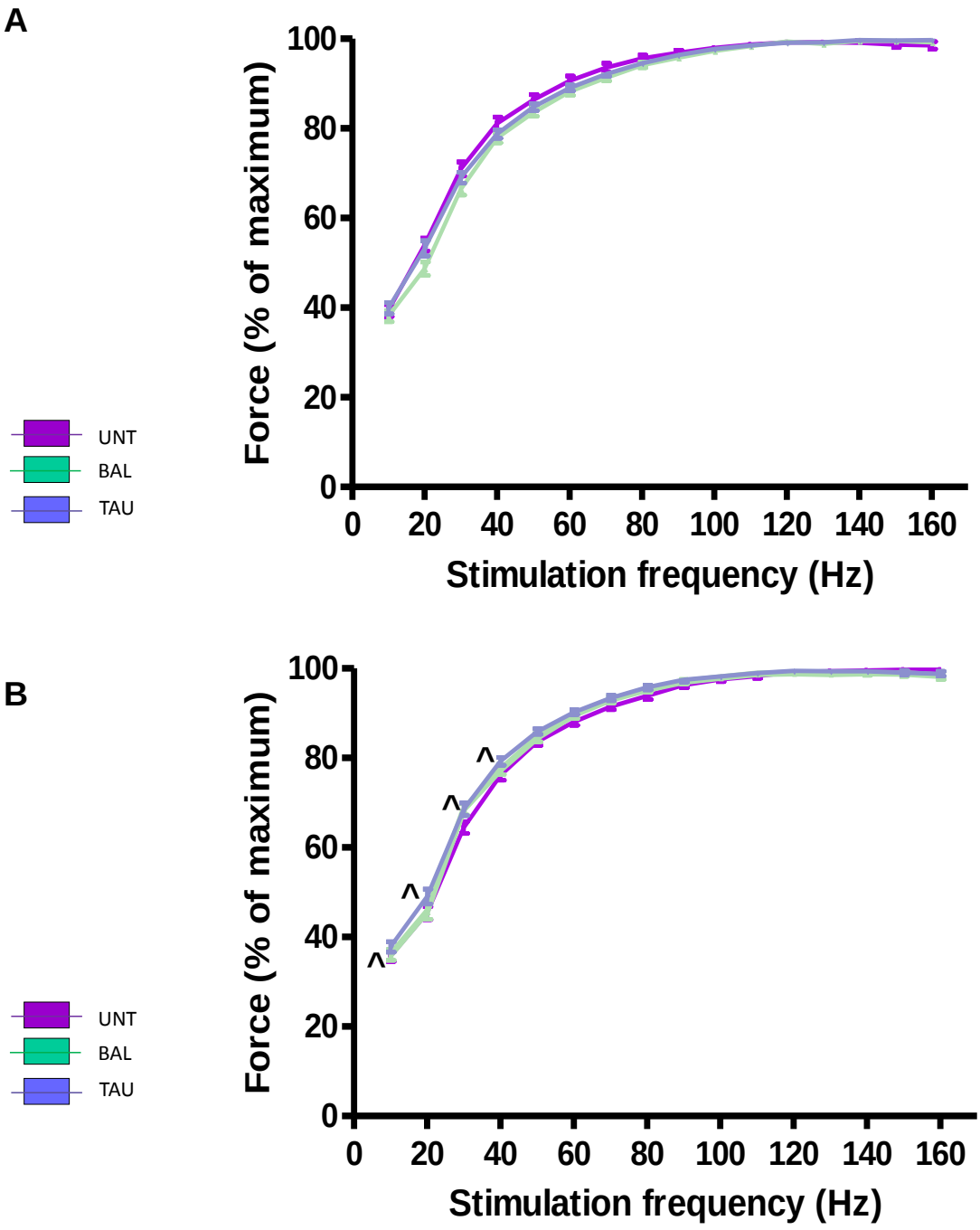


Figure 6.8: The relationship between relative peak force (% of maximum) and stimulation frequency for SOL in control (A) and *mdx* (B) mice in association with treatments

^ significant difference between CON and *mdx* mice across all groups. (n=7-10)

iv) Fatigue

There was no significant difference in fatigue associated with the treatments in CON or *mdx* mice, however, there was a significant difference in fatigue between the strains, with *mdx* mice demonstrating significantly more fatigue ($p < 0.0001$; Figure 6.9) when compared to CON groups.

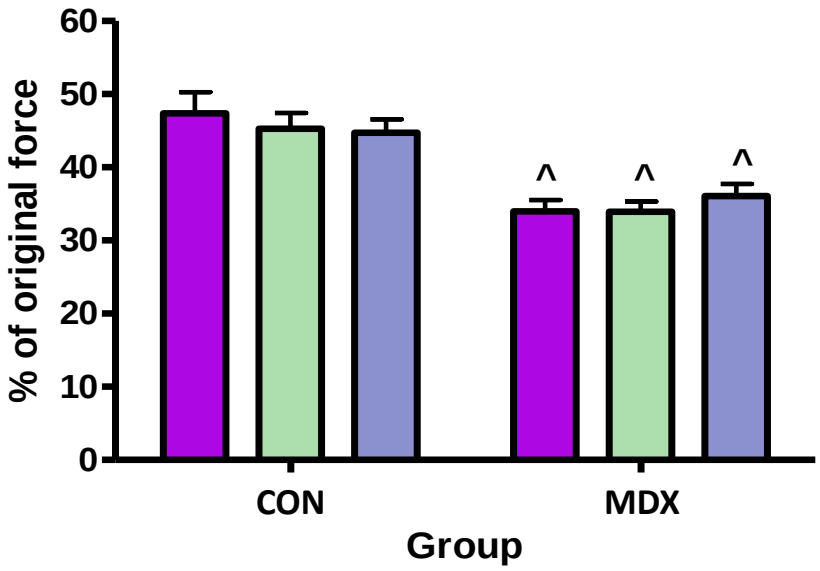


Figure 6.9 Percentage of the original force at the end of a 3 minute fatiguing stimulation at 30Hz for 500 ms every 1s in CON and *mdx* mice in association with treatments

^ significant difference between CON and *mdx* mice across all groups (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue (represents taurine treated groups.

v) Recovery

As shown in Figure 6.10, no significant differences in recovery were observed for CON or *mdx* mice in association with taurine or *β*-alanine treatment. No significant difference in recovery was observed between CON and *mdx* mice at any time point.

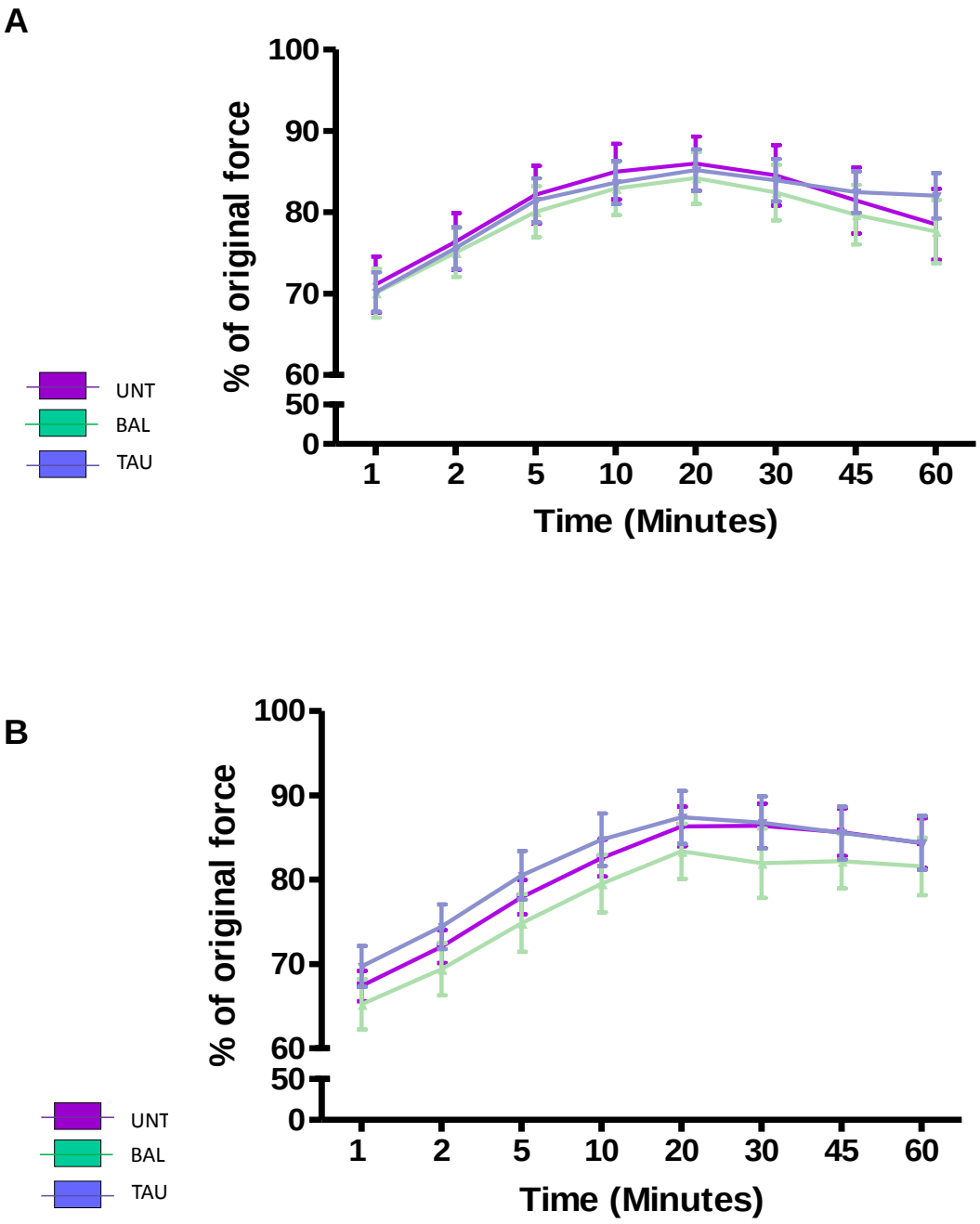


Figure 6.10 Recovery of force for CON (A) and *mdx* (B) SOL muscles after a fatiguing stimulation measured using a stimulation frequency of 80 Hz.

(n=7-10)

6.4.9 Dystrophin expression

Dystrophin was expressed in CON mice but absent in the *mdx*, confirming the presence and absence of the dystrophic condition in the animals used. A representative blot of these results is shown in Figure 6.11.

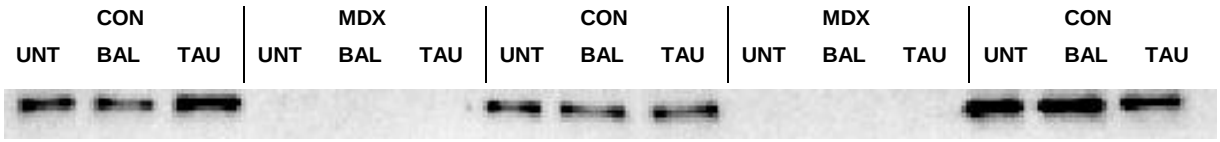


Figure 6.11 Dystrophin expression CON and *mdx* mice across mice in association with treatments

6.4.10 TauT protein expression

As shown in Figure 6.12, There were no significant differences in TauT protein expression in association with either taurine or *β*-alanine treatment, or between the CON and *mdx* mice.

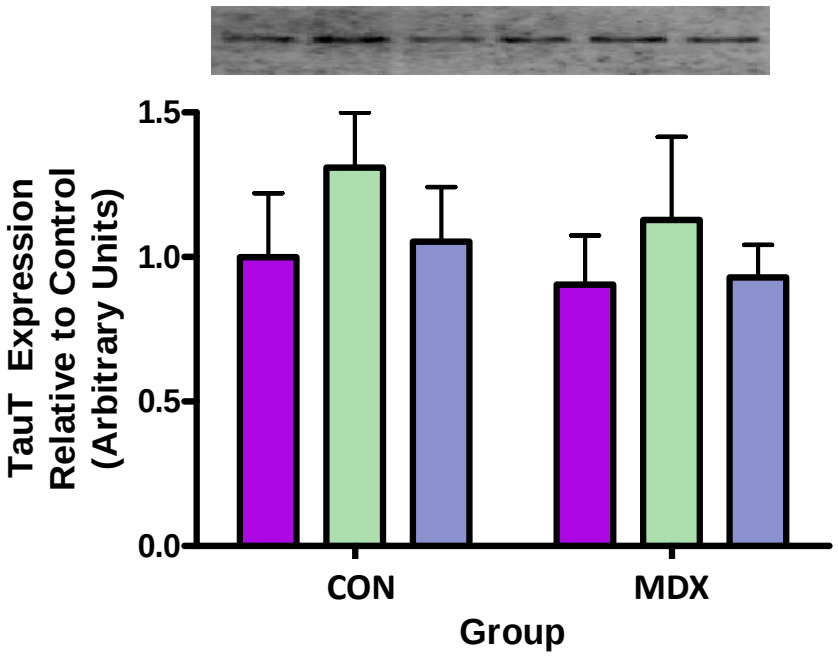


Figure 6.12 TauT expression in CON and *mdx* EDL muscles in association with treatments

Representative Western blot and graphs for TauT protein expression in treatment groups for CON and *mdx* mice. Values are arbitrary units normalised to actin and expressed relative to untreated CON animals. Data is expressed at mean ± SEM (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

6.4.11 E-C coupling and Ca^{2+} handling expression

There were no significant differences in Actin, Myosin, DHPR, RyR, SERCA or CSQ in association with the treatments or between CON and *mdx* mice. See Figures 6.13 to 6.15.

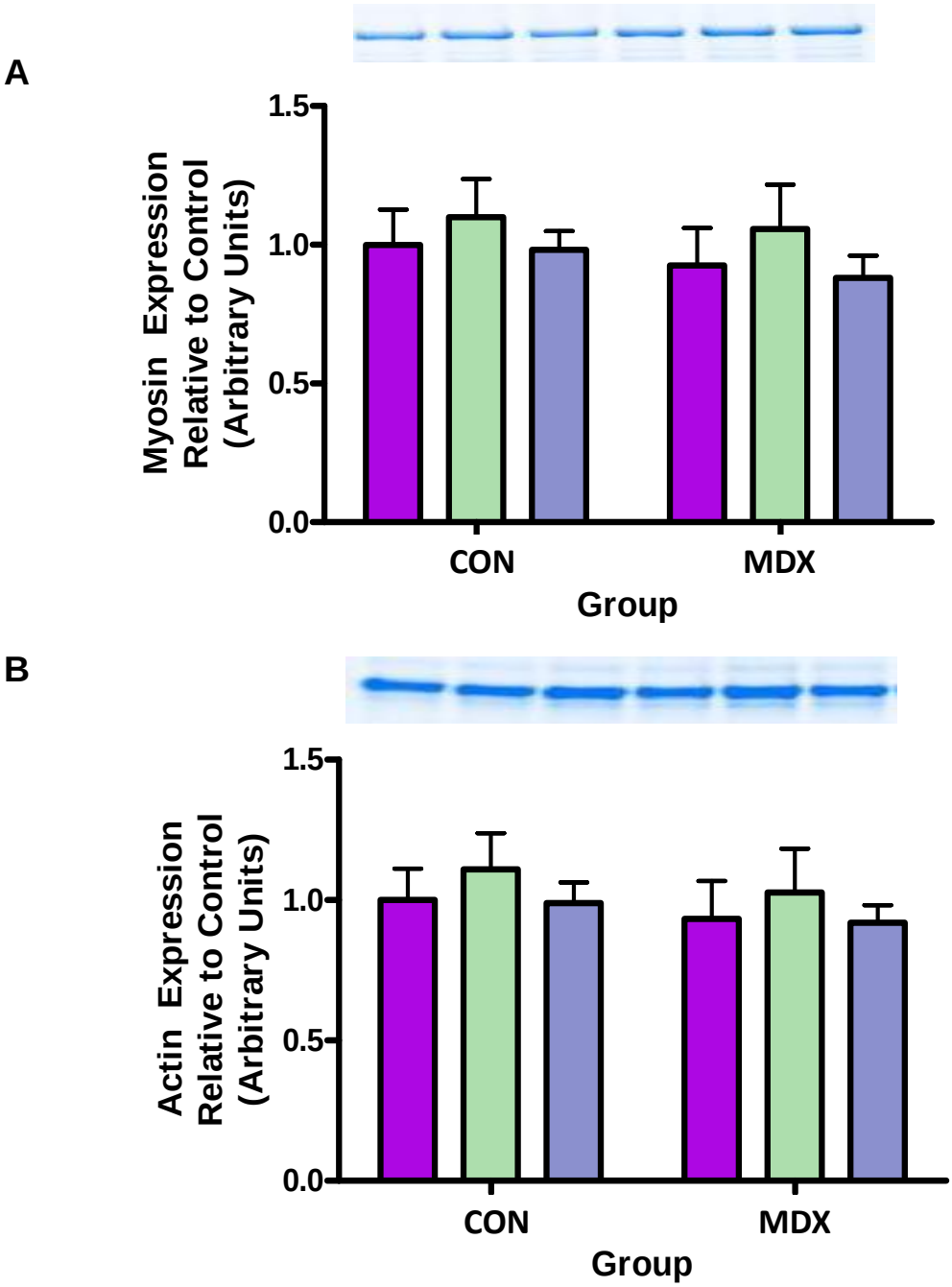
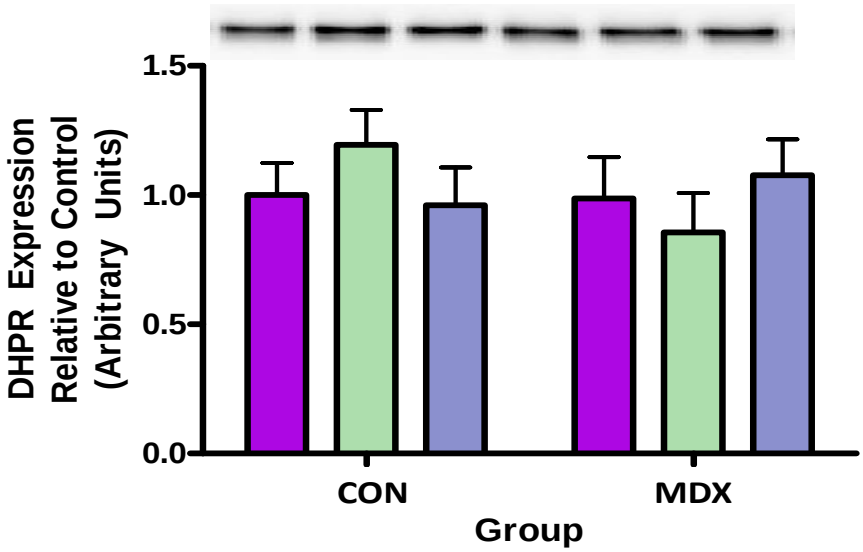


Figure 6.13 Contractile protein expression in EDL in CON and *mdx* mice in association with treatments.

Representative Western blot and graphs for myosin and actin protein expression in treatment groups for CON and *mdx* mice. Values are arbitrary units normalised to actin and expressed relative to untreated CON animals. Data is expressed at mean ± SEM (n=7-10) Purple bars represent untreated groups, green bars epresent *β*-alanine treated groups and blue represents taurine treated groups.

A



B

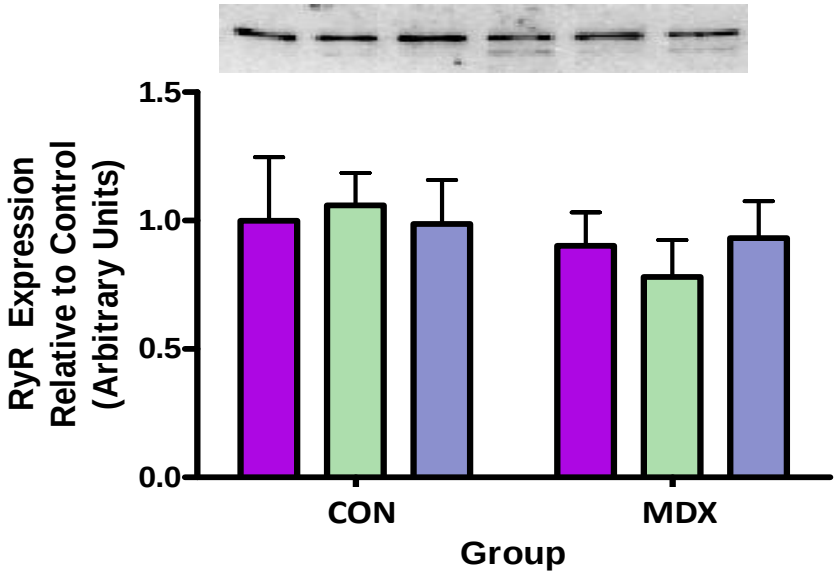


Figure 6.14 DHPR (A) and RyR (B) expression in EDL in CON and *mdx* mice in association with treatments.

Representative Western blots and graphs for DHPR (A) and RyR (B) protein expression in treatment groups for control and *mdx* mice. Values are arbitrary units normalised to actin and expressed relative to untreated CON animals. Data is expressed at mean ± SEM (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

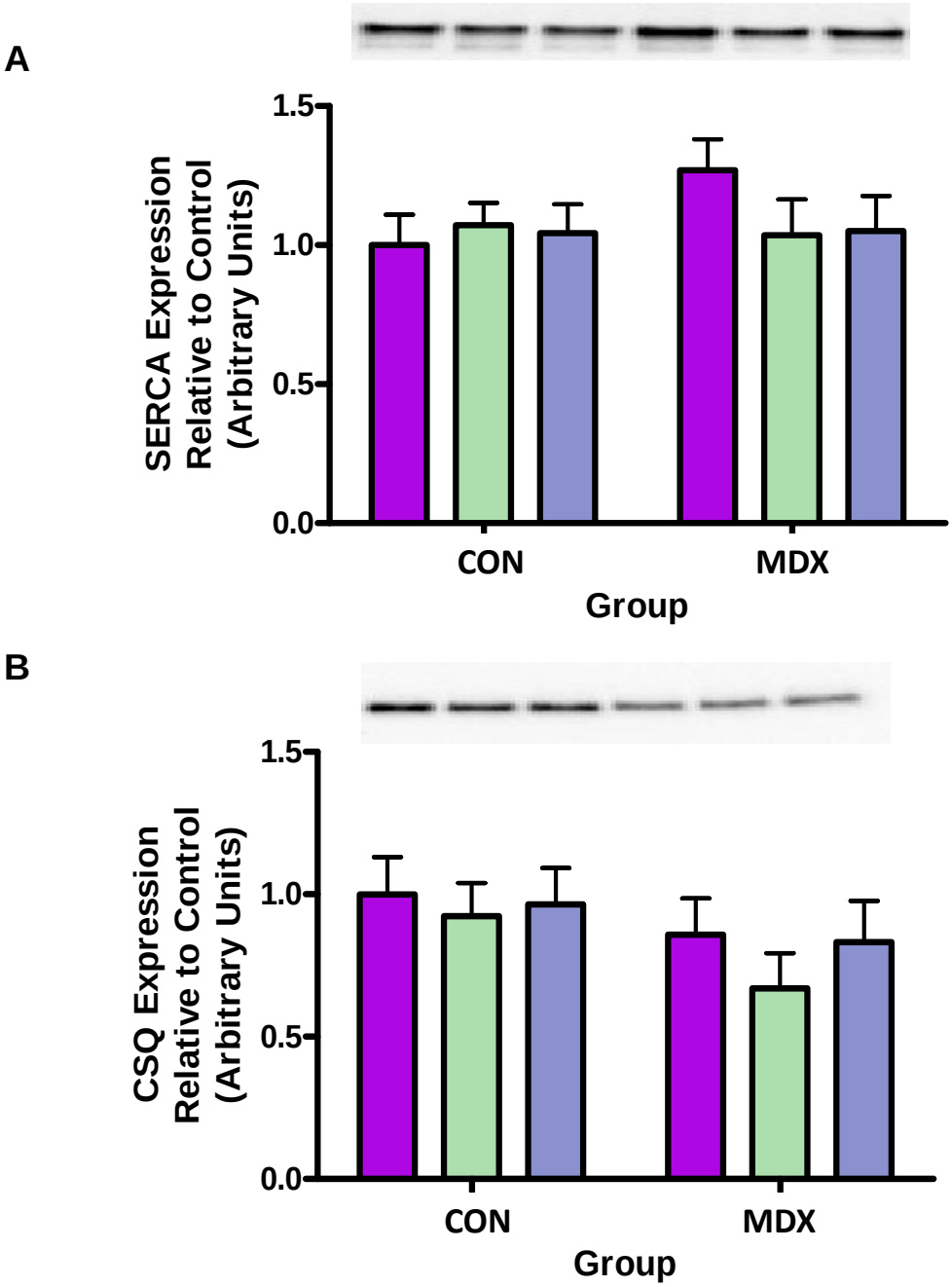


Figure 6.15 SERCA (A) and CSQ (B) protein expression in EDL in CON and *mdx* mice in association with treatments.

Representative Western blots and graphs for SERCA (A) and CSQ (B) protein expression in treatment groups for control and *mdx* mice. Values are arbitrary units normalised to actin and expressed relative to untreated CON animals. Data is expressed at mean ± SEM (n=7-10). Purple bars represent untreated groups, green bars represent *β*-alanine treated groups and blue represents taurine treated groups.

6.5 Discussion

This study has produced several novel and important findings with regard to the effect of taurine on dystrophic skeletal muscle function. This is the first study to show that taurine and *β*-alanine supplementation increases and decreases skeletal muscle taurine content, respectively, in six month old *mdx* and CON mice. Interestingly, despite taurine previously being reported to improve maximum force output (Cozzoli et al., 2011b, De Luca et al., 1998), no significant difference in tetanic force was observed in EDL or SOL muscle of CON or *mdx* mice in association with taurine supplementation, or with *β*-alanine-induced taurine depletion. However, treatment with taurine was able to increase resistance to fatigue in fast-twitch muscle of the *mdx* mouse, although this appears to be specific to dystrophic skeletal muscle, as no such effect on fatigue was observed in CON mice.

Surprisingly, while *β*-alanine resulted in significant depletion of taurine content in skeletal muscle, *β*-alanine also improved resistance to fatigue in CON and *mdx* EDL muscles suggesting that *β*-alanine itself has beneficial effects on the maintenance of muscle force during fatiguing stimulation. In addition, although *β*-alanine decreased fatigue, no improvement in force recovery was observed in the *mdx* mouse. However, in CON mice, *β*-alanine successfully improved resistance to fatigue and also resulted in a higher percentage of pre-fatigue force being maintained during recovery. The present study is also the first to examine E-C coupling and Ca^{2+} handling protein expression in CON or *mdx* mice in association with taurine and *β*-alanine treatment, demonstrating that supplementation has no significant effects on protein expression in older mice in contrast to that found in Chapter 4 in younger *mdx* mice.

6.5.1 The effect of taurine and β-alanine supplementation on fluid intake

Fluid intake was significantly decreased in both CON and *mdx* mice in association with β-alanine treatment in the present study. This is in agreement with Dawson et al. (2002) who showed a significant decrease in the fluid consumption of rats supplemented with the same dosage of β-alanine water. In addition, studies of β-alanine supplementation in humans have indicated that high doses of β-alanine (40 $mg.kg^{-1}$ of body weight) results in unpleasant symptoms of paresthesia (tingling and numbness) in limbs (Harris et al., 2006, Hill et al., 2007).Thus, it is possible that the high dose of β-alanine used in this study also produced unpleasant side effects in mice, causing them to consume less fluid. Moreover, β-alanine supplementation has also been shown to reduce renal fluid and Na^+ excretion (Mozaffari et al., 1997), which could also account for the decrease in the volume of water consumed by the mice in this treatment group.

Interestingly, while taurine supplementation had no significant effect on fluid consumption in CON mice, taurine supplementation in the *mdx* mouse caused a significant increase in fluid intake at some time points. Furthermore, no adverse side-effects were observed with 3 % taurine treatment in CON or *mdx* mice, as was found by Dawson et al. (2002), who reported that rats in the taurine supplemented group experienced diarrhoea as a result of treatment. The supplementation dose used in this thesis were modelled on literature which uses doses of 1-3 % taurine in drinking water, to avoid symptoms such as those that were reported in Dawson et al. (2002). However, results from the present study suggests that mice may be able to withstand higher dosages of taurine supplementation than rats when administered via drinking water. The increase in fluid consumption observed with taurine treatment in *mdx* mice is most likely due to increased urine output, as taurine has been shown to promote diuresis and increase water loss in rodents (Meldrum et al., 1994, Mozaffari et al., 1997).

6.5.2 The effect of taurine and *β*-alanine supplementation on body and muscle mass

As expected, all of the *mdx* mice analysed in the present study demonstrated significantly higher body and muscle mass relative to CON animals at six months of age, likely due to skeletal muscle hypertrophy that occurs as a result of the dystrophic pathology (Consolino and Brooks, 2004, Zammit and Partridge, 2002). Supplementation with *β*-alanine had no effect on body or muscle mass of either CON or *mdx* mice, as has been shown previously with *β*-alanine supplementation in rats and humans (Dawson et al., 2002, Kendrick et al., 2008). Interestingly, and in conflict with findings in Chapter 5 with 2 % taurine supplementation, four weeks of 3 % taurine supplementation resulted in a significant decrease in body and muscle mass in *mdx* mice, with no effect observed within the CON group. This is possibly due to the 50% increase in supplementation in the present study versus the protocol on Chapter 5, where a lower level of taurine supplementation was used for *mdx* mice. Previous studies in exercised *mdx* mice also demonstrate variable results with different taurine supplementation protocols, with DeLuca et al (2003) demonstrating and increase in body mass while Cozzoli et al. (2011) showed no

6.5.3 The effect of taurine and *β*-alanine supplementation on skeletal muscle fluid and taurine content

Consistent with the literature (Goodman et al., 2009, Grindstaff et al., 1997), and the results from Chapter 4, taurine supplementation did not cause any increases in skeletal muscle water content in CON or *mdx* mice. There were also no significant differences in muscle fluid content observed with *β*-alanine supplementation, although all groups of *mdx* mice demonstrated significantly greater muscle water content relative to CON mice. Fluid content in dystrophic skeletal muscle has been shown to be increased in some studies, most likely due to myofibre damage and resulting oedema, while others report no increase or a slight decline in muscle fluid (Dunn and Radda, 1991, Dupont-Versteegden et al., 1995, Selsby, 2011). For example, Decrouy et al. (1993) found a significantly higher fluid content in the *mdx* EDL relative to non-dystrophic controls, while no significant difference in fluid content was observed in the SOL muscles of the same adult mice. In contrast, both Dupont-Versteegden et al. (1995) and Tameyasu et al.

(2002) show decreased muscle fluid content in *mdx* mice relative to controls mice. As factors such as the age of the animal at experimentation, fibre type of the muscle examined, housing conditions (which can influence physical activity) and phenotypic variation within *mdx* colonies can all influence the degree of muscle damage observed (De la Porte et al., 1999, Dunn and Radda, 1991, Grounds et al., 2008b), this is likely to account for some of the variability in results for muscle fluid content within the literature and the current study.

While both the supplements used in the present study had no effect on the water content of skeletal muscle, taurine and *β*-alanine supplementation successfully increased and decreased skeletal muscle taurine content, respectively, in both CON and *mdx* mice. The improvement in taurine content was similar between supplemented CON and *mdx* mice, with an approximate 18 % increase in skeletal muscle taurine content in the PLANT, with similar percentage increases observed for the DIA. This 18 % increase in skeletal muscle taurine content as a result of supplementation is slightly less than was observed in rats with the same treatment protocol, where an increase of ~37 % was found in the EDL with taurine treatment (Dawson et al., 2002). Similarly, Goodman et al. (2009) found that 2.5 % w/v taurine supplementation via drinking water resulted in a 39.5 % improvement in taurine content of rat EDL muscle. This suggests that the same dose of taurine supplementation is less effective in increasing skeletal muscle taurine content in *mdx* mice, and as such, higher doses of taurine may be necessary to increase skeletal muscle taurine content in mice that are comparative to vailues reported with taurine supplementation in rats. Indeed, it appears that 2-3% w/v of taurine in drinking water is close to the maximum tolerable dosage for rats, as adverse side-effects such as diarrhoea have been reported with this supplementation protocol within the literature (Dawson et al., 2002), and through unpublished observations from our lab. However, no such symptoms were observed in association with taurine treatment in CON or *mdx* mice, suggesting that mice may potentially be able to tolerate higher doses of taurine. As no other studies are available in the literature examining the effect of different doses of taurine supplementation on dystrophic skeletal muscle taurine content, or within any mouse model, further investigations are necessary to determine the most effective dosage of taurine treatment to maximally increase skeletal muscle taurine content.

Likewise, there are very few examples in the literature examining the effect of *β*-alanine supplementation on skeletal muscle taurine content (Dawson et al., 2002, Hill et al., 2007). Indeed, this is the first study to examine the effect of *β*-alanine supplementation on taurine content in the dystrophic *mdx* mouse. Treatment with 3 % w/v *β*-alanine in drinking water significantly depleted taurine in the PLANT and DIA muscles by ~16 % (CON) and ~12 % (MDX-BAL), although as was observed for taurine supplementation, this depletion was significantly less effective in mice than has previously been reported in rat skeletal muscle. Dawson et al. (2002) is the only study to examine the taurine content of skeletal muscle after *β*-alanine supplementation in rodents, finding a 40-50 % decrease in taurine content of several muscles, including the EDL, SOL and GAST after four weeks of *β*-alanine supplementation in the rats. A study of *β*-alanine supplementation in humans found no significant difference in skeletal muscle taurine following supplementation, although relative to the dose used in the rat study the amount administered to humans in this study was significantly less (Hill et al., 2007). As suggested for taurine supplementation, it is possible that mice can tolerate higher *β*-alanine treatment, and thus a higher dose of *β*-alanine would be necessary to achieve depletion of taurine to 40-50 % of untreated control.

6.5.4 The effect of taurine and *β*-alanine supplementation on plasma CK concentration

Despite taurine being proposed to protect skeletal muscles from damage (Dawson et al., 2002, Huxtable, 1992, Uozumi et al., 2006b), the few studies that have examined the effect on taurine supplementation on plasma CK concentration in the literature have shown no effect on plasma CK in association with taurine treatment (Cozzoli et al., 2011b, Dawson et al., 2002). In the present study, plasma CK was actually higher in the taurine-supplemented *mdx* group, however this may be due to methodological issues. Blood collection via this method was difficult in such small mice, and may also account for the large variability observed in plasma CK across all groups. Cardiac puncture was used in this study as opposed to cavity blood (in Chapter 5) in order to minimise contamination of the sample, however, this has lead to far more variation in the sample than was observed in Chapter 5, and has introduced another factor that needs to be considered when collecting samples and analysing results. Unfortunately, too many of the *mdx* taurine group were noted as having been difficult to obtain blood from, and thus

insufficient numbers are available for analysis of only those samples that were collected with a single puncture and yielded a large volume of blood.

6.5.5 The effect of taurine and *β*-alanine supplementation on EDL contractile function

Increasing skeletal muscle taurine content has previously been shown to improve maximal force output. Bakker and Berg (2002) demonstrated that introducing taurine to a skinned muscle fibre preparation of a rat EDL caused 20 % greater depolarisation-induced isometric force, while more recently, Goodman et al (2009) also reported a significant increase in peak tetanic force in the EDL of taurine supplemented rats. In the present study, taurine supplementation was unable to significantly increase peak tetanic force in CON or *mdx* mice, even though grip strength has previously been reported to increase with taurine treatment in *mdx* mice (Cozzoli et al., 2011b, De Luca et al., 2003). Moreover, no significant differences in twitch characteristics were observed with taurine treatment, despite factors such as TTP and ½ RT being indicators of Ca^{2+} handling. This was unexpected; as Goodman et al. (2009) found that taurine supplementation resulted in 19 % greater twitch force, longer TTP and a trend for increased ½ RT in fast-twitch skeletal muscle. In addition, Bakker and Berg (2002) suggested that taurine may improve skeletal muscle force production via augmenting SR Ca^{2+} uptake and release, which would alter the twitch characteristics of myofibres. The results in the present study are consistent with the results obtained in Chapter 5, despite differences in the dose taurine supplementation, and the use of a higher temperature in the muscle bath. It is likely that the differences observed between the present study and those that are available in the literature on taurine supplementation and strength in the *mdx* are due to differences in the experimental protocols implemented. Both DeLuca et al.(2003) and Cozzoli et al. (2011) used grip strength meters to assess muscle strength, therefore incorporating the voluntary actions of several limb muscles relative to the single EDL subjected to electrical stimulation. In addition, the dystrophic phenotype of the *mdx* mice in both these studies had been purposely worsened with a chronic treadmill running program. As strength deficits in these mice would have been greater due to the increased skeletal muscle damage resulting from the treadmill exercise, it is possible that the effects of taurine were more pronounced in those studies, as deficits

within the muscle were greater. Certainly, even 6-month old *mdx* mice hind limb muscles still display only small episodes of damage and repair.

Although *β*-alanine supplementation is effective in reducing skeletal muscle taurine content, there is no evidence that it improves maximal strength (Hoffman et al., 2006, Kendrick et al., 2008). Results from the present study are consistent with these findings and the results of Hamilton et al. (2006), who used GES treatment to deplete skeletal muscle taurine content in mice and found no difference in maximal specific force with taurine depletion. In contrast to Hamilton et al. (2006), who showed reduced twitch force with GES treatment, maximum twitch force was unaffected in the present study, with *β*-alanine. In addition, Hamilton et al. (2006) also found a shift in the force frequency relationship with GES treatment, while the present study demonstrated no significant difference in the force frequency relationship with *β*-alanine or taurine supplementation. Considering the contractile protocol in the current study was modelled on the protocol used by Hamilton at al. (2006), the differences observed could not be due to differences in the experimental procedure, and are rather more likely due to the difference in the supplement used to induce taurine depletion. GES has been reported to significantly decrease skeletal muscle taurine concentration,as it competitively inhibits taurine uptake through the TauT (Hamilton et al., 2006). It is more effective in depleting skeletal muscle taurine content than *β*-alanine, with many studies showing a 60-70 % decrease in muscle taurine content after treatment (DeLuca et al., 1996, Hamilton et al., 2006). The expense of this chemical (50 g = $1120 US), however, means that supplementing large numbers of animals with GES can be impractical, and *β*-alanine (50 g = $70AUS) is a more viable alternative. However, as the maximum level of depletion achieved in the present study was 16 %, versus the greater than 60 % depletion achieved with GES treatment by Hamilton et al. (2006), this likely accounts for the variation in results between the studies.

Although taurine and *β*-alanine supplementation had no effect on tetanic and twitch force characteristics, significant differences in muscle fatigue and recovery of muscle force were observed with both treatments in the present study. Interestingly, the effects of the supplements were not consistent in both CON and *mdx* mice, suggesting that the mechanism of action is different in non-dystrophic versus dystrophic muscle. Taurine-supplemented *mdx* mice maintained a significantly higher percentage of their pre-fatigue

force relative to the UNT-MDX group, while taurine had no effect on fatigue in CON mice. Taurine has previously been shown to decrease fatigue, with Dawson et al. (2002) finding a significant increase in running performance, while Yatabe at al. (2009) reported an increase in the running time to exhaustion in taurine treated rats. In fast-twitch muscle specifically, Goodman et al. (2009) showed 6 % greater force in taurine treated rat EDL at the end of a continuous high-frequency fatiguing stimulation. The conflicting results regarding taurine's action on fatigue from the present study in the CON and *mdx* mice may result from the high temperature (30 °C) of the muscle bath in the present study. For example, Wineinger et al. (1998) examined the effect of age and temperature on *in vitro* contractile characteristics of *mdx* mice using the EDL muscle of 8 and 62 week old animals, at bath temperatures of 20 °C and 35 °C. When measured at 20 °C fatigability of the *mdx* EDL was similar to that of control mice however, when the temperature was increased to 35°C, *mdx* mice were significantly more susceptible to fatigue relative to non-dystrophic control EDL (Wineinger et al., 1998). This clearly suggests that there are temperature sensitive mechanisms that make the *mdx* EDL more fatigable (Wineinger et al., 1998). A possible explanation for this phenomenon is increased ROS production at higher temperatures, which has been shown to accelerate muscular fatigue (Reardon and Allen, 2009). As *mdx* mice are inherently more susceptible to oxidative stress and have impaired antioxidant systems (Haycock et al., 1996, Rando, 2002, Selsby, 2011), it is possible in the present study that the high temperature of the muscle bath more severely affected the *mdx* muscle. As such, the protective actions of taurine with regard to oxidative stress may have been more pronounced in the *mdx* mouse compared to CON mice. Indeed, several studies have shown that taurine protects against increases in oxidative stress, including lipid peroxidation, during skeletal muscle contraction (Dawson et al., 2002, Goodman et al., 2009, Sener et al., 2005, Silva et al., 2011).

Interestingly, *β*-alanine treatment caused a significant improvement in resistance to fatigue in both CON and *mdx* mice, suggesting that although *β*-alanine depletes taurine from fast-twitch muscle, it has its own beneficial effects on performance. Indeed, *β*-alanine is known to be an effective ergogenic aid, and has previously been shown to delay fatigue in humans and rodents (Stout et al., 2006, Stout et al., 2007). The beneficial effects of *β*-alanine treatment on performance are largely attributed increases in intramuscular carnosine, as *β*-alanine is required for carnosine synthesis, and four weeks of *β*-alanine supplementation has been shown increase intramuscular carnosine

pools by 40-60 % (Harris et al., 2006). While the most documented physiological role of carnosine is the maintenance of acid-base homeostasis, it has also been reported to have strong antioxidant and free radical scavenging activities (Hartman et al., 1990, Rajanikant et al., 2007), protect proteins from glycation (Hipkiss et al., 1995) and improve the sensitivity of the contractile apparatus to Ca^{2+} (Dutka and Lamb, 2004) particularly in fast-twitch skeletal muscle. These factors alone, or in combination, could explain the significant decrease in susceptibility to fatigue observed in CON and *mdx* mice in the present study.

As expected, given the lower level of fatigue observed, the percentage of original force during recovery (at 100 Hz stimulation frequency) of the *β*-alanine supplemented CON group was significantly higher than that of both the CON-UNT and CON-TAU mice. As taurine supplementation significantly decreased fatigue in the *mdx*, it is not surprising that during recovery, the MDX-TAU group maintained a significantly higher percentage of pre-fatigue force relative to both the MDX-UNT and MDX-BAL groups, and achieving recovery similar to the CON animals. Interestingly, although *β*-alanine also significantly decreased fatigue in the *mdx* mice, no significant difference in recovery post-fatigue was observed in this group. The reason for this is not immediately obvious, although may be related to the higher frequency of stimulation used during the recovery period than during the fatigue period, where the beneficial effect of the extra buffering capacity is more likely to be observed. Further investigation is required to clarify this phenomenon.

Despite *mdx* mice reportedly being more susceptible to fatigue relative to non-dystrophic control mice, no significant difference in fatigue was observed between the CON-UNT and MDX-UNT groups. This finding is not unusual, as there is conflicting data in the literature, with some groups reporting an increased susceptibility of *mdx* muscle to fatigue, while others report no change or even improvements in fatigue resistance (Gregorevic et al., 2002, Sacco et al., 1992, Watchko et al., 2002). It appears that fatigue in the *mdx* mouse is highly dependent on factors such as the age of the animal, conditions of the stimulation protocol and the temperature at which the experiments are conducted (Gregorevic et al., 2002, Hayes and Williams, 1998, Lynch et al., 2001b, Wineinger et al., 1998). For example, Connelly et al (2001) examined grip force in the *mdx* mouse across the lifespan from 3 to 24 weeks of age and demonstrated strikingly higher fatigue in *mdx* muscles of approximately 40-45 % of control values, which was

present at all time points and did not change with age (Connolly et al., 2001). Similarly Gregorevic et al. (2002) found that after four minutes of intermittent stimulation *mdx* muscles fatigued to 45% of their pre-fatigue force, while control muscles reduced by only 25% (Gregorevic et al., 2002). In contrast, Sacco et al (1992), using a lower stimulation frequency (40 Hz) than that used by Gregorevic et al (120 Hz) found that *mdx* muscles were in fact less fatigable than that of controls (Sacco et al., 1992). It has also been suggested that enhanced fatigue resistance could be the result of a shift in the MHC phenotype to a slower isoforms (Watchko et al., 2002). Such a fibre-type switch would leave *mdx* muscles with a greater oxidative capacity, a lower myofibrillar ATPase activity and enhanced fatigue resistance (Watchko et al., 2002). Thus, the lack of difference in the fatigability of EDL muscles from CON and *mdx* mice, may be due, in part, to a fibre-type shift to a slower MHC isoform in the *mdx.* This hypothesis, however, requires further investigation.

6.5.6 The effect of taurine and *β*-alanine supplementation on SOL contractile function

In the present study, no significant differences in SOL contractile function were observed in association with taurine or *β*-alanine supplementation in CON or *mdx* mice. This was not surprising, as slow-twitch skeletal muscle already contains substantial stores of taurine, and other examples within the literature have been unable increase this storage pool with taurine treatment (Dawson et al., 2002). Although taurine content in SOL were not measured in the present study, it is likely that our supplementation protocol was also unable to significantly increase taurine stores in slow-twitch skeletal muscle enough to impact on contractile function. Moreover, while *β*-alanine treatment has been shown to significantly deplete taurine content in the SOL by ~50 %, no changes in muscle function were observed with *β*-alanine treatment either (Dawson et al., 2002). This is surprising, as it has been suggested that intracellular taurine content is more strongly regulated in slow-twitch muscle, possible due to the oxidative nature of this fibre type requiring high intracellular taurine in order to function efficiently (Dawson et al., 2002). However, as already suggested, it is likely that the expected decline in contractile function with the loss of taurine is compensated for by the actions of *β*-alanine itself, possibly due to increased muscle carnosine, which is reported to have several actions that are consistent with those of taurine, such as anti-oxidative effects. In addition, the depletion

of taurine with *β*-alanine, as opposed to GES treatment, would likely see even less depletion in SOL muscles, and thus less effect. Unfortunately, as indicated, analysis of taurine content from slow-twitch muscles specifically in this study could not be undertaken.

Despite no significant differences in contractile properties or fatigue resistance with taurine or β-alanine supplementation, there were several differences in SOL contractile function when comparing CON and *mdx* mice. Non-dystrophic control muscles demonstrated significantly greater peak tetanic and twitch force, as well as an improved resistance to fatigue. The possible mechanisms proposed to cause dystrophic skeletal muscle to be more susceptible to fatigue include elevated ROS production, impaired oxidative metabolism and ionic disturbances (Kobayashi et al., 2008, Reardon and Allen, 2009). The recruitment of slow-twitch fibres, prevalent in the SOL, during mild exercise is supportive of this, and helps to explain why the same response is not necessarily observed in the fast-twitch EDL.

6.5.7 The effect of taurine and *β*-alanine supplementation on TauT protein expression

This thesis is the first to investigate TauT protein expression in the dystrophic *mdx* mouse, and the only study to examine skeletal muscle TauT protein expression in conjunction with taurine supplementation in any mouse model. The expression of the TauT transporter was a key line of enquiry in this thesis, as DeLuca et al. (2001) demonstrated a clear trend toward a decrease in taurine content in hind limb muscles of 6-8 month old *mdx* mice, coupled with an 140% increase in the plasma concentration of the amino acid. The authors suggested that these results indicate a difficultly in maintaining the appropriate concentration of taurine in dystrophic skeletal muscle (De Luca et al., 2001a). Thus, this thesis aimed to determine if the loss of taurine from dystrophic skeletal muscle was, in part, associated with alterations in the expression of the TauT.

While it is well accepted the taurine increases in plasma in association with skeletal muscle damage (Cuisinier et al., 2001, Dunnett et al., 2002, Nanobashvili et al., 2003), and that the pathology of dystrophic muscle would increase the plasma concentration of

taurine (McIntosh et al., 1998a, McIntosh et al., 1998b), it is also possible that the high plasma content of taurine observed in the *mdx* mouse are due to an inability of dystrophic skeletal muscle to take up taurine from the circulation. If alterations to TauT expression were found, it may help to explain some of the findings in the *mdx* mouse within the literature. Findings from Chapter 4 suggested that while TauT protein expression is significantly reduced in *mdx* mice at day 70, the fact that taurine supplementation was still effective in raising skeletal muscle taurine content to above CON levels reveals that it is likely that activity of the TauT is upregulated in dystrophic muscle, in order to compensate for the decreased expression of the TauT transporter. It is also possible that the TauT is less sensitive to extracellular taurine in dystrophy (potentially due to the chronically elevated levels resulting from muscle damage) and thus plasma taurine concentration needs to be elevated even further in order to overcome this insensitivity and increase intramuscular stores. Data from the current chapter shows that by six months of age, TauT protein expression is increased, such that significant differences in TauT expression are no longer observed between CON and *mdx* mice. Despite this 'normalisation' of TauT protein expression, the tendency for a decreased taurine content in the hind limb muscles of the *mdx* reported by DeLuca et al. (2001), coupled with the significantly decreased taurine content in the UNT-MDX relative to CON in the present study, suggests that although TauT protein expression in the *mdx* is not significantly different to non-dystrophic controls, that activity of the TauT may be decreased with age. There is also the potential that taurine efflux increases in dystrophic skeletal muscle with age, which could also account for the lower content of taurine observed in the *mdx* versus CON. However, it is important to note that taurine supplementation is able to significantly increase skeletal muscle taurine content, and thus potentially increases the activity of the TauT in *mdx* mice or by reducing taurine efflux.

6.5.8 The effect of taurine and *β*-alanine supplementation on E-C coupling and Ca^{2+} handling protein expression

Results from Chapter 4 demonstrated that taurine supplementation alters the expression of contractile proteins, preventing the decline in myosin expression in *mdx* mice relative to CON, and also significantly increased actin expression at day 28 and day 70. However, results from the six month old animals in the current study demonstrated no

significant difference in contractile protein expression, not only between CON and *mdx* mice, but also no difference in association with taurine supplementation. Moreover, in Chapter 4 significant differences were found in CSQ expression between CON and *mdx* mice, with CSQ expression being significantly decreased in *mdx* mice at day 70, which was rescued with taurine supplementation. However, in the present study, using adult mice, no significant difference in any E-C coupling proteins or CSQ expression were observed. Thus it appears that E-C coupling, Ca^{2+} handling and contractile protein expression is altered with age in *mdx* mice. This is also a key consideration for any potential therapeutic option for human DMD patients. Furthermore, this study demonstrates that, unlike in human DMD, E-C coupling, Ca^{2+} handling and contractile proteins may be upregulated as part of the recovery that is observed in the *mdx* mouse. This has implications for the testing of therapeutic interventions, and experiments attempting to elucidate the causes of skeletal muscle pathology in dystrophy.

6.6 Conclusions

In summary, this is the first study to show that taurine and *β*-alanine supplementation are able to significantly increase and decrease skeletal muscle taurine content, respectively, in CON and *mdx* mice. While the alterations to taurine content in association with both the supplements have no effect on peak tetanic force or twitch characteristics, taurine supplementation significantly increases resistance to fatigue in *mdx* mice but not in CON mice. Treatment with *β*-alanine significantly improved resistance to fatigue in both CON and *mdx* mice, suggesting that although *β*-alanine depletes skeletal muscle taurine content, beneficial effects of the *β*-alanine supplement itself causes improved muscle function that may compensate for the loss of taurine.

CHAPTER SEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Summary of the major findings

The findings reported in this thesis demonstrate that taurine supplementation is able to increase skeletal muscle taurine content in the dystrophic *mdx* mouse during the early acute damage phase, through to adult animals where a chronic, but low degree of skeletal muscle damage persists. Interestingly, although TauT protein expression is significantly decreased in *mdx* mice at day 70, taurine supplementation still results in an increase in intramuscular taurine stores, suggesting that TauT activity may be upregulated with taurine supplementation. At some point between day 70 and 6 months of age, recovery of TauT protein expression occurs, as there was no difference in TauT expression between non-dystrophic controls and adult *mdx* mice. Moreover, taurine supplementation does not reduce TauT protein expression in skeletal muscle, despite downregulation of TauT expression with taurine supplementation previously being reported in other tissue types (Matsell et al., 1997, Tappaz, 2004). Supplementation with the competitive inhibitor of taurine transport, β-alanine, did not significantly impact on TauT protein expression.

With regard to measures of muscle function, taurine supplementation at 2 % and 3 % w/v via drinking water, and in the long- and short-term, was unable to increase peak tetanic force in six month old *mdx* mice, despite improvements in grip strength previously being reported in association with taurine supplementation (Cozzoli et al., 2011b, De Luca et al., 2003). Peak twitch force, TTP and ½ RT were also unchanged with taurine treatment. This was surprising, as twitch kinetics are often used as a measure of Ca^{2+} movements, which have been shown to be altered with elevated taurine content in skeletal muscle (Bakker and Berg, 2002, De Luca et al., 1998, De Luca et al., 2001a). Interestingly, while no improvements in peak force were observed, taurine

supplementation did significantly reduce fatigue in the EDL of *mdx* mice, especially when examined at physiological stimulation frequencies and bath temperatures closer to typical muscle temperature. This novel finding that taurine is able to reduce fatigue in the *mdx* mouse is important, as dystrophy results not only in reduced strength but also increased fatigability (Frascarelli et al., 1988, McDonald et al., 2010), and thus fatigability is a measure for functional improvement with pharmacological treatments. The effect of depleting taurine content in *mdx* skeletal muscle on contractile function was also examined in this thesis. The results show that although taurine concentration was significantly reduced, no detrimental effects on muscle function were observed. In fact, β-alanine reduced fatigue in the *mdx* EDL to a similar level that was observed with taurine supplementation. While this might initially appear puzzling, this is likely due to the beneficial effects of β-alanine supplementation compensating for any detrimental effects of lowering skeletal muscle taurine . This thesis is also the first to examine the effect of taurine supplementation and depletion on contractile function of the slow-twitch SOL, finding that taurine supplementation and depletion had no effect on contractile function in this muscle. This is likely due to slow-twitch skeletal muscle already having a high concentration of taurine, and thus the effect of supplementation was less, while depletion was also likely to be less with dose of β-alanine used. Several E-C coupling and Ca^{2+} handling proteins were also investigated, early in the *mdx* lifespan and at six months of age. It was shown that taurine supplementation significantly increases the expression of the contractile proteins actin and myosin as well as CSQ, and depressed the expression of the RyR in young *mdx* mice, while no significant effects of taurine supplementation on any of these proteins are apparent when supplementation occurs later in the lifespan. Finally, this thesis is the first to show that taurine supplementation alters the activity of some key metabolic enzymes in dystrophic muscle, lowering PFK and β-HAD activity in the EDL, and increasing CS and CK activity in SOL.

7.2 The effect of taurine supplementation and depletion on skeletal muscle taurine content and TauT protein expression

Taurine supplementation was able to significantly increase skeletal muscle taurine content in mouse EDL, TA, PLANT, GAST and DIA muscles, despite the differences in the fibre-type of these muscles, and the degree of skeletal muscle damage occurring due to the differences in the age of the animals at experimentation. Interestingly, the

ability to increase intramuscular stores of taurine with supplementation was not impaired despite significantly less TauT protein expression being observed early in the *mdx* lifespan (day 28 to day 70), suggesting that the activity of that TauT may be increased in order to compensate for the lower transporter expression seen in dystrophic skeletal muscle. The observation that TauT is not significantly different from control animals at six months of age suggests that there is some recovery of TauT expression as skeletal muscle becomes more functional, and provides a rationale for why taurine content is reported to be not significantly different from non-dystrophic muscle in adult *mdx* mice (De Luca et al., 2001a) but significantly reduced early in the *mdx* lifespan (McIntosh et al., 1998a, McIntosh et al., 1998b). As such, while *mdx* mice appear to have compensatory mechanisms (possibly increased activity) that increase skeletal muscle taurine content in adult mice, supplementation with taurine results in earlier activation of these mechanisms, allowing increased taurine accumulation in muscle earlier in the lifespan and thus potentially affording dystrophic skeletal muscle increased protection from damage. It is possible that TauT becomes insensitive to high levels of taurine within the plasma of the young *mdx* mouse (due to high levels of skeletal muscle damage leaking taurine into the plasma) (De Luca et al., 2001a, McIntosh et al., 1998a, McIntosh et al., 1998b), and that supplementation increases the concentration of taurine to a threshold that is then able to overcome this insensitivity, and increase the activity of the TauT.

Importantly, as was also shown by Goodman et al. (2009) in the skeletal muscle of healthy rats, taurine supplementation does not result in downregulation of TauT protein expression, although it is possible that taurine treatment reduces TauT activity leading to impaired uptake after long treatment periods. TauT expression increased with β-alanine supplementation ~25-30%, however this did not reach statistical significance, possibly due to low sample numbers. This suggests that although taurine supplementation does not alter TauT expression, that β-alanine treatment, if continued for a longer duration, may increase TauT protein expression in response to the drop in taurine stores. Also of interest is the finding that taurine supplementation at 2 % and 3 % w/v was unable to increase skeletal muscle taurine to concentrations that were comparable with rat studies using the same dose and duration of supplementation (Dawson et al., 2002). While 3 % w/v of taurine in drinking water results in deleterious effects in rats, causing diarrhoea and dehydration, no such effects were observed in mice administered the same dose.

Thus, it is possible that similar beneficial effects of taurine on dystrophic skeletal muscle may not have occurred as the dose of taurine was not high enough to raise skeletal muscle taurine content sufficiently to improve all measures, particularly muscle force production. Supplementation with β-alanine significantly depleted taurine content in both non-dystrophic and *mdx* mice, however as was observed with taurine supplementation, the degree of depletion achieved was not as great as previously seen in rat studies.

7.3 The effect of taurine supplementation and depletion on contractile function

Results from Chapter 5 and 6 of this thesis demonstrate that taurine supplementation is able to improve some aspects of contractile function in the fast-twitch muscle of the *mdx* mouse, however the beneficial effects of taurine are best observed under more physiological conditions. Specifically, short-term taurine supplementation was shown to be effective in reducing fatigue when a dose of 3 % w/v of taurine was administered and fatigue was tested with intermittent stimulation (70 Hz 250 ms every 1s for 1 minute) and at a bath temperature of 30°C, while short-term taurine supplementation at 2% w/v tested with a high-frequency continuous stimulation (120 Hz for 10 seconds) at a bath temperature of 24°C yielded no significant improvements in resistance to fatigue. This demonstrates the importance of physiological experimental conditions when measuring whole muscle contractile function, particularly for dystrophic skeletal muscle, and when assessing fatigue.

While the discrepancy between these results could be due to the different dosage of taurine used between these studies, it is most likely that the beneficial effects of short-term taurine supplementation were only observed in the physiological contractile protocol as *mdx* skeletal muscle has been shown to be more prone to fatigue when exposed to higher muscle bath temperatures (Wineinger et al., 1998). At lower temperatures, the difference in fatigability between non-dystrophic control and *mdx* muscle appears to be less distinct (Connolly et al., 2001, Gregorevic et al., 2002, Sacco et al., 1992, Wineinger et al., 1998). This is likely due to elevated ROS production at higher temperatures leading to increased fatigue (Reardon and Allen, 2009), and considering *mdx* skeletal muscle is already more prone to oxidative stress and has impaired antioxidant systems (Haycock et al., 1996, Rando, 2002, Selsby, 2011), higher

muscle bath temperatures may accentuate this impairment. However, it should be noted that the temperature of the muscle bath in Chapter 6 is closer to what skeletal muscles would experience *in vivo*, and thus this protocol is not artificially stressing the muscles more than would be experienced under normal conditions. Interestingly, depletion of taurine stores with β-alanine supplementation did not significantly impact on skeletal muscle function in the *mdx* EDL or SOL, and actually also resulted in improved resistance to fatigue in the EDL. This suggests that although taurine is essential for skeletal muscle function, direct effects of β-alanine are able to compensate for the loss of taurine in dystrophic *mdx* mice, possibly through increasing muscle carnosine. As carnosine has been reported to have some similar effects to taurine, including protection from ROS, as well as increased buffering capacity, it is likely that protection from fatigue with β-alanine supplementation is linked to carnosine's ability to protect *mdx* muscle from oxidative stress and/or the effects of lower pH. Unfortunately, no measures of oxidative stress, antioxidant levels or muscle pH were conducted in the current thesis, thus this hypothesis cannot be confirmed at present. Interestingly, long-term taurine supplementation in the *mdx* EDL did show reduced fatigue when tested with the high-frequency stimulation protocol, demonstrating that long-term taurine supplementation may be more beneficial than short-term treatment regimes, as positive effects on muscle function were observed even under non-physiological conditions. It is unfortunate that due to time constraints and the cost of housing that long-term supplementation of *mdx* mice could not be evaluated with the more physiological contractile protocol, as it is possible that distinctions between the treatment duration would be apparent.

Another key finding of this thesis, which contrasts with the two studies on *mdx* muscle strength after taurine supplementation (Cozzoli et al., 2011b, De Luca et al., 2003), is that peak tetanic force was not significantly increased with any of the taurine treatment protocols. This could be due to the use of grip strength as a measure of force in the previous studies reported within the literature, while the current thesis has examined *in vitro* contractile function of specific muscles. Clearly, grip strength incorporates the voluntary force output of several muscles within the limb and is a functional measure under physiological conditions. In contrast, the current thesis evaluated the function of a electrically stimulated single muscle, rather than a group of muscles working synergistically. Another possible reason for the discrepancy in results is that the dystrophic phenotype of the *mdx* mice in both the Cozzoli et al. (2011) and DeLuca et al.

(2003) studies had been purposely exacerbated with a chronic treadmill running program. As strength deficits in these mice would have been greater due to the increased skeletal muscle damage resulting from the treadmill exercise, it is possible that the effects of taurine were more pronounced as a result of the exercise program (Cozzoli et al., 2011b, De Luca et al., 2003). Certainly, even 6-month old *mdx* mice hind limb muscles still display only small episodes of damage and repair, and the greatest effect of taurine supplementation on taurine content and protein expression in the present thesis was observed in Chapter 4, where young *mdx* mice during the peak degenerative phase were examined. Indeed, based on the findings from Chapter 4 of this thesis and the studies on exercised *mdx* mice, it appears that taurine is most beneficial when the muscle is in a significantly compromised functional state. This is of particular interest with regard to DMD, as it is possible that taurine supplementation may result in the most functional gains when administered during peak degeneration, with the aim of preserving muscle function. Considering to date there is no clinical evidence that beginning steroid use after the loss of ambulation has any beneficial effects on patient outcome (Bushby et al., 2004, Manzur et al., 2004), it is possible that taurine could be used as a therapeutic treatment once the disease has progressed and still improve muscle function.

7.4 The effect of taurine supplementation on metabolic enzyme function, E-C coupling and Ca^{2+} handling proteins

Taurine supplementation altered the enzyme activity in dystrophic skeletal muscle, lowering PFK and β-HAD activity in the EDL, and increasing CS and CK activity in SOL. These changes occured despite no significant changes in contractile function occurring within SOL, and the suggestion that taurine stores in slow-twitch skeletal muscle, which are already high compared to fast-twitch muscle, cannot be significantly increased with supplementation. As taurine cannot be directly used for energy production, the effects of taurine on enzyme activity could be due to increased protection from Ca^{2+} entry and ROS production in dystrophic skeletal muscle, which would then protect the mitochondria. Indeed, the reduced ROS production that has been suggested as a possible mechanism for taurine's effect on fatigue also supports the changes to enzyme function observed in the SOL, as excessive ROS directly impacts mitochondrial function as well as triggering increased Ca^{2+} entry into myofibres which further impairs energy

metabolism. As these positive effects were not seen in the EDL, it is likely that the protective effects of taurine with regard to energy metabolism may be different in slow versus fast-twitch skeletal muscle.

In addition to the changes in enzyme function, this thesis showed that taurine supplementation can modulate the expression of contractile proteins, RyR and CSQ early in the *mdx* lifespan. However, as supplementation with taurine at 5 months of age has no effect on these measures, this suggests that the effect of taurine is largely dependant on the phenotype of the skeletal muscle being investigated and the rate of skeletal muscle turnover. The increase in contractile proteins and CSQ, but depression of RyR, expression is a particularly interesting finding; it appears that when high levels of skeletal muscle damage are occurring (likely through Ca^{2+} driven effects), taurine increases CSQ expression to increase the ability of the SR to store Ca^{2+} while reducing RyR expression to reduce Ca^{2+} release, and also minimise leak. Taken together with the increase in actin and myosin, this may mean that taurine is able to improve muscle function, while limiting Ca^{2+} leak into the myoplasm. This, however, requires further investigation.

7.5 Limitations

As there is so little information currently available in the literature on taurine and dystrophic skeletal muscle function, the current thesis focused on determining if taurine supplementation was able to significantly increase skeletal muscle taurine content (as it has been suggested that there is an impaired ability to retain taurine in dystrophic muscle), and if altering taurine content (increasing or decreasing) impacted on measures of muscle function. These two key aims were chosen because although taurine had been shown to improve grip strength in *mdx* mice, no study has examined the effect of taurine supplementation on other key contractile measures that are relevant to dystrophy, such as fatigue and recovery, and the effect of taurine on fast- versus slow-twitch dystrophic muscle had also not been evaluated. Moreover, if no improvements to muscle function with taurine treatment were observed, there would be little point in investigating other alterations with taurine treatment. Thus, the impact of taurine supplementation on dystrophic muscle contractile function had to be a key line of

enquiry, and as such, investigation into several other effects of taurine supplementation were not evaluated, creating several limitations within this thesis.

The first limitation to the present group of studies is that only indirect measures of muscle damage were made (plasma CK), so the effect of taurine supplementation on damage levels in dystrophic skeletal muscle cannot be definitively determined from the present work. As reduced skeletal muscle damage is an important outcome for DMD treatments, direct measures of skeletal muscle damage in dystrophy via histological examinations need to be made. A second key limitation to the current work is that all measures of contractile function were obtained from adult *mdx* mice, where chronic but low levels of skeletal muscle damage are present. The use of *mdx* mice at this age, while common in the literature, does raise the question as to whether they are a good model for DMD at this stage of the lifespan, as skeletal muscle pathology is far milder than that observed in human DMD. Exercised *mdx* mice might have been a better model for these studies. However, as the equipment required to accurately control exercise training is expensive, and was unavailable in our laboratory, this was not possible for the current thesis. Moreover, while exercise exacerbates the dystrophic condition, it also initiates a number of repair and adaptive mechanisms that would also influence the effects of taurine supplementation. Further, all contractile measures were conducted *in vitro* with the normal blood and nerve supply to the muscle cut, and as significant differences in the effect of taurine with varying stimulation frequencies and bath temperatures were observed, the transferability of these results to *in vivo* function may be limited. From a methodological perspective, the fact that taurine concentration was not measured in the same muscle as the contractile and biochemical testing due to the small muscle mass available is a limitation. It would be beneficial to develop a technique that measures taurine content in muscle using only small amounts of tissue as this would strengthen the results. Finally, while the greatest change in contractile proteins, E-C coupling proteins and CSQ were observed in young *mdx* mice, the functional effects of these changes were not evaluated in the present study.

7.6 Future directions

The results from this thesis have raised several key lines of enquiry that need to be investigated with further research. As taurine supplementation did not increase skeletal muscle taurine content to the same magnitude that has previously been observed in rats

with the same dose, additional research is required to find the optimum dose of taurine that can be administered to mice, which will maximally increase skeletal muscle taurine content without any detrimental side-effects. In addition to optimising the dose of taurine, duration of treatment (acute versus chronic) also needs to be examined, as no measures of skeletal muscle taurine content, TauT or E-C coupling protein expression after life-long (long-term) treatment were made in the present thesis. Contractile function after taurine supplementation should also be measured *in vivo*, preferably in young and/or exercised *mdx* mice. Due to the differences in contractile proteins, RyR and CSQ that were observed in young *mdx* mice after taurine supplementation, single fibre investigations of E-C coupling in *mdx* mice at this age would also be interesting, to see how these changes affect force production and Ca^{2+} handling. In addition, direct histological measures of skeletal muscle damage in *mdx* muscle after taurine supplementation needs to be examined, to determine whether taurine is able to attenuate skeletal muscle damage. Finally, as TauT protein expression in young *mdx* mice is decreased, but taurine supplementation still results in significant increases in taurine stores, activity of the TauT and possible post-translational modifications should be evaluated in *mdx* mice throughout the lifespan.

7.7 Conclusion

In conclusion, the results from this thesis have shown that taurine supplementation in the *mdx* mouse is able to significantly increase taurine stores within skeletal muscle, despite downregulation of the TauT early in the lifespan and varying levels of skeletal muscle damage. TauT protein expression is not decreased in association with taurine supplementation in skeletal muscle, as has been reported for other tissue types. While peak tetanic and twitch force were not altered with taurine treatment, significant improvements in fatigue and recovery were observed in dystrophic skeletal muscle after taurine supplementation. Interestingly, depletion of taurine stores with β-alanine did not result in detrimental effects on function in dystrophic skeletal muscle, most likely as beneficial effects of β-alanine compensate for the loss of taurine stores. Taurine supplementation during peak degeneration of skeletal muscle appears to have the most beneficial effects, increasing contractile proteins and the SR Ca^{2+} buffer CSQ, while depressing the expression of the RyR. The activity of metabolic enzymes were also altered with taurine supplementation in both fast- and slow-twitch muscle, suggesting

that taurine has the ability to modulate energy metabolism in dystrophy, albeit via indirect effects.

Cumulatively, the results from this thesis demonstrate that taurine supplementation can significantly increase skeletal muscle taurine content in the *mdx* mouse at multiple points in the lifespan, and has beneficial effects on dystrophic skeletal muscle function, particularly with regard to reduced fatigability and improved recovery. Further research is required to determine the mechanisms behind the effect of taurine on dystrophic skeletal muscle, however the results presented in this thesis suggest that taurine remains a possible therapeutic treatment for dystrophy that warrants further enquiry.

CHAPTER EIGHT

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