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The effect of insulin treatment and exercise modality on skeletal muscle fiber size in streptozotocin-induced type 1 diabetic rats

John Z. Nickels
The University of Western Ontario

Supervisor
Dr. Earl Noble
The University of Western Ontario

Graduate Program in Kinesiology

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ABSTRACT

Severe Type 1 Diabetes Mellitus (T1DM) is known to have several negative effects on skeletal muscle mass, a condition known as diabetic myopathy. One of these effects is the atrophy of the glycolytic muscle fibers. However, the role of intensity of insulin treatment and exercise modality in attenuating this loss in fiber cross-sectional area (CSA) has yet to be determined. The purpose of this investigation was to examine the effects of 12 weeks of differing intensity of insulin therapy and exercise modality on the CSA of plantaris muscle fibers of STZ-induced T1DM rats. Fibers were identified by myosin heavy chain (MHC) composition. Rats were divided into control sedentary (CS), T1DM-sedentary (DCT - conventional versus DIT-intensive insulin therapy, blood glucose 9-15mmol/l and 7-9mmol/l, respectively) and T1DM-exercised (DRE- resistance, DHE-endurance, DCE-combined training, blood glucose 9-15mmol/l) groups. Exercises consisted of repeated weighted ladder climbs (50%, 75%, 90%, and 100% pre-determined max. carrying capacity, 1.1 m, 80°) or treadmill running (27 m/min, 6% grade, 1hr) 5 days/week for 12 weeks. DCE animals performed resistance and endurance exercise on alternating days. Following 12 weeks, diabetic sedentary (DCT, DIT) and diabetic exercised (DRE, DHE, DCE) groups did not differ in body weight (BW) or plantaris whole-muscle weight, except where DIT BW was significantly greater than DRE ($P<0.05$). The moderate hyperglycemia in DCT did not adversely affect muscle fiber CSA suggesting insulin supplementation can protect against muscle fiber atrophy. In fact, intensive supplementation (DIT) resulted in larger fast twitch MHC's than either the control (CS) or conventional diabetic (DCT) conditions ($P>0.05$) or aerobic exercise (DHE) in some instances (all fibers expressing fast MHC's except MHCIIx/IIb and MHCIIb – $P>0.05$). Surprisingly, DIT exhibited

significantly larger MHCIIa and fewer hybrid fibers than any other group. MHCI fibers were unaffected by either diabetes or exercise, however, exercised animals displayed changes in percent fiber composition, including a significant proportion of hybrid fibers. These changes suggested a shift in fiber type particularly from MHCIIx to MHCIIa. Resistance exercise, whether alone (DRE) or in conjunction with aerobic training (DCE) resulted in significantly larger MHCIIx and MHCIIb than CS. Treadmill running (DHE) resulted in less hypertrophy of fibers expressing fast MHC than the other exercised groups. These findings indicate that conventional insulin therapy is sufficient to maintain muscle mass and fiber CSA, while intensive insulin therapy and exercise with conventional insulin therapy induce differential changes in fiber percent composition and fiber CSA increases.

Keywords: type 1 diabetes mellitus, diabetic myopathy, skeletal muscle, myosin heavy chain insulin, exercise, resistance training, endurance training, muscle fiber cross-sectional area, muscle mass

CO-AUTHORSHIP:

Dr. Earl Noble was involved in project design, interpretation of the results and thesis revisions.

DEDICATION:

For my parents, thank you for your endless love and support.

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List of Abbreviations

a/P_0	Relationship of Maximum Force and Max Shortening Velocity
AGE	Advanced Glycation End Product
AR	Aldose Reductase
ARI	Aldose Reductase Inhibitor
ATP	Adenosine Triphosphate
BMR	Basal Metabolic Rate
Ca_2^+	Ionic Calcium
CSA	Cross Sectional Area
ddH2O	Double distilled water
dH2O	Distilled water
DHEA	Dehydroepiandrosterone
ECM	Extracellular Membrane
EDL	Extensor Digitorum Longus
GADA	Glutamic Acid Decarboxylase
GLUT4	Glucose Transporter Protein 4
GPD	α-Glycerophosphate Dehydrogenase
IA2A	Insulinoma-associated Auto-Antibody 2
IAA	Insulin Reactive Auto-Antibody
IGF-1	Insulin-like Growth Factor 1
IMCL	Intramyocellular Lipid
MHC	Myosin Heavy Chain
MSTN	Myostatin
mTOR	Mammalian Target of Rapamycin
MUNE	Motor Unit Number Estimation

MuRF1	Muscle Ring Finger Protein-1
O_2	Oxygen
P_0	Maximum Isometric Force
PAI-1	Plasminogen Activator Inhibitor-1
PCr	Phosphocreatine
PFK	Phospho-Fructo Kinase
PI-3 K	Phosphatidyl-inositol-3 Kinase
ROS	Reactive Oxygen Species
SC	Satellite Cell
SDH	Succinate Dehydrogenase
SERCA	Sarcoplasmic Reticulum Ca2+-ATPase Pump
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TA	Tibialis Anterior
TCA	Citric Acid Cycle AKA Kreb's Cycle
Ub-P	Ubiquitin Proteasome
Vmax	Maximum Unloaded Shortening Velocity
ZnT8A	Zinc Transporter 8

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CHAPTER 1

1.1 Introduction: Diabetes Mellitus Overview

Diabetes mellitus is a term used to typically describe two distinct pathophysiological states, which although differing in the underlying physiological basis of their dysfunction, have in common a condition in which blood glucose levels are elevated (hyperglycemia) due to impaired insulin response, which ultimately leads to microvascular, neurological and macrovascular complications [1,2] . Over time, these complications often manifest themselves in the form of retinopathy, nephropathy, peripheral and autonomic neuropathy, atherosclerotic cardiovascular and cerebrovascular disease, and skeletal muscle myopathy [2–4]. In fact, in the United States diabetes mellitus is the leading cause of blindness, end stage renal failure, and non-traumatic limb amputation, and is also associated with a two-to-seven-fold increase in risk of developing cardiovascular and cerebrovascular disease [5]. Indeed, T1DM is a disease that not only carries dire health consequences if proper management measures are neglected, but also impacts a vast breadth of individuals globally.

Of the complications commonly associated with the disease, diabetic skeletal muscle myopathy has historically received less research attention comparatively. However, the role of maintaining a healthy skeletal muscle mass across the lifespan, in both healthy and diabetic populations, has more recently become an area of intensive investigative efforts [6–9]. In the context of diabetic myopathy, the importance of maintaining healthy skeletal muscle mass and function begins in adolescence and is vital in improving overall metabolic control, and consequently reducing the progression rate of other complications. The role of exercise and physical activity in not only the prevention of chronic disease, but in improving health and the

amelioration of various complications associated with diabetes mellitus has been identified as an important therapeutic strategy in the management of the disease [8,10–12]. As skeletal muscle exhibits a truly remarkable capacity to adapt to the various stressors exerted on it by exercise, identifying the specific effects of differing modalities of exercise (i.e. aerobic, resistance, combined) on skeletal muscle in individuals with diabetes provides a unique opportunity to examine the effectiveness of exercise in the prevention and treatment of myopathy. The aim of this chapter is to provide an overview on type 1 diabetes mellitus specifically, describe the heterogeneity and plasticity of skeletal muscle fibers, and the pathology and mechanisms underlying diabetic myopathy.

1.2 Classification and Diagnosis Criteria

Diabetes mellitus diagnoses generally fall into two etiopathogenetic categories: Type 2 diabetes mellitus (T2DM) in which elevated fasting blood glucose develops as the result of resistance to insulin action, and Type 1 diabetes mellitus (T1DM) in which an autoimmune mediated destruction of the pancreatic beta-cells in the Islets of Langerhans results in an absence of insulin secretion, and in-turn elevated blood glucose [13]. Symptoms that commence prior to a diagnosis of Type 1 diabetes often include polydipsia, polyuria, weight loss and excessive hunger, and are believed to be the result of the insulin secretion deficiency induced hyperglycemia [13,14]. The diagnosis of T1DM generally involves two measurements of a fasting blood glucose level of 7 mmol/L or higher or any blood glucose level over 11 mmol/L, and has been expanded to include glycated haemoglobin (HbA_{1C}; a measure of glycemic levels over 3 months) of 6.5% or greater [15,16]. One of the primary distinguishing features between T1DM and T2DM that has garnered predictive diagnostic consideration in the clinical setting is the presence of serological auto-antibodies specific to beta-cell auto-antigens. Over 90% of individuals with

T1DM express one or more of the following auto-antibodies in the months or years prior to onset: insulin reactive (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated auto-antigen 2 (IA2A) and zinc transporter 8 (ZnT8A)[17–19]. Of particular concern is the increasing number of individuals with T1DM who have developed insulin resistance, the primary pathology underlying T2DM [20]. It has been hypothesized that this may be a result of the intensive insulin therapy program (that most individuals with T1DM partake in) driving the increase in average body weight of T1DM patients that has been observed in recent decades [21,22].

1.3 Epidemiology

T1DM represents 5-15% of all diabetes mellitus cases, and it is estimated that there are over 33 million individuals with T1DM worldwide, and although uncommon, as noted above, individuals can be diagnosed with T1DM and T2DM simultaneously [14,23]. It has also been proposed that 5-15% of adults diagnosed with T2DM may actually have T1DM, due to difficulties in discerning between T2DM and T1DM diagnoses, and thus the actual number of T1DM cases may be underestimated [24]. T1DM has also been referred to as *juvenile-onset diabetes* as it commonly develops during adolescence in genetically susceptible individuals as the consequence of an environmental trigger [14,25,26]. However, it should be noted that although the age-specific incidence rate rises in the years between infancy and puberty and then tends to decline, nearly 30% of T1DM patients are diagnosed after age 20 [5].

Alarmingly, T1DM incidence rates have been steadily increasing annually on a global scale, reported to be as high as by 5.3% , 3.3%, 2.6% and 2.4% in the United States, Norway, Germany and Finland, respectively [5,14,27–29] It is also of note to consider that the most substantial increases in incidence seem to have been amongst children; in Europe, for example, children

under 5 years have experienced the greatest increment [30]. Based on the current trajectory for T1DM incidence trends, the number of children under 5 years of age in Europe is expected to double between 2005 and 2020 [27]. Considering the fact that the majority of individuals that are diagnosed during childhood will likely face a life-time of erratic fluctuations in glycemic levels and in turn an increased risk of developing future complications, the importance of early and effective management is critical [31].

1.4 Complications:

Type 1 diabetes has long been associated with a greater risk of mortality due to complications, with estimates ranging from 5 – 10 times greater risk compared to individuals without the disease [23]. The majority of the complications associated with T1DM are classified as either microvascular or macrovascular in etiology. Since the introduction of insulin therapy in the management of T1DM, long-term complications such as retinopathy, neuropathy, nephropathy and cardiovascular disease have represented the greatest contributors to morbidity and mortality [1]. The systemic nature of the metabolic dysregulation associated with T1DM and thus chronic hyperglycemia results in pathological complications that are extensive in range. As such, additional debilitating complications that arise as a consequence of T1DM include blindness, end stage renal failure and non-traumatic limb amputation, all of which can contribute to a chain of circumstances which accelerate the deterioration of health [5]. In 1993, the Diabetes Control and Complications Trial provided critical evidence for the role of intensive insulin therapy (and thus strict glycemic control) in reducing the progression of these complications, and identification of the underlying mechanism of hyperglycemia-induced damage has provided insight into the underlying pathogenesis of macrovascular disease, establishing a basis for the goals of T1DM management [2]. Despite the benefits of intensive insulin therapy in attenuating

the progression of the aforementioned complications, one of the drawbacks of maintaining rigid glycemic control is the potential development of insulin resistance, which has been observed in T1DM populations [32].

Of the many organs detrimentally affected by T1DM, skeletal muscle has received less research attention. Skeletal muscle is the largest metabolically active organ by weight, the body's primary glucose sink, and central to insulin sensitivity and basal metabolic rate and experiences a myriad of dysfunctions in response to T1DM collectively known as myopathy [33,34]. Diabetic myopathy is often clinically defined by lower muscle mass, weakness, a reduced physical and regenerative capacity, as well as several other histopathological, hormonal, and metabolic abnormalities [4,33–39]. Interestingly, pathological alterations such as muscle fiber atrophy and mitochondrial morphological abnormalities can occur in recently diagnosed T1DM patients (1 – 28 weeks duration) prior to the manifestation of diabetic neuropathy, indicating that skeletal muscle is acutely sensitive to the T1DM milieu [34,40]. When the array of physical and functional consequences associated with diabetic myopathy are considered in conjunction with the predominance of T1DM manifestation in adolescence, a significant period of muscle growth and development, the importance of addressing diabetic myopathy is quite clear. A more detailed discussion of the pathology and potential underlying mechanisms exacerbating diabetic myopathy will be presented later in this chapter, after sufficient elucidation of skeletal muscle physiology and muscle fiber heterogeneity is provided.

1.5 Skeletal Muscle Overview:

Skeletal muscle is a truly remarkable tissue, having occupied a fundamental role in human evolution and survival, and continuing to act as a critical determinant of health and disease. In humans it represents 40% of total body weight, 50-75% of all body proteins, and 30-50% of all protein turnover [41,42]. Skeletal muscle is essential to locomotion, stability/postural maintenance, thermogenesis and whole-body metabolism; the heterogeneous nature of the individual muscle cells/fibers also permits muscles to perform a wide variety of tasks spanning the spectrum of motor control demands [33,43,44]. The impressive variation in muscle fiber structural and functional properties is mediated by different amounts and specific kinds of proteins (of which there are a multitude of isoforms) which function together to define fiber phenotype, and as such enable an exquisite adaptability to physical demands [42,44] . In Hochachka's "*Muscles as Molecular and Metabolic Machines"* the author provides an extensive list of specific proteins (albeit only including the minimal requirements), which outline the functional and structural muscle fiber components that describe a typical fast glycolytic fiber phenotype. As such, the author fixates on the fact that this brief list of components alone would require the regulated expression of over 100 genes, and thus the adaptive plasticity exhibited by muscle fibers is theoretically only limited by the number of ways in which the contributing isoforms are assembled [42].

Human skeletal muscle displays a remarkable capacity to adapt to the physiological stresses imposed on it by physical activity and exercise demands. For example, an examination of the vastus lateralis skeletal muscle properties of a world-champion sprint runner revealed a high abundance of pure MHC IIX fibers (24%) that were highly transcriptionally responsive to high intensity exercise, and a total glycolytic fiber population of 71% ; the power output of

which were higher than any reported human values to date [45]. Conversely, as East African black athletes have long been regarded as dominant in endurance athletic events, investigation of vastus lateralis muscle biopsies of Xhosa compared to Caucasian endurance athletes (matched for training and racing distances) revealed that Xhosa runners exhibited lower plasma lactate concentrations at 80% Peak Treadmill Speed (PTS) and significantly higher percentage composition MHC IIa and lower MHC I compared to Caucasian runners, although oxidative enzyme activities did not differ [46]. The skeletal muscle phenotypic adaptations found in humans occur along a broad spectrum and are implicated in not only athletic performance, but play a significant role in the maintenance of health across the life span.

1.6 Fiber Type Nomenclature and Classification:

The process in which the heterogeneous properties of skeletal muscle fibers have been elucidated and classified has been gradual, mirroring advancements in available scientific techniques capable of investigating the differing functional, metabolic, and structural properties. As such, the nomenclature has evolved accordingly, in order to appropriately catalogue these findings and incorporating more information in each definition. Spangenburg and Booth provide a description of this process, beginning as early as 1873 when Ranvier distinguished "red" muscles that contracted slowly from "white" muscle that contracted fast [47]. In the 1960's these muscles/fibers (type I and II) were further classified by their contractile properties as well as their oxidative capacity, introducing a new sub-classification of type IIA and type IIB [48,49]. The refinement of analyses of metabolic properties of muscle fibers through microdissection of single fibers also allowed the identification of widely varied enzyme activities between fiber types [50–52]. In the 1980's, the use of electrophoretic separation techniques allowed correlation between MHC composition, contraction speed and myosin ATPase to be identified [53,54]. The MHC composition

based classification of muscle fibers was furthered expanded with the identification of a third unique MHC, MHC IIx/d [55–57]. The subsequent improvements in immunohistochemical techniques and comparison with histochemical myosin ATPase fiber typing permitted the observation of human fibers histochemically staining positive for type IIB myosin ATPase to actually expressing MHC IIx/d, and as such delineating a complete lack of MHC IIb in human muscle [58]. One of the more recent observations of importance to the MHC centered approach to fiber phenotype nomenclature was the identification of fibers displaying heterogeneous MHC composition; hybrid muscle fibers [59,60]. Although not all muscle proteins associated with specific MHCs are necessarily expressed in parallel, the central role of myosin as the molecular motor and the differing MHC composition across fiber types suggest MHC expression as the best available marker for fiber typing and communication purposes [44,47]. Thus, according to the MHC based classification and nomenclature of fiber types, fiber phenotype appears to occur along a spectrum that could be described as MHC I ←→MHC IIa ←→ MHC IIx/d ←→ MHC IIb with hybrid MHC co-expression occurring "between" pure MHC fibers. This MHC expression sequence is not requisite however; fibers co-expressing MHC I and IIx while lacking MHC IIa have been identified [44,61].

1.7 Skeletal Muscle Fiber Diversity:

The diverse nature of the functional, structural, metabolic, and adaptive properties of muscles fibers is essential to the ability of skeletal muscles to perform tasks of varying physical demands. Phenotypic changes in response to neural and hormonal influences involve a myriad of different molecular mechanisms responsible for mediating the activation of specific gene programs, and in turn, specific isoform expression [44,47,62]. The significant role of muscle activity in the prevention and amelioration of chronic disease and complications suggests the importance

of further investigation in elucidating activity (and inactivity) dependent signaling pathways in order to identify potential therapeutic targets [44]. As mentioned previously, MHC IIb is only expressed in some mammals (i.e. mice, rats etc.). The complete absence of MHC IIb fibers in human skeletal muscle is likely the consequence of body size's role in functional demands, where the energy metabolism for unit body mass is inversely related to body size (Kleiber's law) and basal metabolic rate (BMR) per unit mass varying with three-fourth power of body mass [44,63]. The thickness (diameter) of individual muscle fibers also appears to scale allometrically; although surprisingly human cyclists have been shown to possess significantly larger vastus lateralis fibers than those obtained from a single lion (*Panthera leo*) specimen, indicating exceptions to this observation exist [64]. Additionally, the maximum shortening velocity of each fiber type appears inversely allometrically scaled, although this relationship is not fixed as horse type I and IIa fibers maintain greater shortening velocities than humans despite significant body mass disparity [44,65,66].

1.8 Effective Motor Neuron – Muscle Fiber Coupling… the Motor Unit:

As the primary function of fiber heterogeneity can be simplified to a necessity of coupling specific isoforms in such a way that the physical requirements stipulated of a muscle can be achieved efficiently, one of the most basic pairings is that of a motor neuron with appropriate fiber constituents. Efficiently matching fiber phenotypes with specific neural activity patterns is an essential aspect of utilizing the distinct properties of different fiber types and plays a primary role in fiber remodeling in adult skeletal muscle [44]. As such, the neuromuscular system is organized into discrete units (motor units) consisting of a motor neuron and the homogeneous muscle fiber population it innervates [67]. In 1985 Henneman proposed the "size principle" which theorized the orderly recruitment of motor units based on nerve cell size, where

slow twitch motor units are recruited before larger fast twitch units [68]. The activation of motor units of increasing size is critical to the performance of fine motor skills, and therefore muscles in the human hand /forearm tend (abductor pollicis longus) to contain a high number of motor units despite a smaller absolute size comparatively, as determined by motor unit number estimation (MUNE). As one of the health consequences of T1DM is neuropathy, MUNE has been recommended as a measure to predict motor unit dysfunction in animals, and MUNE has even been found to be lower in children with T1DM [69,70].

1.9 Myosin Isoform Contractile Properties:

The myosin isoforms present in skeletal muscle are the primary determinants of fiber contractile properties, ATP consumption, and the fiber's ability to respond to the neural demands imposed on it. In accordance with the diversity of motor unit activity patterns, the contractile performance and ATP consumption requirements of the different myosin isoforms occur across a spectrum. The mechanical power generated by muscle fibers defined in terms of power is characterized by the mechanical energy generated per unit of time, and as such is directly related to the chemical energy released by ATP hydrolysis via myosin ATPase [44]. Peak power output values increase from slow MHC type I fibers to IIb fibers, which possess the highest peak power values of all mammalian species; indeed, this range across fibers can be as high as 10 fold when measured in vitro at low temperatures [44,71].

Mechanical power generation is determined by the interaction of the maximum isometric force (P_o) and the maximum unloaded velocity (V_{max}), and the curvature describing this relationship (a/P_o) essentially describes the force sustained at a particular velocity [44]. As such, these parameters determining power output display differences between fiber phenotypes. The

V_{max} of muscle fibers are determined exclusively by myosin isoform, and show a pattern of increasing velocity from slow fibers to fast fibers, with IIb fibers exhibiting the highest maximum velocity [64,72,73]. In fact, the higher overall proportion of pure MHC IIx fibers that have been observed in world champion human sprinters and the fastest terrestrial species on earth, the cheetah (*Acinonyx jubatus*), provide excellent examples for the superior speed of contraction parameters bestowed by these fiber phenotypes [45,74,75]. Maximum isometric force and specific force is also higher in fast compared to slow fibers and this disparity has been suggested to be the result of a greater number of strongly bound myosin heads compounded by greater force production per head, comparatively [64,76–78]. Thus appropriately, studies examining MHC isoform content in the vastus lateralis muscle homogenates of body builders have reported higher proportion (~15%) of IIx isoform compared to active young men (~5%) [79]. However, it should be noted that inferences made from muscle homogenates with the regular occurrence of hybrid fibers co-expressing MHC isoforms, an assumption of a greater number of pure IIx fibers is inappropriate unless detected at the single fiber level [75].

The myosin-ATPase activity levels of the different MHC isoforms also show marked variation in rate of energy release and consumption, and thus contribute to the contractile properties of muscle fibers. During isometric contraction in vitro, steady-state ATPase activity has revealed that ATP hydrolysis rate also increases across the spectrum of fiber types, with IIb fibers possessing the highest rate [80–82]. However, the amount of ATP consumed per unit time per unit tension (tension cost) is lower in slow fibers, thus contributing to their role in maintaining repetitive contractions and posture [80]. Taken together, these functional parameters support the role of different MHC fiber types being expressed in response to specific neural activity patterns while permitting a wide range of dynamic movements.

1.10 Metabolic Control:

The supply and regeneration of ATP during muscle activity is achieved through complex metabolic regulation involving the integration of several different biochemical pathways. Indeed, ATP hydrolysis provides the basis of all cellular energy supply, with 1 mol of ATP providing ~50-60 kJ energy, and during muscle contraction over 70% of ATP consumption is related to myosin ATPase activity and the remaining 30% ionic transport [83,84]. These pathways involve different metabolic substrates and are most simply differentiated as anaerobic (O_2-independent) or aerobic (O_2-dependent), and their relative utilization and efficiency of energy production shows marked variation between fiber types in response to their respective contractile and functional properties. ATP is generated anaerobically primarily through the phosphagen system substrate creatine phosphate (PCr) and through anaerobic glycolysis/glycogenolysis, which partially catabolizes glucose to rapidly supply energy while transitioning from rest and in the early stages of physical activity. Conversely, aerobic generation of ATP is maintained by aerobic glycolysis and mitochondrial oxidation of both glucose and free fatty acids, the complete catabolism of which enables huge increases in yield of ATP/mol of substrate and a greater capacity for sustained energy production [42]. The delay in activation of aerobic metabolic systems compared to the immediate reliance of anaerobic PCr and subsequently anaerobic glycolysis is termed "metabolic inertia", and this appears to be a pattern in which a decline in anaerobic ATP production/capacity is matched with an inversely parallel increase in oxidative mitochondrial ATP generation [85]. It has been suggested that in exercise conditions *in vivo* in humans, the limitations of the "cardiac pump" and thus O_2 delivery to muscle mitochondria is a major determinant of the activation of aerobic metabolic control [42,86]. In accordance with the impressive heterogeneity displayed in the mechanisms underlying neural activity patterns,

sarcolemmal excitability, EC coupling and myosin contractile properties, fiber types display pronounced differences across a continuous metabolic spectrum.

The different MHC fiber types exhibit considerable variation in fiber size and thickness, generally following a trend of increasing cross-sectional area from type I fibers being the smallest to the robust IIx and IIb fibers. This increase in fiber size is inversely related to the expression of the oxidative enzyme succinate dehydrogenase (SDH) and positively correlated with the glycolytic enzyme α-glycerophosphate dehydrogenase (GPD) [87]. The reliance of smaller fibers on primarily oxidative means of ATP generation is further supported by the presence of an increased absolute number and ratio of capillaries to fibers, as well as an increased relative proportion of mitochondrial volume (6%, 4.5% and 2.3% in MHC I, IIa, IIx respectively) [88,89]. Additionally, the mitochondria of slow oxidative fibers also exhibit morphological differences in the form of densely packed cristae [90]. From an enzymatic perspective, citric acid cycle (TCA) enzyme activities (essential to aerobic metabolism) are over twice as high in slow fibers compared to fast fibers in rabbit muscle [91]. The fundamental involvement of aerobic metabolism in sustaining muscle activity during repetitive contractions is further complemented by increased glucose uptake capacity, GLUT4 (glucose transporter) protein expression, and myoglobin content in slow compared to fast fibers [92–94]. Interestingly, myoglobin is nearly absent in IIb fibers, and in humans oxidative fibers exhibit ~50% higher content than fast glycolytic fibers [94,95]. In terms of mitochondrial oxidation, FFA's contribution to TCA is also higher in slow fibers compared to fast fibers, and slow fibers have also shown greater O_2 consumption when maximally stimulated (0.048 mM/s and 0.022mM/s in soleus and gastrocnemius, respectively) [96].

The larger, glycolytic fibers exhibit enzymatic and metabolic properties that contrast those of the smaller, oxidative fibers. The function of anaerobic metabolism in rapidly producing

energy is apparent when comparing the glycolytic power output ; during short duration, intense activity in vivo in humans fast fibers consume 2.5 mM/s of ATP compared to 1.2 mM/s in slow fibers [97]. This rapid anaerobic glycolytic production of energy is aided by the fact that at rest, fast fibers display slightly higher PCr content as well as much higher glycogen content [42,97,98]. Interestingly, the amount of PCr available for use as metabolic substrate appears to be more dependent on fiber hypertrophy and not adjustments in concentration; this finding corresponds with the greater potential for hypertrophy of MHC type II glycolytic fibers [99]. In terms of activation time-course, Bangsbo et al. demonstrated the initial reliance on PCr metabolism followed by gradual increases in anaerobic and finally aerobic glycolytic and mitochondrial metabolism in healthy human subjects, with PCr supplying 80% of power output for initial 30s, 45% for 60-90s, and ~30% at 120s onwards [85]. Similar to PCr metabolism, anaerobic glycolysis is capable of producing high rates of power output and heavily relied upon by fast fibers [97]. Indeed, all of the enzymes involved in glycolytic metabolism, including the rate-limiting phosphofructokinase (PFK) show much higher activity levels in fast compared to slow fibers [51,52]. The sympathetic nervous system stimulated hormonal release of epinephrine also displays a greater effect on glycogen breakdown in fast fibers, likely indicative of epinephrine's role in the "fight-or-flight" response necessitating rapidly available energy [100]. As would be expected, the varied metabolic properties of specific fiber types results in a range of energy consumption demands during maximal isometric contraction in humans [101]. As such, MHC type I fibers have been found to consume 6.5 mmol/kg/s, while IIa and IIx fibers have been measured consuming 17.6 mmol/kg/s and 26.6 mmol/kg/s, respectively [44,102].

1.11 Satellite Cells, Growth, Regeneration and Fiber Type:

A unique feature of skeletal muscle is the post-mitotic, multi-nucleated nature of individual muscle fibers. As a result of this terminal differentiation state, the capacity for growth (hypertrophy) and regeneration exhibited by muscle fibers is largely due to the presence of a small population of progenitor cells found between the basal lamina and sarcolemma, known as satellite cells [103–108]. First identified in 1961, satellite cells (SC) normally exist in a quiescent state, but in response to a number of different stimuli these cells become activated, proliferate, and fuse to existing fibers becoming new myonuclei [106]. A number of molecular markers differentially expressed by SC's have been identified, and include but are not limited to: Pax 7, Pax 3, MyoD, Myf5, NCAM/CD-56; however, myogenic regulatory factors such as MyoD and Myf5 are not expressed by quiescent SC [108,109]. In terms of SC activation and signaling, a number of contributing mechanisms have been proposed, all of which have been described as contributing to the specific "niche" in which SC exist outside of the muscle fiber [108]. Factors that have been identified to contribute to the maintenance of this specific niche and SC activation include the extracellular matrix (ECM), vascular and neural networks, myofiber secreted factors, mechanical tension, Wnt and Notch signaling, and hormones such as IGF-1 [108,110–123]. Although the exact mechanisms and interactions of these niche components is beyond the scope of this review, the topic has been reviewed extensively elsewhere [106–108]. In fiber hypertrophy, the contribution of SC to the existing myonuclei pool is believed to be driven by increases in fiber cytoplasm, as myonuclei are unable to divide, and the nuclei to cytoplasm ratio or "myonuclear domain" appears to be constant in adult fibers. Furthermore, SC have been shown to play a critical role in fiber regeneration in response to exercise and myotrauma, where ablation of Pax7+ SC abolishes the regeneration capacity in adult muscle [124–126]. Physical exercise such as

resistance training has also been shown to be highly effective at increasing the density and activation of satellite cells, as determined by immunohistochemistry and electron microscopy [109,127–130].

SC content appears to vary based on muscle fiber type, with slow fibers generally possessing a greater number of SC's than fast fibers and potentially maintaining intrinsically different properties based on fiber type of origin [109,131–133]. For example, the percentage of satellite cells in the soleus has been found to be two to four times higher than that of the EDL and tibialis anterior (TA) muscles, composed predominately of slow and fast twitch fibers, respectively. Interestingly, SC content in sedentary elderly compared to young individuals has been shown to be reduced in fast fibers specifically, and comparatively elderly subjects tend to experience reduced increases in SC density in response to resistance training [107,134–136]. This fiber-type specific SC dysfunction does not appear to be exclusive to aged individuals; satellite cell abnormalities have also been observed in models of diabetes, in which fast fibers are the most susceptible to diabetic myopathy [137]. For example, in steptozotocin (STZ) rodent models of T1DM, SC activation has been found to be greatly reduced, and overall regeneration capacity attenuated following myotrauma, as well as SC exhibiting reduced MRF expression and impaired differentiation [137–141]. Although the combined effects of oxidative stress, a chronic low-grade inflammatory profile, and impaired ECM remodeling as a result of the diabetic milieu have been suggested to contribute to these dysfunctions, the T1DM specific expression of plasminogen activator inhibitor-1 (PAI-1) and myostatin (MSTN) are particularly interesting [137]. MSTN is a known negative regulator of muscle mass and a strong repressor of satellite cell activation which shows elevated protein and gene expression in T1DM [142–146]. PAI-1 is a hormone involved in the regeneration process and ECM turnover rate, impedes fiber recovery

following injury, contributes to SC regulation and is found to be elevated in STZ diabetic rats [34,141,147–151]. Although both of these regulatory mechanisms contribute to diabetic SC dysfunction independently, the positive correlation between glucocorticoid levels (which are elevated in diabetic myopathy), PAI-1 expression and MSTN gene expression would seem to indicate an inter-related SC dysfunction pathogenesis [34,147,148,152,153]. Furthermore, the fast fiber specific atrophy in both diabetic and aged populations with the presence of SC abnormalities could also suggest some overlap in the pathogenesis of the two atrophic states, providing further impetus for additional fiber specific myopathy and sarcopenia research investigation.

1.12 Diabetic Skeletal Muscle Myopathy Characterization:

As stated previously, diabetic myopathy is a condition typically characterized by reduced muscle mass, muscular weakness, diminished physical work capacity and decreased maximal force production [4,34,36,37,154]. Furthermore, the reduced muscle mass and force production that manifest in diabetic myopathy seem to be related to muscle fiber atrophy, with fast glycolytic fibers (MHC IIx, IIb) appearing to be the most severely affected whereas slow fibers are relatively unaffected [33,34,155–159]. Additionally, children with T1DM have also been found to have reduced motor unit number estimates (MUNE) compared to non-diabetic children, indicating the possible role of neuropathy in diabetic myopathy progression [70]. However, recently diagnosed (1 to 28 weeks) T1DM patients have also been shown to exhibit fiber atrophy, Z-disk disruptions and mitochondrial abnormalities prior to the onset of any neuropathy, indicating that skeletal muscle is acutely and detrimentally sensitive to the diabetic milieu [34,40,160]. In addition to these complications, diabetic myopathy reduces the regenerative capacity of muscle fibers, with elevated levels of MSTN and PAI-1 negatively impacting SC activation and recruitment [141,145].

As such, it is quite apparent that diabetic myopathy embodies a myriad of dysfunctions that collectively contribute to functional and physiological consequences.

Although diabetic myopathy has historically not received the same level of research attention as other complications associated with T1DM, maintaining a healthy level and quality of muscle mass is central to the maintenance of overall physical health. As skeletal muscle is responsible for enabling locomotion, as well as serving as the primary glucose sink and metabolic regulator, investigating the underlying pathological mechanisms and determining intervention strategies such as specific exercise modalities is crucial to attenuating the progression of complications related to diabetic myopathy [33,34]. As T1DM is most often diagnosed in children and adolescents, and skeletal muscle undergoes significant development and growth during childhood and puberty, early intervention in preventing the onset of the dysfunctions associated with diabetic myopathy is of critical importance [34]. The findings of several studies investigating the physical work capacity of adolescents and adults with T1DM are somewhat challenging, as many studies have indeed shown reduced capacities compared to non-diabetic cohorts, while other studies have reported no apparent physical impairment in performance [34,161–165] Additionally, some of the studies in which reduced submaximal and maximal workload were observed found that the degree of capacity detriment corresponded inversely to metabolic control (HbA1c), introducing question as to whether poor metabolic control is the result or the cause of this reduced physical capacity [34,161,162]. In fact and somewhat surprisingly, some human T1DM studies have even reported an increase in the relative percent composition of fast fibers, as well as an increase in glycolytic enzymes [34,38,39]. It should be noted that these findings could be the result of the individuals in these studies maintaining excellent glycemic control and insulin therapy management [34]. Further complicating this, the use of

functional measures such as VO_2 max and PWC170 (submaximal exercise) do not necessarily indicate muscle and fiber specific dysfunctions, as such measures involve the interaction of both the cardiovascular and skeletal muscle systems [34].

The use of animal models such as the alloxan, STZ or *Ins2*$^{Akita+-}$ diabetic mice and rats have provided a wealth of information regarding the impact of diabetic myopathy on whole skeletal muscle and individual fibers. For example, a study comparing the STZ and *Ins2*$^{Akita+/-}$ diabetic mice models to control animals after 8 weeks of diabetes found significantly reduced gastrocnemius-plantaris-soleus mass (control 0.16 +- 0.005g, *Ins2*$^{Akita+/-}$ 0.12 +- 0.003g, STZ 0.12 +- 0.01g) and IIB/D fiber size (control 2241 +- 144 μm^2, *Ins2*$^{Akita+/-}$ 1294 +- 94 μm^2, STZ 1768 +- 163 μm^2) in the diabetic animals compared to controls [166]. STZ rodents have also shown muscle specific loss in force, where soleus or slow fibers show no loss in force and EDL or fast fibers show impaired force production [155,156,167]. Another study using a 90% partial pancreatecomy rat model of diabetes found that after 8 weeks of diabetes both type I and II fibers occupied ~25% and ~45% less area, respectively, as well as diabetic animals exhibiting 45% lower gastrocnemius-plantaris-soleus complex mass and significantly lower absolute force production [168]. Surprisingly, some studies have actually reported increases in relative force production [34,169,170]. This finding may seem antithetical when compared to other studies, Krause et al. have postulated that this finding may be caused by altered Ca^{2+} transients in the fibers, as STZ rats have been shown to exhibit hyperactive Ca^{2+} kinetics and SERCA activity [34,171] Although a number of other animal studies display findings similar to these, the differential severity of atrophy and loss of force production could be a result of the duration of diabetes, different experimental glycemic ranges due to the presence or absence of insulin, or even the dosage of diabetes-inducing compounds like STZ administered. For example, the addition of

various doses of STZ (0.25 – 3.0 mg/ml of a glucose-analogue used to induce pancreatic beta-call destruction) to myoblasts in vitro was found to show that both chronic and acute exposure resulted in reduced proliferative capacity in a dose- dependent manner, implicating STZ in fiber dysfunction independent of systemic hyperglycemia [172]. Furthermore, studies investigating the effect of different dosages of STZ (60 mg/kg, 80 mg/kg, 100 mg/kg and 150 mg/kg) found alterations in metabolic enzymes in higher dosage groups, and rats administered lower doses responded better to insulin treatment [173]. As such, some researchers have adopted lower concentration, multi-dose STZ administration methods in an attempt to avoid complications resulting from STZ independent of beta-cell destruction [174,175]. Another issue amongst studies using rodent models of diabetes is the fact in many cases the secretion or administration of exogenous insulin is absent, and as a result these animals often have extremely high blood glucose levels for extended periods of time, likely further exacerbating muscle myopathy and making translation to human muscle difficult.

1.13 Pathological Contributors and Underlying Mechanisms:

Although the underlying mechanisms believed to primarily contribute to the atrophy and reduced force production observed in cases of diabetic myopathy are hyperglycemia and an abnormal protein synthesis-degradation balance, a number of additional pathological contributors have also been identified [33,34]. For example, the metabolic hormones adiponectin and leptin may not directly contribute to muscle fiber atrophy *per se*, but the metabolic effects of their abnormal expression patterns in T1DM may contribute to diabetic myopathy. Adiponectin and leptin are adipokine hormones that are known to increase insulin sensitivity and fat metabolism, and have been found to be increasingly and decreasingly expressed, respectively, in individuals with T1DM [33]. Although leptin levels may be decreased in individuals with poorly controlled T1DM,

insulin therapy has been shown to recover leptin expression in children and STZ rats [33,176,177]. Interestingly, the elevated expression of adiponectin found in individuals with T1DM appears to arise independent of glycemic control, and has been shown to be positively correlated with intramyocellular lipid (IMCL) accumulation in the skeletal muscle fibers of non-diabetic mice [20,33,178,179]. Higher levels of IMCL have also been observed in the skeletal muscle of humans with T1DM and STZ rodents, and have been suggested as contributing to altered insulin sensitivity [20,166,180]. Coleman et al. have theorized that increased IMCL content associated with elevated adiponectin expression in T1DM could be the result of a metabolic compensatory mechanism in which lipids are removed from the circulation and stored within muscle fibers [33].

Similar to other T1DM complications such as vascular disease and neuropathy, hyperglycemia resulting from a lack of pulsatile insulin release is considered a primary pathological mechanism in diabetic myopathy. Hyperglycemia resulting from T1DM accelerates the process of glycation, which occurs normally with aging, and results in the formation of cross-linkages between proteins known as advanced glycation end products (AGE) [181,182]. AGE accumulation in both serum and muscle has been established as contributing to diabetic complications, and has even been associated with reducing motor function and grip strength in non-diabetic elderly individuals [2,181,183–186]. In T1DM muscle, AGE accumulation appears to occur primarily in fast fibers, but increases in the AGE content of muscle fiber SERCA pumps has also been reported [183,187,188]. In essence, increased AGE content in myofibrillar protein represents a post-translational modification in which myosin structure and function is negatively affected; for example, glycation has been shown to reduce in vitro myosin motility and modify molecules in the ECM [2,189].

Hyperglycemia also promotes the activation of the polyol pathway, in which the enzyme aldose reductase (AR) inadvertently depletes NADPH through the reduction of excess glucose to sorbitol, and in turn reduces the regeneration capacity of the intracellular anti-oxidant glutathione [2]. As such, chronically elevated levels of reactive oxygen species (ROS) and oxidative stress develops and is believed to contribute to the diabetic myopathic condition [34,190]. Support for this concept is provided by the findings that the use of aldose reductase inhibitors (ARI) have been shown to ameliorate contractile function and muscle fiber area in STZ diabetic rats [34,191,192]. In terms of the preferential atrophy of fast fibers, fast fibers have been reported to exhibit a high degree of mitochondrial free radical leak, and the reduction in anti-oxidant defense due to polyol pathway activation could be considered to contribute to this atrophy [193]. In fact, a study investigating the fiber-specific responses to cachectic stimuli found that mice injected with lipopolysaccharide endotoxin displayed muscle/fiber type specific responses; the fast vastus lateralis muscle expressed significantly higher levels of ROS and E3 ubiquitin ligases atrogin-1 and muscle ring finger-1 (MuRF1) compared to the slow soleus muscle [194]. Conversely, the slow soleus muscle was found to express enhanced nitric oxide (NO), inducible NO synthase (iNOS) and antioxidant genes in response to the oxidative stress "insult", which the researchers suggested could be a mechanism by which slow fibers display resistance to oxidative stress induced atrophy [194]. The E3 ubiquitin ligases atrogin-1 and MuRF1 have been identified as playing an important role in skeletal muscle atrophy, and have been reported to be inactivated by IGF-1, which happens to be reduced in adolescents and adults with T1DM [195–201]. In addition to increased atrogene expression, 21 days of STZ diabetes in rats has been reported to reduce expression of MRF's myogenin, MyoD and Jun D concomitantly with decreases in antioxidant levels and synthesis of CK and myosin light and heavy chains [139]. Indeed, the administration of

the steroid hormone dehydroepiandrosterone (DHEA) and subsequent amelioration of MRF and antioxidant expression, as well as protein synthesis, has been suggested by the authors to support the atrophic role of oxidative stress in STZ diabetes [139].

The dynamic nature of muscle mass, undergoing frequent fluctuations of both protein synthesis and degradation activity, suggests that an imbalance in protein anabolism and catabolism likely contributes to the diabetic myopathy state. Indeed, the presence or absence of insulin has a substantial effect on the balance between protein synthesis and proteolysis in T1DM muscle [202–204]. However, most evidence supports the notion of insulin playing a role in inhibiting proteolysis instead of actually increasing protein synthesis in TIDM [34,205,206]. This matter is somewhat complicated by reports in which insulin has been shown to stimulate increased rate of protein synthesis in STZ diabetic rats, but not in human adults and adolescents with T1DM [207–209]. However, in cultured C2C12 myotubes, the addition of insulin and IGF-1 have been shown to increase protein synthesis by 62% and 35%, respectively, although these results would seem to indicate some inherent difference between insulin action in vivo versus in vitro models [210]. Accordingly, the aforementioned researchers indicated that insulin and IGF-1 both acted to stimulate the phosphorylation/activation of the phosphatidylinositol-3 kinase (PI3K) – Akt – mammalian target of rapamycin (mTOR) pathway, a signaling pathway strongly associated with muscle growth and hypertrophy [195,210]. The activation of the PI-3 K signaling pathway by insulin acts to inhibit the ubiquitin-ligase atrogin-1, in turn negatively regulating the expression of ubiquitin and thus the proteolytic ubiquitin-proteasome (Ub-P) pathway [206,211]. Furthermore, the fact that components of the Ub-P proteolytic pathway are often found to be upregulated in T1DM corresponds with the atrophic condition commonly observed in diabetic myopathy [34,139,206,212,213]. Interestingly, Akt expression and activation in STZ rats has been found to be

lower in fast muscles, but not in slow muscles compared to controls, and thus may indicate another aspect of the fiber specific atrophy in diabetic myopathy [34,214]. The infusion of human recombinant IGF-1 in STZ rats has also been shown to partially restore growth rate in diabetic growth-arrested rats, and further addition of insulin resulted in completely restored growth [34,215]. As IGF-1 and insulin are structural homologues which act through similar receptors, increasing IGF-1 content local/autocrine to skeletal muscle through interventions such as resistance exercise could provide promising results for diabetic myopathy[216,217]. In a model of diabetes in which rats were partially pancreatectomized (~90%) and subjected to 8 weeks of progressive resistance training, exercise was found to increase soleus and gastrocnemius-plantaris complex wet weight compared to sedentary rats and partially restored IGF-1 concentration [204,218]. Interestingly, the same increases in IGF-1 concentration in the non-diabetic acutely exercised animals was not observed, and the authors of these studies suggest that the increase in circulating IGF-1 in diabetic rats may act in a compensatory manner [218].

1.14 Role of Exercise in T1DM Management:

Engaging in regular physical activity and exercise is an important component of the health regimen in T1DM, and is associated with an increased life expectancy and reduced risk of developing complications, and even an inversely proportional relationship to mortality risk [11,219–222]. Some of the additional health benefits that exercise confers to individuals with T1DM include (but are not limited to) improved cardiorespiratory and vascular health, decreased insulin requirements, improved endothelial function and overall quality of life [220,223,224]. Despite these established benefits, it has been reported over 60% of adults with T1DM do not partake in the recommended levels of physical activity, and due to a scarcity of specific research in the area, the appropriate type, duration, frequency and intensity of physical activity necessary to induce

glycemic improvements still remains somewhat unclear [219,220,225]. A contributing factor to the poor adherence to a therapeutic physical activity routine in individuals with T1DM is the fear of post-exercise induced and late-onset (nocturnal) hypoglycemia [226,227]. Interestingly, contrary to the known risk of post-exercise hypoglycemia in moderate-intensity aerobic exercise, it has been observed that anaerobic exercise (such as resistance training) can actually induce post-exercise hyperglycemia, further highlighting the benefits of characterizing the divergent effects of differing exercise modalities in T1DM [228]. It follows that the focus of a large number of exercise interventions in T1DM patients has typically been on improving glycemic control measuring markers like glycated hemoglobin (HbA_{1c}) or fructosamine in order to reduce the risk of cardiovascular disease and related complications [219,228]. Although these studies do not necessarily examine the effects of exercise on skeletal muscle mass and fiber size specifically, improving glycemic control through exercise and thus restoring euglycemia could confer improvements in muscle mass in T1DM patients.

In terms of preventing diabetic myopathy, or maintaining/ improving muscle mass with exercise in T1DM patients, research investigating the effects of different modes of exercise on skeletal muscle/fiber size is somewhat sparse. The molecular and physiological effects of progressive resistance training on skeletal muscle and the corresponding strength gains and muscle hypertrophy that occurs in response has been studied extensively in healthy humans and rodents, and has shown promise in attenuating the deleterious effects of diabetic myopathy [229–231]. In one rodent study, partially pancreatomized (~90%) diabetic rats subjected to 8 weeks of progressive overload resistance training were shown to exhibit significant soleus and gastrocnemius-plantaris muscle hypertrophy despite diabetes resulting in lower muscle wet weight compared to healthy animals [232]. Another study using STZ-induced T1DM rats

performing a 1 meter weighted-ladder climb progressive resistance training protocol for 5 weeks (5 sets of 4 repetitions/climbs) resulted in significant hypertrophy of the flexor hallucis longus (FHL) muscle as determined by increased FHL weight-to-body weight in trained versus untrained animals [233] In a human study investigating progressive resistance training in Hispanic older adults with T2DM, 16 weeks of resistance training (3 times per week, 3 sets of 8 repetitions) was found to significantly increase mean upper and lower body strength, whole-body lean mass, muscle quality (strength per unit volume of muscle) and the CSA of type I and type II muscle fibers (vastus lateralis) [234]. It appears that despite the diabetic milieu's propensity for decreasing muscle mass, resistance training is still capable of eliciting skeletal muscle hypertrophy; however, closer examination of the MHC fiber-specific responses to resistance training in T1DM is still warranted in order to better characterize the differential responses of the heterogeneous fiber population to resistance training in T1DM.

Similarly, although aerobic endurance training is typically associated with increasing skeletal muscle mitochondrial content, oxidative enzymes, capillary density and overall oxidative capacity, it has also been shown to play a role in muscle hypertrophy and muscle atrophy prevention [229,230,235,236]. Hence, aerobic endurance training may also be a candidate for the prevention and treatment of diabetic myopathy in addition to its beneficial effects on insulin sensitivity and glycemic control [237]. Several studies on healthy humans have shown that 12 weeks of cycle ergometer endurance training (20-45 mins, 60-80% HRR, 3 sessions per week) is capable of inducing both whole-muscle and fiber hypertrophy in young men and older men and women [235,238,239]. In STZ-induced T1DM rodents, 8 weeks of progressive treadmill training (5 days per week, 30 min – 1.5 hr per day) was found to significantly increase gastrocnemius whole muscle weight and fiber size compared to sedentary diabetic animals, and this anti-atrophic

effect of endurance exercise was shown to be at least partly due to the downregulation of MuRF-1 mRNA in diabetic exercised animals [240]. Further support for the notion of aerobic exercise conferring protection from fiber atrophy in T1DM is provided by the finding that PGC-1α, the master regulator of mitochondrial biogenesis significantly upregulated in response to endurance exercise, appears to also inhibit skeletal muscle atrophy through inhibition of FOXO3which activates atrogin-1 and MuRF-1 [236,241]. Indeed, the fact that glycolytic fibers contain lower levels of PGC-1α than oxidative fibers has been proposed as one of the factors which contributes to glycolytic fiber susceptibility to fasting and glucocorticoid induced atrophy [242,243].With these skeletal muscle specific benefits of aerobic endurance training considered alongside the post-exercise hyperglycemia inducing capacity of anaerobic resistance training, the concept of combined exercise training to prevent diabetic myopathy is particularly intriguing. In healthy adults, combining resistance and aerobic endurance training elicit improved muscle fiber CSA hypertrophy after 5 weeks in the vastus lateralis compared to resistance training alone (17% versus 9%) in young men, while other studies in older men have shown no difference in muscle hypertrophy (11%) between the two [244,245]. Interestingly, 14 weeks of concurrent lower body endurance and upper body resistance training in active elderly has been shown to induce similar increases (38%) in satellite cell counts in muscle samples from both deltoid and vastus lateralis muscle samples, primarily in type II muscle fibers [135]. As such, investigating the potential benefits of combining resistance training with aerobic endurance training in T1DM could provide insight into whether these aforementioned training effects are preserved in the diabetic milieu and could represent an ideal exercise training modality by reducing the risk of post-exercise hypoglycemia and improving insulin sensitivity while increasing whole muscle and fiber hypertrophy.

1.15 Concluding Remarks:

Diabetic myopathy represents a complication associated with T1DM that warrants further research for a number of reasons. The rate of T1DM incidence has been reported to be increasing on a global scale, and the greatest increment increases have been observed in children under the age of 5 years old [5,14,27–30]. Since skeletal muscle undergoes significant growth and development during childhood and adolescence, the increase in children affected by T1DM at a young age has potential to increase the severity of the negative effects of diabetic myopathy, manifesting in serious health and functional consequences. Furthermore, in an increasingly aging society, sarcopenia or the muscle loss (predominately in glycolytic fibers) associated with old age has been shown to be more prevalent in older adults with latent autoimmune diabetes and T2DM compared to non-diabetics [246–249]. Improving our current understanding of how to prevent the development or attenuate the progression of diabetic myopathy may prove to be critical in maintaining skeletal muscle health across the lifespan of patients with T1DM.

Independent of the epidemiological relevance of researching diabetic myopathy, the current body of literature is subject to some criticism in terms of translational ability to humans with T1DM. The STZ-induced T1DM rodent model that has been used for the majority of diabetic myopathy studies to date presents a few challenges in terms of experimental design that make it difficult to compare to humans with T1DM. Firstly, the majority of STZ T1DM rodent studies have utilized single high-dose injections of STZ in order to induce diabetes, usually between 60-70 mg/kg in rats and 120-250 mg/kg in mice [157,166,169,180,250–258]. As high doses of STZ (100 mg/kg – 150 mg/kg) injected in rats has been found to result in premature death, and STZ both *in vivo* and *in vitro* has been shown to have deleterious effects on skeletal muscle independent of hypoinsulinemia-hyperglycemia in a dose dependent manner, attempts to reduce

the myotoxic role of STZ *per se* on skeletal muscle should be a priority in STZ T1DM models investigating diabetic myopathy [172,173]. A second issue with STZ T1DM models of diabetic myopathy is that the majority of these aforementioned studies do not include insulin therapy in their experimental design, general resulting in a severe diabetic state. Insulin therapy is foundational to the management of T1DM in humans, and a near absence of circulating insulin in rodent models of diabetic myopathy limits the degree to which results can be extrapolated to humans with T1DM. Moreover, insulin is known to contribute to the maintenance of muscle mass primarily through the inhibition of skeletal muscle proteolysis, and even small amounts of circulating insulin can significantly inhibit protein breakdown; extreme hypoinsulinemia likely exacerbates the diabetic myopathic condition in STZ T1DM models [259,260]. A consequence of the chronic hypoinsulinemia exhibited by STZ T1DM rodents is of course, hyperglycemia, which in many rodent models has been reported to be between 20 and 38.8 mmol/l, with some studies reporting blood glucose concentrations as high as 44.4 and 56.5 mmol/l [157,166,168,180,250,252,253,255–257]. Once again, these extremes in resting blood glucose concentration are unrepresentative of the general T1DM population (aside from during the weeks-months prior to diagnosis/insulin therapy), and thus detract from the translational capacity of these models. With these limitations in mind, one of the aims of the present study was to investigate the role of conventional (blood glucose 9-15 mmol/l) compared to intensive (7 – 9 mmol/l) insulin therapy on skeletal muscle in multiple low-dose STZ (20 mg/kg) injected T1DM rodents through the use of subcutaneously implanted insulin pellets. The blood glucose range for conventional insulin therapy was selected in order to provide a comparable level of glycemic control to the general T1DM population, whereas intensive insulin therapy represents T1DM patients with tightly controlled blood glucose levels [174,261].

As exercise is known to produce significant adaptations in skeletal muscle with adaptive responses varying in response to specific exercise modality and muscle fiber type, the role of exercise training on the skeletal muscle in T1DM specifically also warrants further research attention. As previously discussed, resistance, aerobic endurance and combined exercise training are particularly interesting potential therapeutic avenues in treating and preventing the development of diabetic myopathy, largely due to their beneficial muscle-specific effects. However, the majority of studies on exercise training in T1DM have focused primarily on improving indices glycemic control rather than examining the effects of exercise modality on skeletal muscle and specific muscle fiber populations [219,223,228,237,262]. Moreover, the few exercise training studies that have examined muscle hypertrophy in response to training have either utilized extreme experimental conditions such as synergistic ablation to induce chronic overload, or have neglected to incorporate insulin therapy and representative blood glucose ranges, limiting their translational capacity to humans [232,253,263]. With consideration of the myriad of adaptive responses of skeletal muscle to exercise modality, comparing the effects of different exercise modalities within the same model of T1DM could provide insight into how specific skeletal muscle fibers adapt despite T1DM. Furthermore, characterizing these differential adaptations in T1DM in response to specific exercise modalities could also allow for the determination of exercise training protocols designed to optimize the maintenance of skeletal muscle mass while conferring improved glycemic control. Hence, the other primary aim of the present study was to examine the effects of resistance, aerobic endurance and combined exercise training on MHC muscle fibers in a multiple low-dose STZ (20 mg/kg) rodent model of T1DM receiving conventional insulin therapy (blood glucose 9-15 mmol/l). The conventional blood glucose range in exercise trained animals, in addition to better representing the general T1DM

population, was selected in order to also permit comparison of exercised animals with intensively treated sedentary animals to identify whether exercise in moderately hyperglycemic conditions is comparable or superior to intensive insulin therapy in maintaining/increasing skeletal muscle mass. This model of T1DM in which experimental conditions may allow improved translation of results to humans in conjunction with submitting animals within the same model of T1DM to a range of exercise modalities should produce novel insights into the diabetic myopathy and its treatment.

1.16 References:

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CHAPTER 2

2.1 Introduction:

Type 1 diabetes mellitus (T1DM) is a condition in which a selective destruction of the insulin producing β-cells of the pancreas results in a loss of insulin production, necessitating treatment with exogenous insulin in order to control blood glucose levels. The loss of the pulsatile insulin response that typically counters increases in blood glucose results in chronically elevated blood glucose, or hyperglycemia, which ultimately leads to microvascular, macrovascular and neurological complications [1,2]. Over time, these complications eventually manifest in the form of retinopathy, nephropathy, neuropathy, atherosclerotic cardiovascular and cerebrovascular disease [3]. A less studied complication associated with T1DM is diabetic myopathy, where the skeletal muscle of individuals with T1DM typically exhibit lower muscle mass, weakness, reduced regenerative and physical capacity as well as a number of hormonal, metabolic and histopathological alterations [4–12]. Another common finding in T1DM is that glycolytic muscle fibers (MHC IIb, MHC IIx) appear to be more prone to the atrophic effects of the diabetic milieu compared to oxidative muscle fibers (MHC I, MHC IIa), and often exhibit a reduced cross-sectional area (CSA) as well as altered metabolic, ultrastructural and regenerative properties [13–24]. Muscle fiber atrophy as well as morphological and ultrastructural abnormalities have also been observed in recent onset diabetes in humans prior to the administration of insulin and independent of indications of neuropathy [11,25,26]. The effects of diabetes mellitus on the skeletal muscle of rodents has also been studied extensively, where induction of T1DM has been primarily achieved through the use of streptozotocin (STZ), a glucosamine-nitrosurea compound that acts as a powerful alkylating agent methylating the DNA of β-cells resulting in DNA-strand breaks, cell death and in-turn a loss of insulin production [18,27–30]. However, the use of STZ to

induce T1DM in rodent models of diabetic myopathy is somewhat problematic, as STZ has been shown to have deleterious effects on muscle mass and muscle fiber size independent of hypoinsulinemia/hyperglycemia *in vivo*, and has been shown to impair myoblast proliferation in a dose-dependent manner *in vitro* [27]. Moreover, the vast majority of STZ-induced T1DM rodent models to date have utilized a single, high dose (60-70 mg/kg in rats, 120-250 mg/kg in mice) STZ injection protocol, raising concerns about the myotoxic role of STZ *per se,* in addition to the diabetic milieu in the severity of diabetic myopathy progression [13,14,16–19,21,31–35]. Furthermore, the majority of STZ-induced T1DM studies investigating diabetic myopathy have not included insulin therapy in their experimental design, resulting in hypoinsulinemic/hyperglycemic extremes that are generally poor representations of the human T1DM population [2,36]. Aside from the likely mortal consequences that extremely low insulin/high blood glucose levels would hold in humans with T1DM, both hypoinsulinemia and hyperglycemia have independently been shown to negatively regulate muscle mass, and as such the severity of these factors may limit the applicability of results from previous rodent models to T1DM patients [11,15,37–40].

Skeletal muscle serves as the body's primary glucose sink and occupies a central role in insulin sensitivity. Considering that T1DM predominately arises during adolescence, a period of significant muscle growth and development, it is important to prevent or treat the progression of diabetic myopathy at an early age [8,11]. Engaging in physical exercise, which is known to induce profound and varied training adaptations in skeletal muscle, has been proposed as a therapeutic strategy in the amelioration of the deleterious effects of diabetic myopathy [41,42]. However, the majority of studies investigating the role of exercise (resistance and aerobic endurance) in humans with T1DM to date have focused primarily on the role of exercise in maintaining healthy blood glucose levels (i.e. glycated hemoglobin or HbA_{1C}), typically with the aim of preventing

the development of cardiovascular disease and related complications [43–47]. Yet, focusing on increasing muscle mass in patients with T1DM may afford benefits that extend beyond improving functional capacity and muscle quality across the lifespan; as skeletal muscle is highly metabolically active, increased muscle mass may improve insulin sensitivity and glycemic control. Previous rodent studies have specifically investigated whole-muscle and muscle fiber hypertrophic capacity in T1DM through the use of synergistic ablation and progressive overload resistance training protocols [31,33,48,49]. While these studies have found that skeletal muscle maintains a capacity for hypertrophy despite T1DM, the drastic nature of synergistic ablation induced chronic overload is difficult to compare with the training effects of whole-body voluntary exercise. Moreover, these studies do not address the muscle fiber-type specific effects of T1DM or overload in terms of MHC isoforms, considered the best available marker for fiber type, which is likely necessary to fully understand the fiber-type specific nature of training adaptations in T1DM [50].

Aerobic endurance training has typically been associated with increases in indices of oxidative capacity in the skeletal muscle of humans and rodents (i.e. increased mitochondrial content, oxidative enzymes and capillarization), however, some research has indicated that aerobic training alone or in conjunction with resistance training can be advantageous in terms of conferring muscle and fiber hypertrophy as well as preventing T1DM induced muscle atrophy [42,51–56].Indeed, in human subjects it has been reported that performing a combination aerobic endurance and resistance training results in greater increases in muscle fiber CSA compared to resistance training alone [54,55]. Conversely, higher intensity resistance exercise may mitigate the potential for hypoglycemia that is often observed after aerobic endurance training [44,57,58]. As such, characterizing the effects of different modalities of exercise (resistance, aerobic endurance

and combined) on MHC muscle fiber size is important in order to understand how specific muscle fiber-types respond to different types of exercise in T1DM. The aim of the present study was to investigate the role of insulin therapy and exercise modality in maintaining or increasing muscle fiber CSA in a multiple low-dose STZ, insulin-treated rodent model of T1DM. Firstly, we hypothesized that intensive insulin therapy (blood glucose 7-9 mmol//l) would result in superior maintenance of muscle fiber CSA compared to conventional therapy (9-15 mmol/l) in sedentary animals. Secondly, we hypothesized that exercised animals (resistance, aerobic endurance or combined) receiving conventional insulin therapy (9-15 mmol/l) would display increased muscle fiber CSA compared to control and sedentary diabetic animals despite moderate hyperglycemia, and that animals subjected to resistance/combined training would display the greatest increases in fiber CSA.

2.2 Materials and Methods:

Ethics approval

The protocols used in the present study were approved by the University Council of Animal Care of Western University (London, Ontario, Canada) in accordance with the standards of the Canadian Council on Animal Care (APPENDIX B)

Animals

Thirty-six, 8 week old, male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). All rats were caged in pairs and held on a constant 12 hour dark/light cycle, temperature (20 ± 1°C) and relative humidity (50%) for the duration of the study. All rats were allowed access to standard rat chow and water *ad libitum*.

Experimental groups

The rats were randomly assigned to one of six treatment groups. The resulting groups were as follows: control sedentary (CS; n=6), diabetic conventional therapy (DCT; n=7), diabetic intensive therapy (DIT; n=7), diabetic resistance exercise (DRE; n=7), diabetic high intensity aerobic exercise (DHE; n=5) and diabetic combined exercise (DCE; n=4). Although all diabetic groups began with a similar number of animals, severe freeze artifact in some plantaris muscle samples resulted in the reduced number of animals in DHE and DCE groups.

Experimental Procedures

Diabetes Induction and Insulin Pellet Implantation

After a 5 day acclimatization period following arrival, T1DM was induced in rats through the use of intraperitoneal (IP), low-dose streptozotocin 20mg/kg (STZ; Sigma-Aldrich) injections over 5 consecutive days [36,59,60]. All STZ injections were dissolved in a 0.1 M citrate buffer (pH 4.5) and performed within five minutes of preparation. The development of T1DM was confirmed by measures of non-fasted blood glucose (BG) of $\geq$ 18 mmol/L on two consecutive days. If necessary, subsequent injections were performed until diabetes was confirmed (APPENDIX A1). Following confirmation of diabetes, insulin therapy was provided through surgical implantation of abdominal-subcutaneous insulin pellets (1 pellet; 2U insulin/day; Linplant, Linshin) (APPENDIX A2). Hyperglycemic range was controlled through adjustment (addition or removal ±0.5 pellet) of insulin pellets; DCT rats were adjusted to maintain a BG range of 9-15 mmol/L while DIT rats maintained a BG range of 7-9 mmol/L.

Exercise Training Protocols

The resistance training protocol used was adapted from Hornerberger and Farrar, and has been implemented previously in our lab to simulate resistance exercise parameters and physiological adaptations observed in resistance training in humans [59,61]. Following confirmation of diabetes and insulin pellet implantation, rats were required to climb a ladder (1.1m, 80°) with weight secured to the proximal portion of the tail contained in a small fabric bag. Familiarization involved a total of 10 ladder climbs, with progressive increases in weight up to 35% body mass for 5 days. Following each climb rats were allowed to rest in a dark box on top of the climbing apparatus for ~2min. Pre-training maximal carrying capacity was determined by initial loading of 75% body mass, with each subsequent climb adding 30g of weight until animals were unable to successfully complete the climb. Resistance training took place 5 days per week for 12 weeks. During training sessions rats were loaded with 50%, 75%, 90% and 100% of pre-determined maximal carrying capacity performing single climbs, and then completed climbs at 100% until exhaustion or unwillingness to climb despite pressurized air/tactile stimulation of the haunches. New maximal carrying capacity was determined every 3 days.

The high-intensity aerobic exercise protocol was one used by our lab to elicit levels of 70-80% of VO_2max [59,60,62]. Following diabetes confirmation and insulin pellet implantation, rats were familiarized for 5 days by running for 15 mins on a motorized treadmill by running at progressively higher speeds up to 30 m/min, 0° grade. Following familiarization, high-intensity aerobic training involved running for 1 hour at 27 m/min on a 6% gradient, 5 days per week for 12 weeks and motivated to run through the use of pressurized air/tactile stimulation of the haunches. Combined exercise training involved alternating the aforementioned resistance and high-intensity aerobic exercise training protocols 5 days per week for 12 weeks, where resistance training would be performed 3 times one week (high-intensity aerobic exercise 2 times) and

high-intensity aerobic exercise would be performed 3 times the following week (resistance training 2 times).

Experimental Measures

Body Weights, Blood Glucose

Body weights (BW) were measured and recorded on a weekly basis throughout the course of the study. Blood glucose (BG) measurements were made weekly by collecting a blood droplet (~50 µL) from the manually occluded saphenous vein, and analyzed via the Freestyle Lite Blood Glucose Monitoring System (Abbot Diabetes Care, Inc.). The corresponding BG concentration was reported in millimoles per liter (mmol/L).

Tissue Collection, Immunohistochemistry

Rats were sacrificed 72 hours after the last training session by anaesthetization with isoflurane followed by cardiac exsanguination. The lower limbs were dissected and the plantaris muscles were removed and wet weight recorded. Plantaris muscles were then mounted in Cryomatrix™ embedding medium (Lot No. 225229, Thermo Fisher Scientific) and rapidly frozen in isopentane cooled to -70°C by liquid nitrogen. Serial 10µm-thick cryosections were cut at -20°C with a Leica CM350 Cryostat (Leica Biosystems) and adhered to VWR Superfrost® Plus Microslides (Cat. No. 48311-703, VWR International). All sections were stored at -30°C until immunohistochemical analysis.

The immunohistochemical methods used were adapted from previously established protocols [63,64]. Prior to staining, sections were allowed to come to room temperature in a humidity chamber for ~10 mins. Slides were then washed (5 mins in Tween-PBS, followed by 2

x 5 mins in PBS) and then incubated in 3% H_2O_2 for 15 mins at room temperature to quench endogenous peroxidases. Slides were washed again (Tween-PBS 5 mins, PBS 5 mins) and then blocked in either 10% (v/v) horse serum with 1% BSA (w/v) in Tween-PBS + Avidin D (Vector Laboratories, Inc.) (BS-1), or 10% (v/v) goat serum with 1% BSA (w/v) in Tween-PBS + Avidin-D (BS-2) depending on the primary antibody to be applied (Table). After blocking for 1 hour at room temperature, excess blocking solution was removed from slides and the appropriate anti-myosin heavy chain primary antibody dilutions in 1% BS-1 or BS-2 and Biotin (Vector Laboratories, Inc.) of BA-D5, SC-71, 6H1 and BF-F3 were applied to detect MHC I, IIa, IIx and IIb, respectively (Table 2.1)(Plate 2.1). Slides were then incubated overnight at 4°C. The monoclonal antibodies BA-D5 and SC-71 were generously donated by C. Putman, while monoclonal antibodies BF-F3 and 6H1 developed by S. Schiaffino and C. Lucas, respectively, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology (Iowa City, IA, 52242). The following day slides were washed again (Tween-PBS 5mins, PBS 5 mins) and secondary antibody diluted in appropriate blocking solution were applied (Table 2.1). After 1 hour of incubation at room temperature, slides were washed again (Tween-PBS 5 mins, PBS 5 mins) and subsequently incubated for 45 mins at room temperature in ABC Peroxidase Staining Kit solution (Thermo Scientific). Following another wash (Tween-PBS 5 mins, PBS 5 mins) slides were then developed for 6-8 mins in 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Inc.), washed in distilled water, dehydrated through graded ethanol washes, and finally cleared with xylene. Slide coverslips were adhered with toluene-based mounting resin (Krysalon™ Harleco, EM Science).

Primary Antibody	Blocking Solution	Dilution	Secondary Antibody	Dilution
BA-D5 (MHCI)	BS-1	1:20	biotinylated horse-anti-mouse IgG (H+L)	1:200
SC-71(MHCIIa)	BS-2	1:10	biotinylated goat-anti-mouse IgG (H+L)	1:200
6H1(MHCIIx)	BS-2	1:10	biotinylated goat-anti-mouse IgG (H+L)	1:200
BF-F3(MHCIIb)	BS-1	1:100	biotinylated horse-anti-mouse IgG (H+L)	1:400

Table 2.1: *Primary antibody dilutions, blocking solution, and secondary antibody dilution. BS-1, BS-2 = blocking solution made with horse and goat serum, respectively.*

The antigen specificity of the primary antibodies used was confirmed by the use of an adjusted metachromatic dye-based myosin ATPase stain originally developed by Ogilvie et al. and adapted for use in rat muscle by Walsh et al. [65,66]. The method consisted of an 8 min incubation in an acidic (pH 4.4) pre-incubation solution, followed by three 2 min washes in Tris buffer (pH 7.8). Slides were then incubated for 30 mins in an ATP-incubation solution (pH 9.4), dipped 4 times in three changes of 1% calcium chloride dihydrate solution and allowed to incubate for 90s in a 0.1% toluidine blue solution. Slides were then promptly rinsed in running $_{dd}H_2O$ for 30s, and rapidly dehydrated by 5 dips in 95% ethanol, two sets of 5 dips in 100% ethanol, and 1 min in xylene. Slide coverslips were adhered with toluene-based mounting resin (Krysalon™ Harleco, EM Science). (Plate 2.1)

Microscopy Image Collection and Muscle Fiber Cross-Sectional Area Analysis

All microscopic imaging was performed on a Zeiss Axio Vert.A1 microscope equipped with an AxioCam ICc 5 camera, linked to the Zen 2.3 Lite computer-based image analysis program (Carl Zeiss AG). Five distinct regions on each section were captured at 10x magnification; care was taken to ensure that the same five regions were captured on each of the MHC stains per animal. Regions were chosen to represent all quadrants and central area of the muscle.

Muscle fiber cross-sectional areas were measured using Adobe Photoshop® CC (Adobe Systems). Scale was determined through the use of a 25mm stage micrometer (KR-814) and the "set measurement scale" option to convert area in pixels to microns squared (μm^2). The "quick selection" tool was then used to record the cross sectional areas of individual muscle fibers and exported from the "measurement log" as a delimited text file to Microsoft Excel.

Data Analysis

Body weight, blood glucose, plantaris wet weight, and muscle fiber cross-sectional areas within and between groups were compared using a one-way analysis of variance (ANOVA) with GraphPad Prism 5 (GraphPad Software, Inc.). On determination of significant differences, pairwise post-hoc analysis was performed using the Tukey test. Differences were considered significant at $P<0.05$. Fiber CSA frequency distributions were generated with GraphPad Prism 5 by taking frequency distribution values for number of fibers per bin size and dividing that number of fibers by the total fiber number for that specific MHC and group, resulting in frequency distribution of fiber size expressed as a fractional percentage (i.e. 0.3 = 30%) of total fiber population.

2.3 Results:

Physical Characteristics:

Physical characteristics (terminal body weight (g), plantaris whole-muscle weight (mg) and terminal blood glucose (mmol/l) are presented in Table 2.2. Terminal body weights of all groups except were similar, except for DIT which was significantly greater than DRE ($P< 0.05$). Plantaris whole-muscle weights were not significantly different between any treatment groups ($P>0.05$). Although frequent adjustments to the number of implanted insulin pellets were made over the course of the study in an effort to maintain conventional (9-15mmol/l) and intensive (7-9mmol/l) blood glucose ranges as closely as possible, terminal blood glucose concentration was not different between diabetic groups (DCT, DIT, DRE, DHE, DCE), and CS terminal blood glucose was significantly lower than all diabetic groups ($P<0.05$). It should be noted that terminal blood glucose concentrations were determined on unfasted animals. That combined with the variability in blood glucose range over the conventional and intensive conditions may have led to the lack of differences.

Resistance Training Load:

Resistance training load over the course of 12 weeks of training for both DRE and DCE animals is presented in Figure 2.1. Both groups demonstrated significant and near linear increases in weight carried over the training period.

Fiber Type Percent Composition, Cross-Sectional Area and Cross-Sectional Area Frequency Distribution:

Myosin heavy chain fiber type percent composition and mean CSA +- SD, as well as percent area occupied by the heterogeneous fiber population are presented in Table 2.3.Between groups, DRE, DHE and DCE MHCIIa/IIx fiber percent composition was significantly greater than DIT animals ($P<0.05$). CS and DIT groups displayed significantly greater percent composition of MHCIIx compared to DCE animals ($P<0.05$). CS, DCT and DCE animals display significantly greater MHCIIx/IIb percent composition than DIT animals ($p<0.05$). In Figure 2.2, mean fiber CSA +- SE differences are presented within experimental treatment groups. Within all groups, a general trend of increasing mean fiber CSA occurred from smaller oxidative fibers to larger glycolytic fibers, and in all groups the mean CSA of MHCIIb fibers was significantly greater than all fiber types.

Comparisons of mean fiber CSA +- SE between groups and specific fiber type CSA frequency distributions are presented in Figures 2.3-2.9. CS MHCIIx/IIb fibers were significantly larger than DCT MHCIIx/IIb fibers ($P<0.05$). DIT animals exhibited MHCIIa and IIa/IIx CSA's that were significantly larger than all other treatment groups ($P<0.05$), MHCIIx CSA that was greater ($P<0.05$) than CS, DCT and DHE, and MHC IIx/IIb as well as MHCIIb CSA's that were greater ($P<0.05$) than CS and DCT animals. DRE animals displayed MHCIIa CSA that was greater ($P<0.05$) than DHE, MHCIIa/IIx that was greater ($P<0.05$) than CS, DCT and DHE, MHCIIx CSA that was greater ($P<0.05$) than CS, DCT, DIT and DHE groups, as well as MHCIIx/IIb and MHCIIb CSA's that were greater ($P<0.05$) than CS and DCT treatment groups. DHE animals exhibited MHCIIx/IIb fiber CSA that was greater ($P<0.05$) than DCT and MHCIIb CSA that was greater ($P<0.05$) than CS and DCT treatment groups. DCE animals displayed MHCI/IIa CSA that was greater ($P<0.05$) than CS, DCT and DIT animals, although these fibers were very few in number. DCE animals also exhibited MHCIIa CSA that was greater ($P<0.05$)

than DCT and DHE, MHCIIa/IIx CSA greater (P<0.05) than CS, DCT and DHE, MHCIIx CSA greater (P<0.05) than CS, DCT and DHE, MHCIIx/IIb CSA greater (P<0.05) than CS and DCT and MHCIIb CSA that was significantly larger (P<0.05) than all other treatment groups.

Group	**CS (n=6)**	**DCT (n=7)**	**DIT (n=7)**	**DRE (n=7)**	**DHE (n=5)**	**DCE (n=4)**
Terminal BW (g)	613.0 ± 83.2	560.0 ± 69.7	621.1* ± 45.8	519.7 ± 56.1	513.2 ± 51.6	536.5 ± 54.6
Plantaris Wt. (mg)	547. 9 ± 72.7	546.5 ± 88.3	609.6 ± 73.5	521.6 ± 47.3	570.7 ± 55.8	522.3 ± 55.1
Terminal BG (mmol/l)	3.983** ± 0.3	16.43 ± 1.9	11.81 ± 3.6	12.40 ± 5.2	15.54 ± 1.3	16.93 ± 2.1

Table 2.2: *Physical characteristics for experimental treatment groups. All data presented as mean ± SD. *=sig. > DRE. ** = sig. < all other groups.*

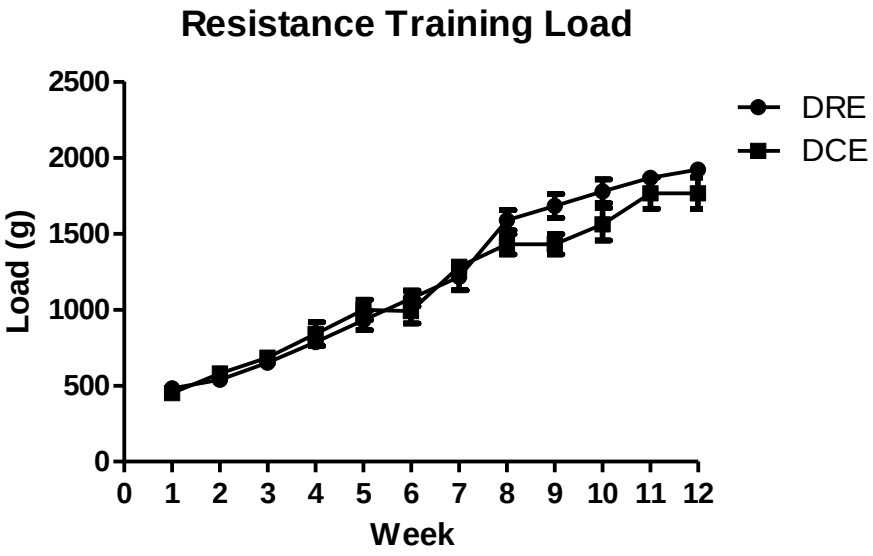
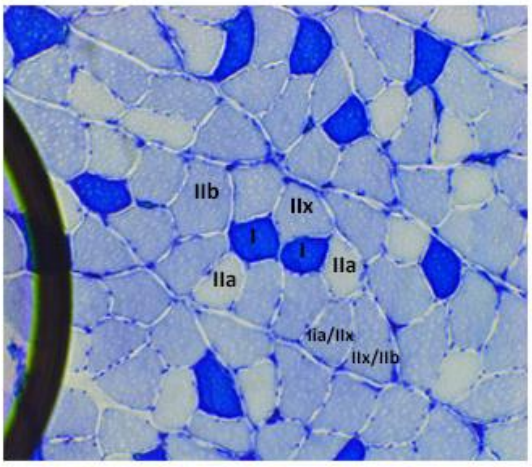


Figure 2.1: *Mean maximal resistance training load for DRE and DCE groups over course of 12 weeks of training. All data points expressed as mean ± SE.*

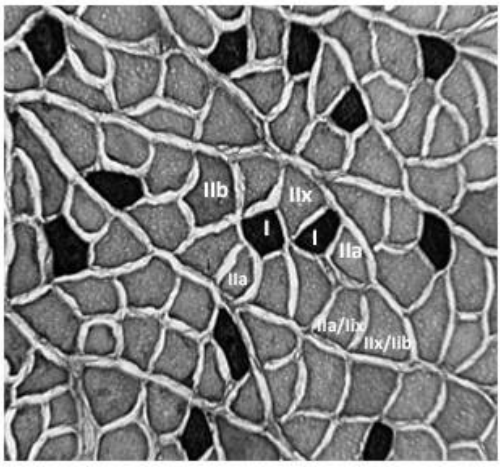
Condition		MHCI	MHCI /IIa	MHCIIa	MHCIIa /IIx	MHCIIx	MHCIIx /IIb	MHCIIb	% Hybrid
CS	**#**	267	16	610	267	1004	501	569	
n = 3234	**%**	8.26	0.49	18.86	8.26	31.04	15.49	17.59	24.24
	cross	2757 ±830.4	2215 ±581.7	2439 ±687.6	2784 ± 831.6	3306 ±888.7	4216 ±1264	4489 ±1190	
	% area	6.78	0.3	13.5	6.96	29.46	18.52	24.47	25.78
DC	**#**	258	39	566	172	798	424	759	
n = 3016	**%**	8.55	1.29	18.77	5.7	26.46	14.06	25.17	21.05
	cross	2930 ±893.6	2211 ±636.2	2420 ±759.1	2907 ±705.1	3340 ±997.5	3740 ±1044	4330 ±1263	
	% area	7.48	0.87	13.44	4.90	26.16	15.56	32.30	21.33
DIT	**#**	306	15	788	33	879	104	763	
n = 2888	**%**	10.6	0.52	27.28	1.14	30.44	3.60	26.42	4.26
	cross	2889 ±1161	2201 ±572.2	2792 ±897.5	4296 ±933.3	3633 ±1024	4657 ±1159	5034 ±1606	
	% area	8.38	0.3	20.5	1.31	29.54	4.43	35.54	6.04
DRE	**#**	278	66	532	350	780	259	655	
n = 2920	**%**	9.52	2.26	18.22	11.99	26.71	8.87	22.43	23.12
	cross	2920 ±1151	2643 ±817.5	2540 ±828.9	3298 ±1229	3821 ±1257	4657 ±1491	5229 ±1420	
	% area	7.3	1.57	12.16	10.37	26.82	10.91	30.87	22.85
DHE	**#**	167	13	483	317	413	208	384	
n = 1985	**%**	8.41	.66	24.33	15.7	20.81	10.48	19.34	26.84
	cross	2941 ±866.9	2940 ±638.7	2325 ±646.9	2893 ±989.2	3201 ±1332	4394 ±1596	5280 ±1748	
	% area	7.24	.56	16.37	13.14	19.35	13.42	29.92	27.12
DCE	**#**	154	32	318	371	192	257	317	
n = 1641	**%**	9.38	1.95	19.38	22.61	11.7	15.67	19.32	40.23
	cross	2748 ±703.8	3117 ±1034	2574 ±824.9	3219 ±1158	3654 ±1136	4689 ±1655	6161 ±1481	
	% area	6.65	3.03	12.84	18.67	10.93	18.8	30.56	40.5

Table 2.3: *Total fiber number, percent fiber composition, fiber CSA and percent area occupied by fiber population. All fiber CSA values are reported as mean ± SD.*

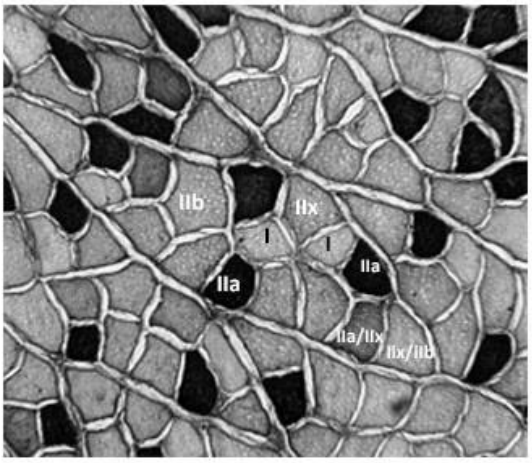
A Myosin ATPase



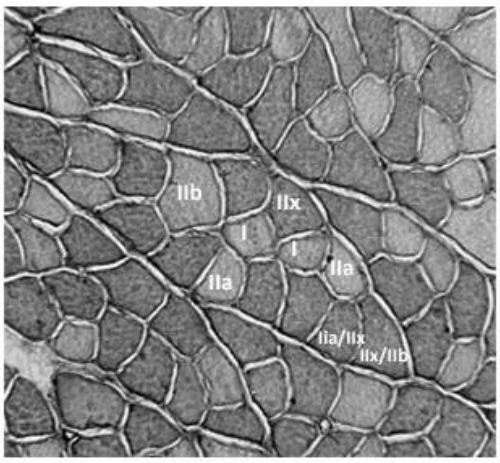
B MHCI (BA-D5)



C MHCIIa (SC-71)



D MHCIIx (6H1)



E MHCIIb (BF-F3)

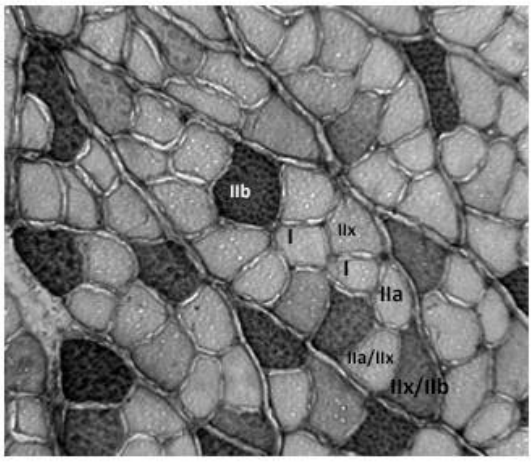


Plate 2.1: *Representative images of fiber types in a trained diabetic rat which underwent resistance training with I = fibers expressing only MHCI, IIa = fibers expressing only MHCIIa, IIx = fibers expressing only MHCIIb, IIB = fibers expressing only MHCIIb. IIa/IIx and IIx/IIb fibers expressing both IIa/IIx MHC's and IIx/IIb MHC's respectively. A) metachromatic myosin ATPase. B) BA-D5 antibody specific to MHCI. C) SC-71 antibody specific to MHCIIa. D) 6H1 antibody specific to MHCIIx. E) BF-F3 antibody specific to MHCIIb.*

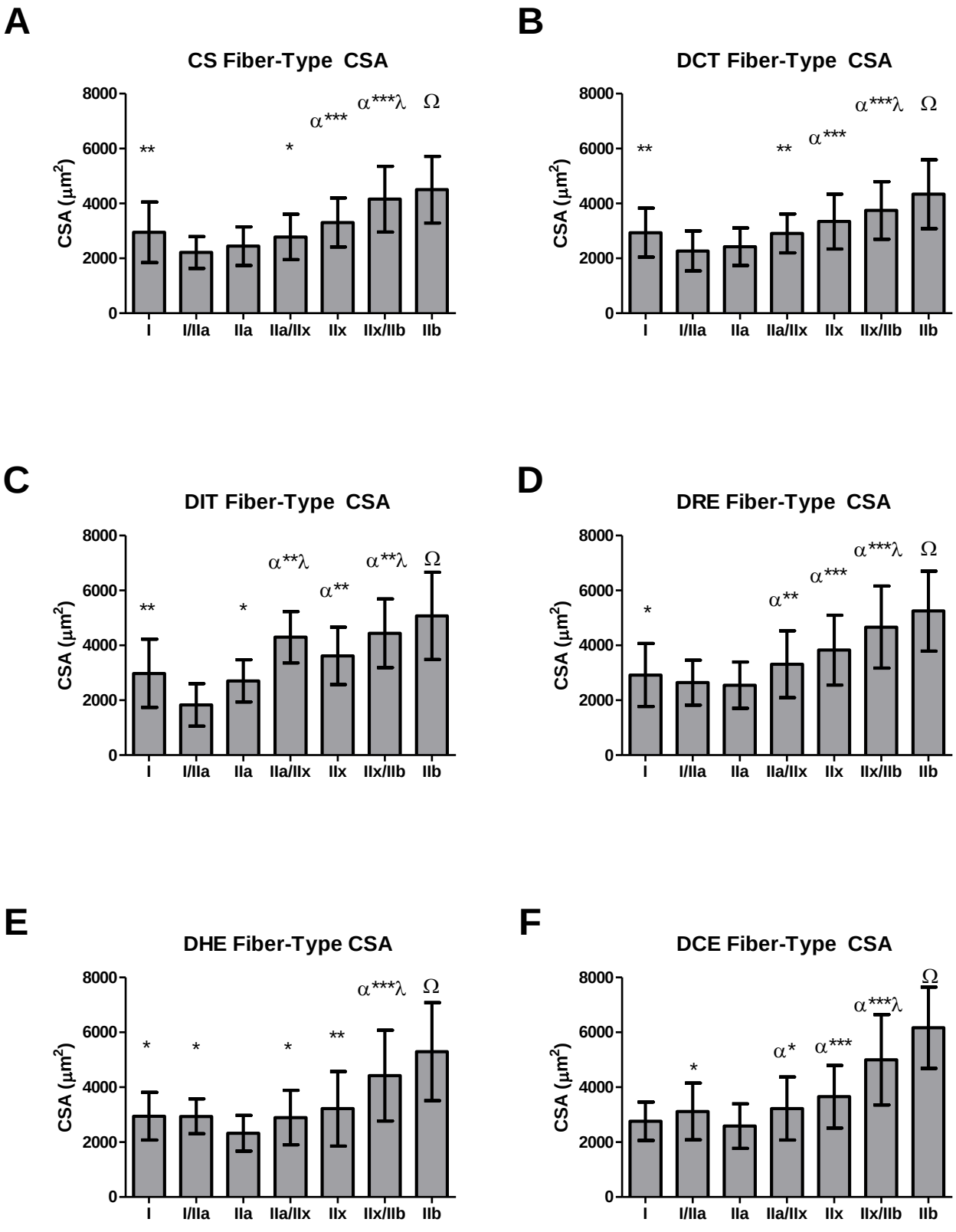

A
CS Fiber-Type CSA
B
DCT Fiber-Type CSA
C
DIT Fiber-Type CSA
D
DRE Fiber-Type CSA
E
DHE Fiber-Type CSA
F
DCE Fiber-Type CSA
CSA (μm²)
0
2000
4000
6000
8000
I
I/IIa
IIa
IIa/IIx
IIx
IIx/IIb
IIb

Figure 2.2: *MHC fiber CSA differences within groups. All data presented as mean ± SD. Differences were considered were considered significant where P<0.05.*

** = sig. > MHCIIa, ** = sig. > MHCI/IIa, MHCIIa, *** = sig. > MHCI/IIa, MHCIIa, MHCIIa/IIx, α = sig. > MHCI, λ= sig. > MHCIIx, Ω = sig. > all MHC. Exceptions where DIT MHCIIa is only sig. > MHCI/IIa, DHE MHCIIx only sig. > MHC IIa, MHC IIa/IIx and DCE where MHCIIx only sig. > MHCI, MHCIIa and MHCIIa/IIx.*

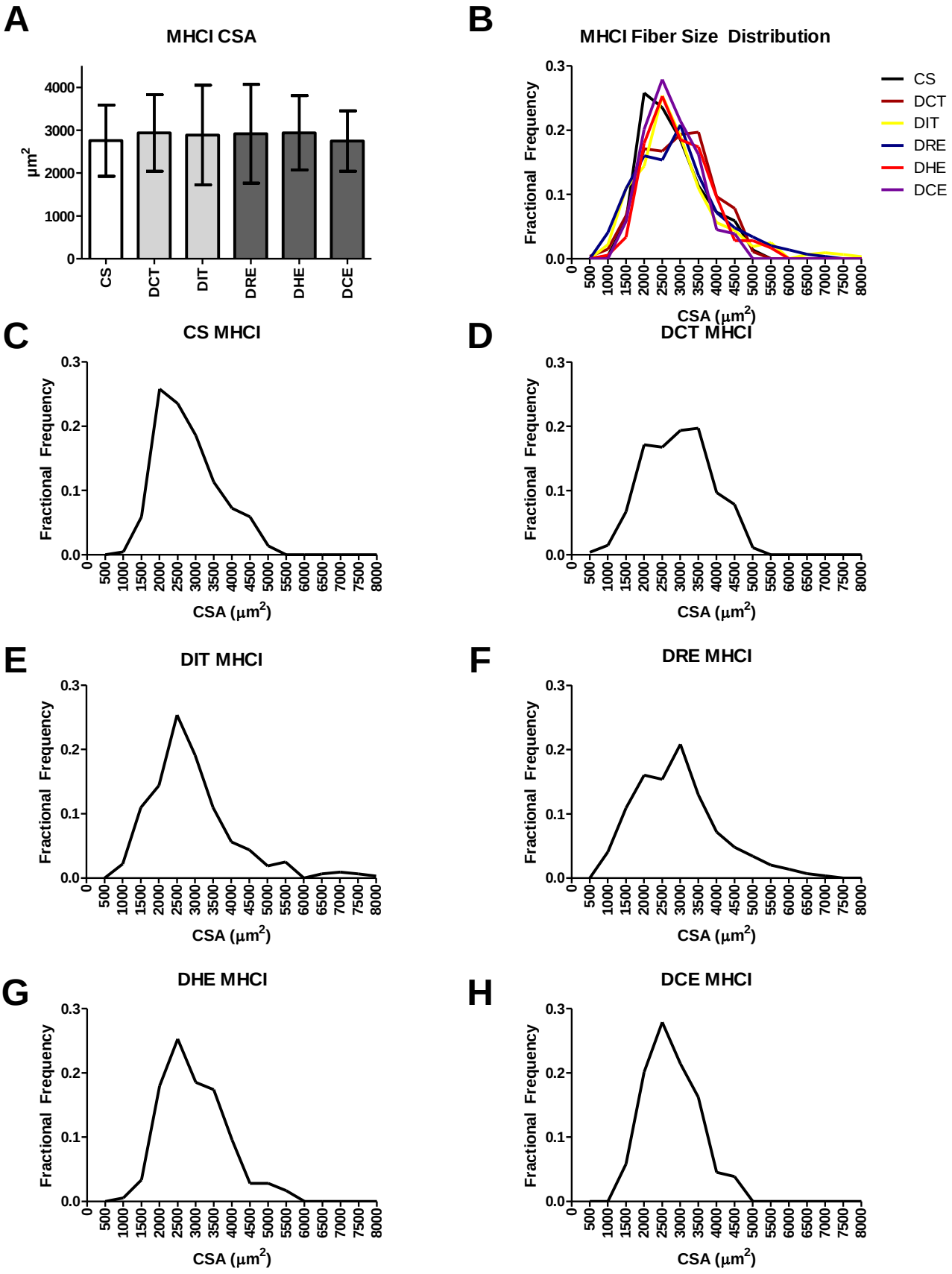

A
MHCI CSA
μm2
4000
3000
2000
1000
0
CS
DCT
DIT
DRE
DHE
DCE
B
MHCI Fiber Size Distribution
Fractional Frequency
0.3
0.2
0.1
0.0
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
6500
7000
7500
8000
CSA (μm2)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCI
D
DCT MHCI
E
DIT MHCI
F
DRE MHCI
G
DHE MHCI
H
DCE MHCI

Figure 2.3: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCI. A) MHCI CSA differences between groups, data expressed as mean ± SD, no statistically significant differences. B – H) Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.*

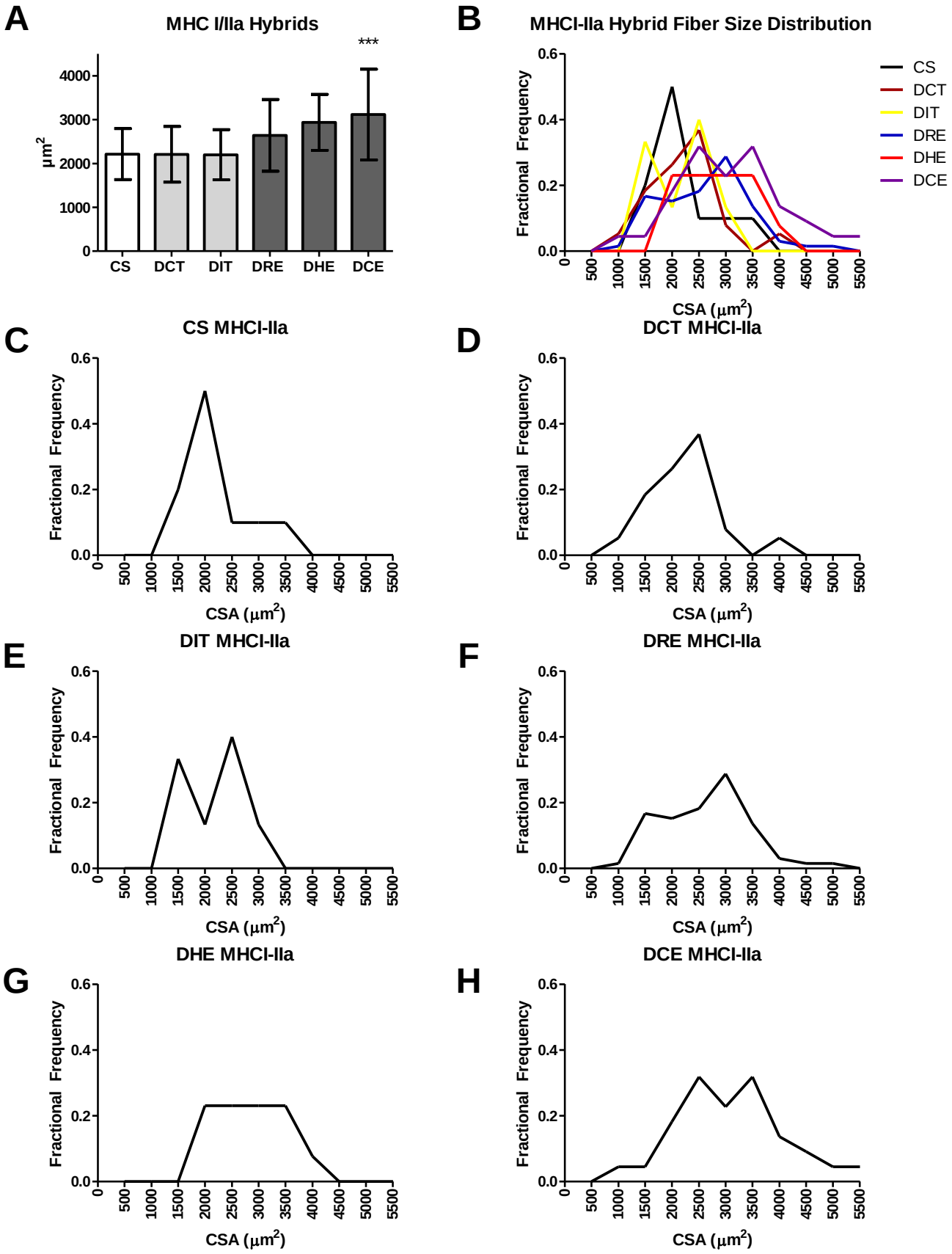

A
MHC I/IIa Hybrids
***
μm2
4000
3000
2000
1000
0
CS
DCT
DIT
DRE
DHE
DCE
B
MHCI-IIa Hybrid Fiber Size Distribution
Fractional Frequency
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
CSA (μm2)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCI-IIa
D
DCT MHCI-IIa
E
DIT MHCI-IIa
F
DRE MHCI-IIa
G
DHE MHCI-IIa
H
DCE MHCI-IIa

Figure 2.4: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCI/IIa* ***A)*** *MHCI/IIa CSA differences between groups, data expressed as mean ± SD.* ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig. > CS, DCT, DIT.*

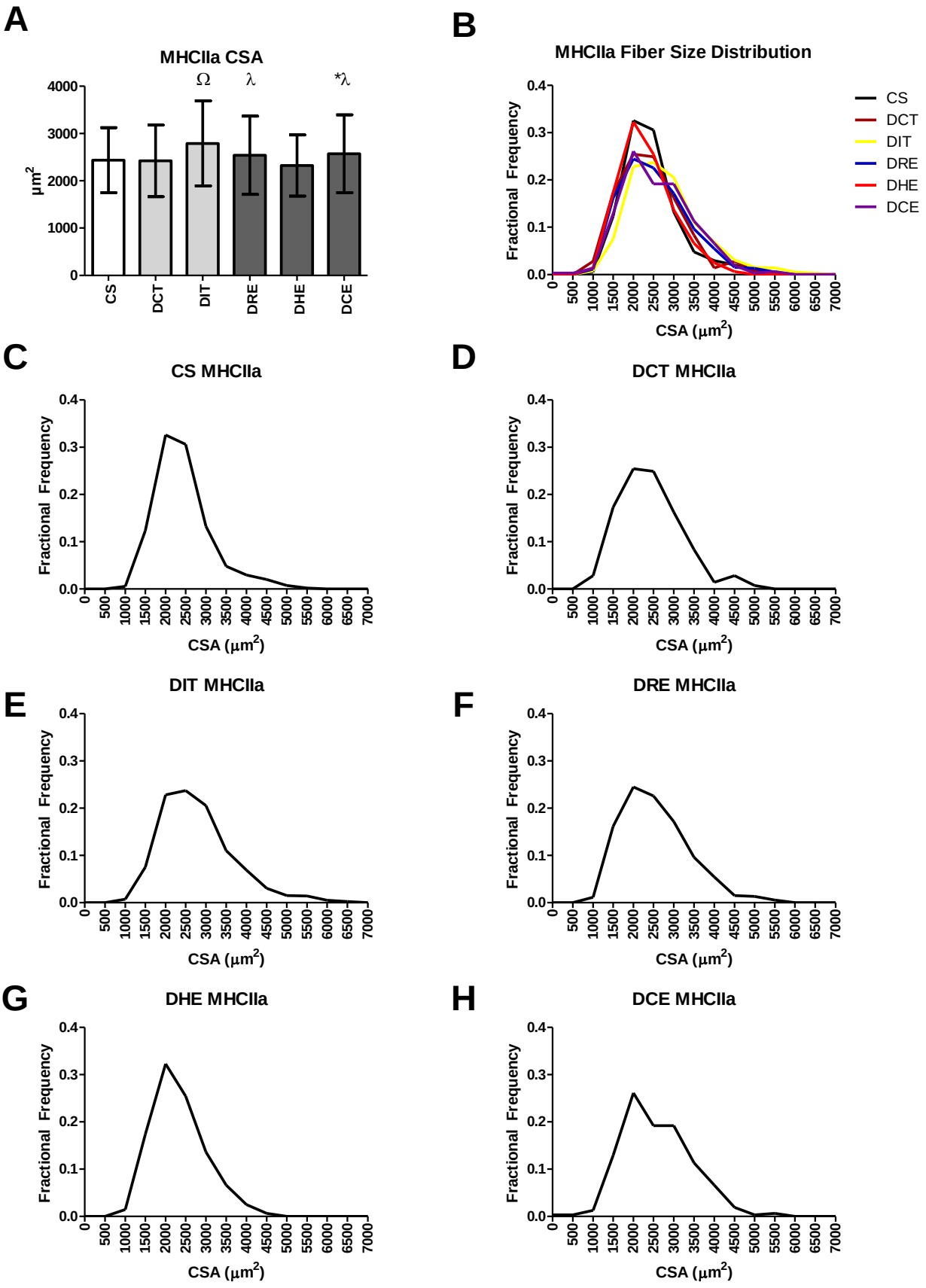

A
MHCIIa CSA
Ω
λ
*λ
μm2
4000
3000
2000
1000
0
CS
DCT
DIT
DRE
DHE
DCE
B
MHCIIa Fiber Size Distribution
Fractional Frequency
CSA (μm2)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCIIa
D
DCT MHCIIa
E
DIT MHCIIa
F
DRE MHCIIa
G
DHE MHCIIa
H
DCE MHCIIa

Figure 2.5: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCIIa* ***A****) MHCIIa CSA differences between groups, data expressed as mean ± SD.* ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig.> DCT,* ***λ****= sig. > DHE,* ***Ω*** *= sig. > all groups.*

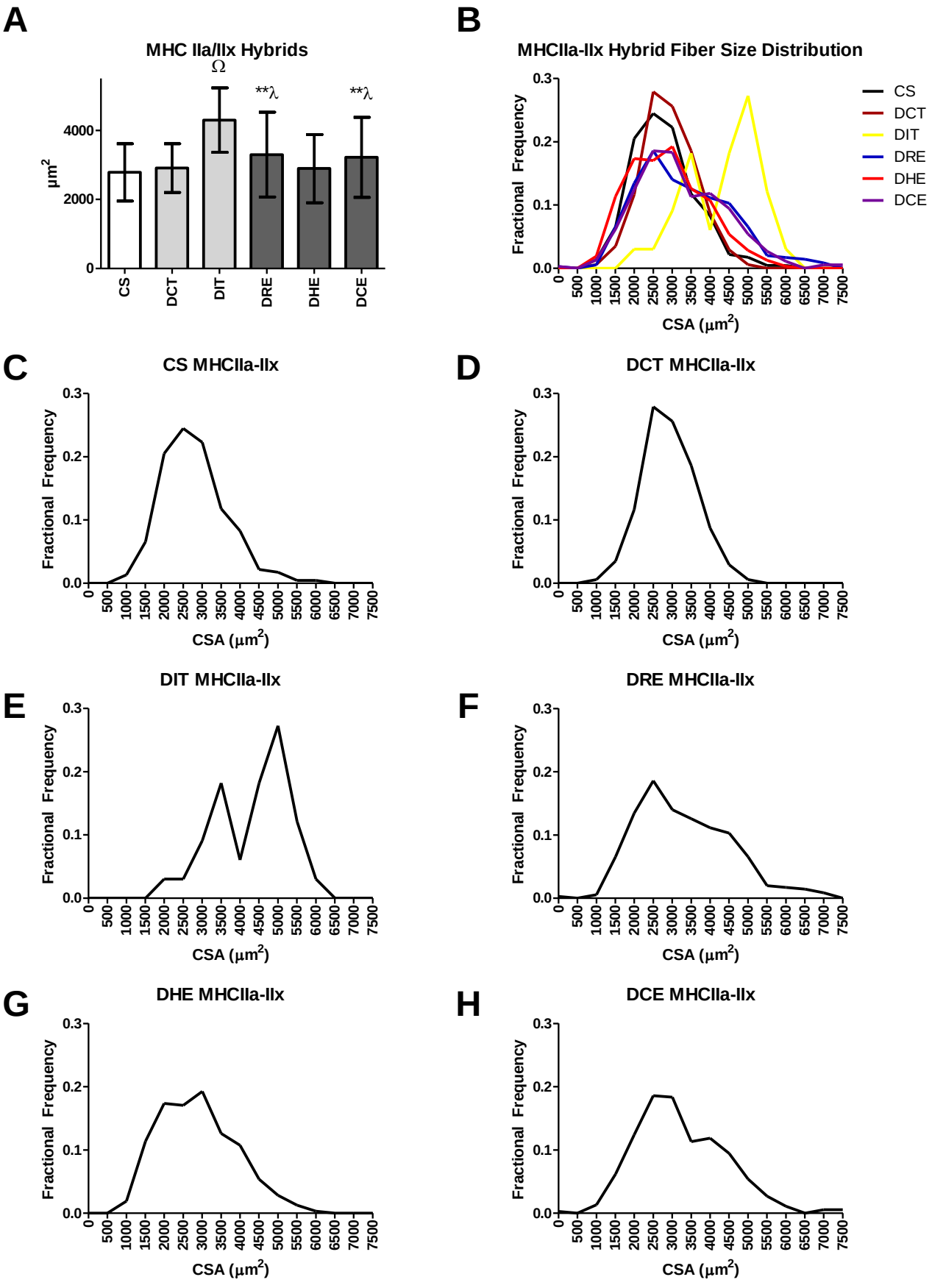

A
MHC IIa/IIx Hybrids
Ω
**λ
**λ
μm²
4000
2000
0
CS
DCT
DIT
DRE
DHE
DCE
B
MHCIIa-IIx Hybrid Fiber Size Distribution
Fractional Frequency
0.3
0.2
0.1
0.0
0 500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500 6000 6500 7000 7500
CSA (μm²)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCIIa-IIx
D
DCT MHCIIa-IIx
E
DIT MHCIIa-IIx
F
DRE MHCIIa-IIx
G
DHE MHCIIa-IIx
H
DCE MHCIIa-IIx

Figure 2.6: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCIIa/IIx* ***A)*** *MHCIIa/IIx CSA differences between groups, data expressed as mean* ± SD. ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig. > CS, DCT,* ***λ****= sig. > DHE,* ***Ω*** *= sig. > all groups.*

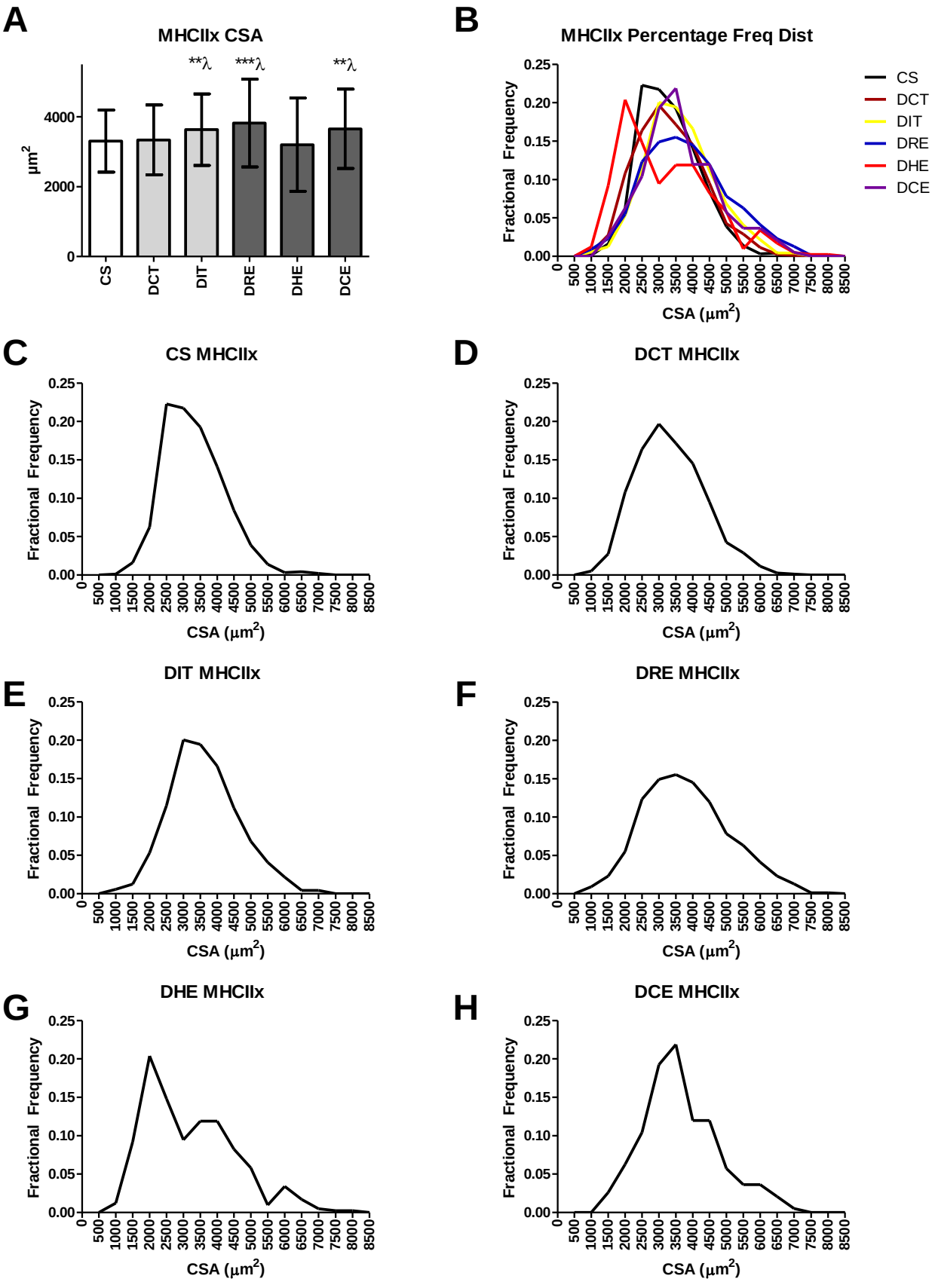

A
MHCIIx CSA
**λ
***λ
**λ
μm2
4000
2000
0
CS
DCT
DIT
DRE
DHE
DCE
B
MHCIIx Percentage Freq Dist
Fractional Frequency
0.25
0.20
0.15
0.10
0.05
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
6500
7000
7500
8000
8500
CSA (μm2)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCIIx
D
DCT MHCIIx
E
DIT MHCIIx
F
DRE MHCIIx
G
DHE MHCIIx
H
DCE MHCIIx

Figure 2.7: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCIIx* ***A)*** *MHCIIx CSA differences between groups, data expressed as mean ± SD.* ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig. > CS, DCT,* ******** *= sig. > CS, DCT, DIT,* ***λ****= sig. > DHE*

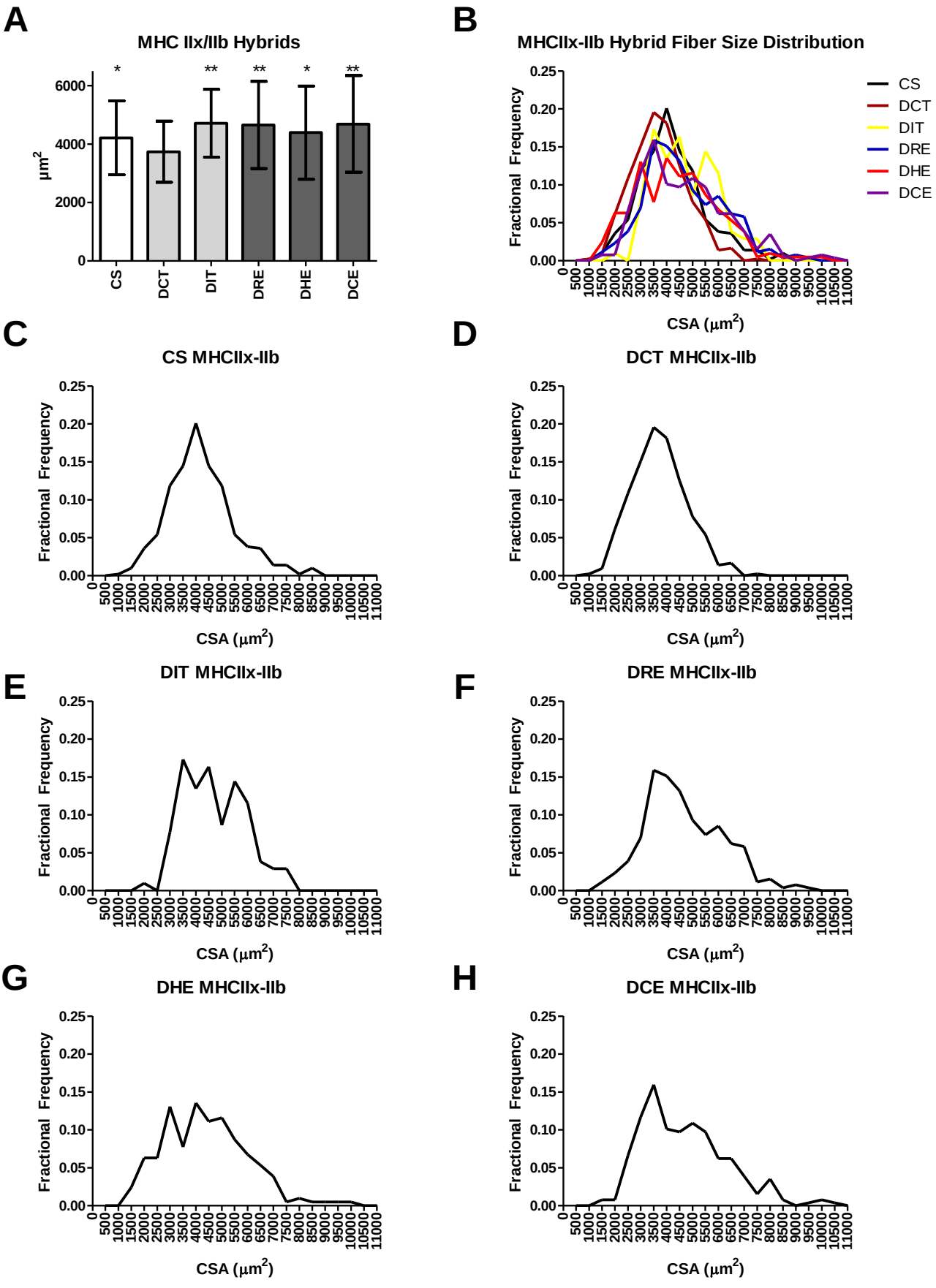

A
MHC IIx/IIb Hybrids
μm2
6000
4000
2000
0
*
**
**
*
**
CS
DCT
DIT
DRE
DHE
DCE
B
MHCIIx-IIb Hybrid Fiber Size Distribution
Fractional Frequency
CSA (μm2)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCIIx-IIb
D
DCT MHCIIx-IIb
E
DIT MHCIIx-IIb
F
DRE MHCIIx-IIb
G
DHE MHCIIx-IIb
H
DCE MHCIIx-IIb

Figure 2.8: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCIIx/IIb* ***A)*** *MHCIIx/IIb CSA differences between groups, data expressed as mean ± SD.* ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig.> DCT,* ******** *= sig. > CS, DCT.*

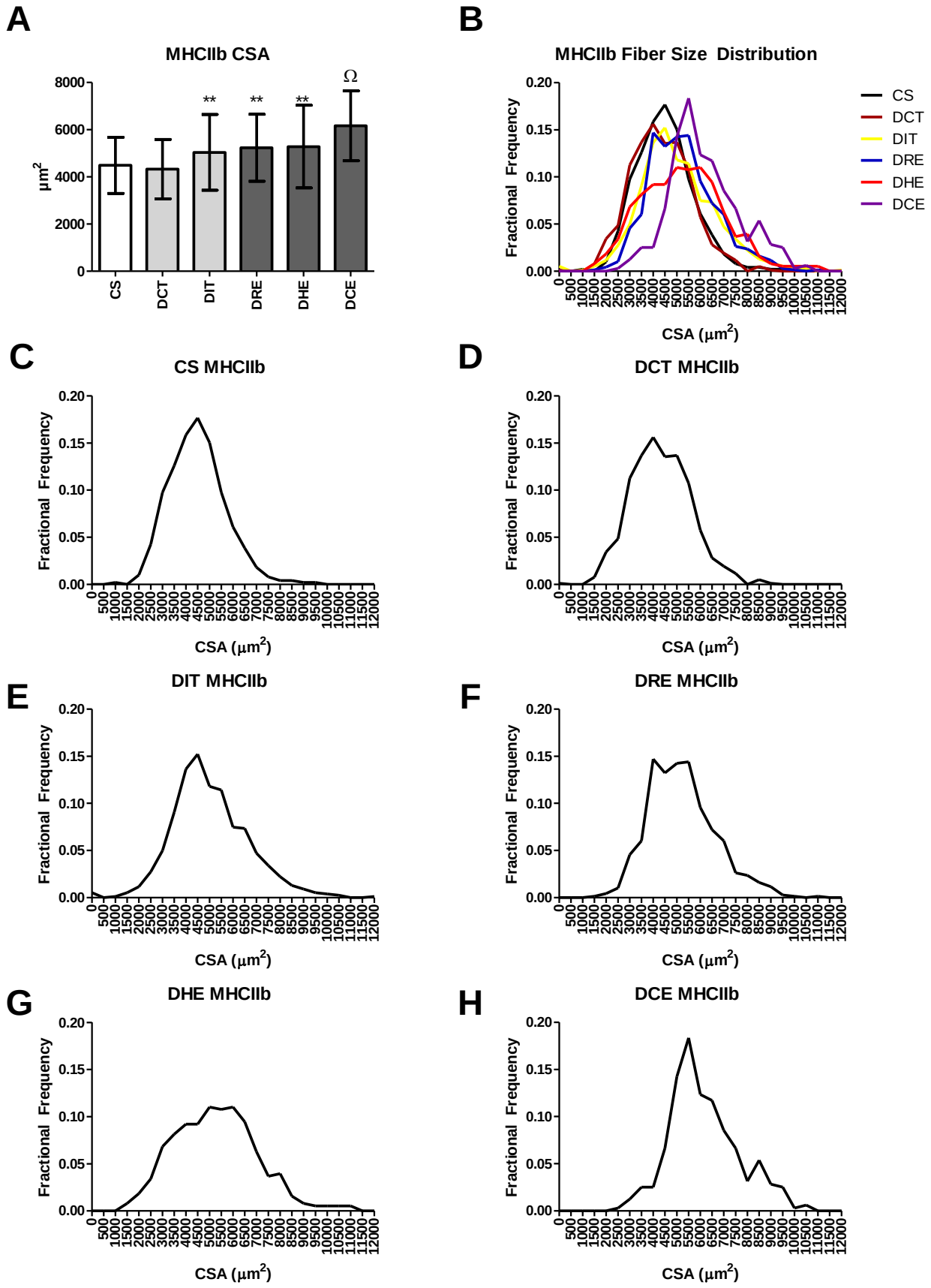

A
MHCIIb CSA
μm²
8000
6000
4000
2000
0
**
**
**
Ω
CS
DCT
DIT
DRE
DHE
DCE
B
MHCIIb Fiber Size Distribution
Fractional Frequency
CSA (μm²)
CS
DCT
DIT
DRE
DHE
DCE
C
CS MHCIIb
D
DCT MHCIIb
E
DIT MHCIIb
F
DRE MHCIIb
G
DHE MHCIIb
H
DCE MHCIIb

Figure 2.9: *MHC CSA differences between groups and fiber CSA frequency distributions for MHCIIb* ***A****) MHCIIb CSA differences between groups, data expressed as mean ± SD.* ***B-H)*** *Fiber CSA frequency distributions for CS, DCT, DIT, DRE, DHE and DCE groups.* ******* *= sig. > CS, DCT,* ***Ω*** *= sig. > all groups.*

2.4 Discussion:

The present study demonstrated that both exercise modality and intensity of insulin therapy induce differential effects on the cross-sectional area (CSA) of muscle fibers identified by myosin heavy chain in multiple low-dose STZ-diabetic, insulin treated rats. Furthermore, this study provided support for the protective effect of both exercise and insulin in the attenuation of the reduced body weight, whole-muscle weight and glycolytic muscle fiber size commonly observed in diabetic myopathy [8,11,13,67]. Although several studies have examined the effects of T1DM and STZ-induced T1DM on skeletal muscle in both humans and rodents, there is a paucity of research examining the long-term effects of different types of exercise training and insulin therapy regimens on the CSA of muscle fibers identified specifically by their MHC content. As muscle fibers expressing different MHC display differential sensitivity to various atrophic stimuli, analyzing fiber size in the context of each of the four MHC's (as well as "hybrid" fibers co-expressing MHC's) in the present model of T1DM has allowed for a characterization of how these fibers respond to exercise and insulin therapy in a diabetic milieu [23]. In addition, studies utilizing STZ to induce the development of T1DM have been considered contentious in regards to diabetic myopathy, as often, the dose of STZ administered has been shown to have atrophic and metabolic effects on muscle fibers independent of hyperglycemia [27,36,68,69]. Hence, a multiple low-dose STZ injection protocol was used to induce T1DM in the present study in an attempt to restrict the atrophic effects of STZ *per se* as opposed to hyperglycemia and/or hypoinsulinemia associated with T1DM. Further, like many rodents studies of STZ induced T1DM, subcutaneously implanted insulin pellets and specific blood glucose range clamps were employed in an attempt to provide a more realistic model of the average T1DM human population [70]. The use of an appropriate insulin supplemented STZ

model, reminiscent of human T1DM, largely maintained body mass, and muscle fiber size despite chronic, moderate hyperglycemia. All of the exercise models employed in the present study appeared, at minimum, to maintain muscle fiber size (aerobic endurance) or result in increased fiber CSA (resistance, combined). Exercise did differentially influence both muscle fiber composition and fiber size of specific fiber types, however, collectively, the use of multiple low-dose STZ induction of T1DM, efforts to control blood glucose in physiologically relevant ranges via insulin supplementation, long term exercise training, and IHC identification and analysis of MHC specific fiber CSA have afforded novel insights into the muscle fiber specific nature of diabetic myopathy.

A common finding in many STZ induced T1DM rodent models investigating diabetic myopathy are physical characteristics including significantly reduced body weight and whole-muscle weight in diabetic animals compared to non-diabetic controls [11,14–16,18,20,22,31,34,71]. In the present study, the finding that mean terminal body weight and whole-muscle plantaris weight in all groups were not significantly different from one another (except for where DRE body weight was significantly smaller than DIT) is likely due to a number of different factors including the nature of the experimental design (STZ dose, insulin treatment, blood glucose levels), muscle-specific factors (muscle glycogen and lipid levels, H_2O content) and exercise training. Unlike the present study, the majority of previous STZ T1DM rodent models reporting significantly reduced body and whole-muscle weight in diabetic animals tend to have utilized a single, high-dose STZ injection, typically between 60-70 mg/kg in rats and 120-250 mg/kg in mice [13,14,16,18,21,22,31–34,71–73]. As previously mentioned, STZ has been shown to exhibit negative effects on skeletal muscle growth and development independent of the resultant hyperglycemia and/or hypoinsulinemia that follows STZ-induced pancreatic β-cell apoptosis. Johnston et al. examined these muscle-specific

effects of STZ *in vivo* and *in vitro* using young Sprague-Dawley rats and C2C12 myoblasts, respectively and the changes were determined to be dose-dependent in nature and due to a G2/M phase cell cycle arrest [27]. As the repair of DNA damage primarily occurs during the G_2 phase prior to entering the M (mitotic) phase of the cell cycle, and STZ treatment is generally understood to cause DNA alkylation through an increase of free radicals and ROS, the G_2/M phase cell cycle arrest observed *in vitro* was suggested to support the idea that STZ exposure induces significant DNA damage in myoblasts [27]. Hence, the multiple, low-dose STZ (20mg/kg) model employed in the present study may have played a role in the relative maintenance of body and whole-muscle weight observed treatments by reducing the dose-dependent myotoxic effects of STZ in all of all of the experimental treatment groups. It should be noted, however, that STZ transportation relies on the presence of the glucose transporter isoform GLUT2, which although largely expressed in the liver and pancreas has been shown to be expressed only in small quantities in skeletal muscle, and thus may actually indicate a limited capacity of STZ for myotoxicity However, as measured, the terminal body weights of the animals in the present experiment did not allow for distinguishing between the relative contributions of lean body mass (i.e. muscle) versus fat mass. Body composition analysis would have been helpful in this regard, as observation suggested that DIT animals had significantly more abdominal fat (as has been observed in intensively treated human subjects) than their exercise trained counterparts.

Most studies using STZ to assess the effect of T1DM on muscle mass have reported reduced whole-muscle weight and a preferential atrophy of glycolytic muscles/fibers. However, they have neglected to control blood glucose through insulin supplementation, and as such the blood glucose concentrations exhibited by these animals are far greater than would be observed in most human patients with T1DM [36]. Blood glucose concentrations throughout the course of

experimentation or at the time of sacrifice, ranged anywhere from 20 mmol/l to 38.8 mmol/l, with some studies reporting blood glucose levels as high as 44.4 and 56.5 mmol/l [13,14,16,18–21,31,33,34,74]. In the present study, effort to clamp blood glucose ranges (7-9 mmol/l for intensive, 9-15 mmol/l for conventional therapy and exercised groups) was used in order to model both the tightly regulated/monitored and moderately hyperglycemic conditions that are more representative of the general human population living with T1DM [36,70]. The disparity between blood glucose levels typically observed in humans with T1DM and those reported in rodent models of diabetic myopathy is an important consideration as hyperglycemia *per* se has been shown to have deleterious effects on skeletal muscle. Krause et al. have indicated that some of the negative outcomes of hyperglycemia related to the diabetic myopathic condition and negative regulation of muscle mass likely includes the formation of advanced glycation end products (AGE), chronic oxidative stress, and the polyol pathway [11]. Hypoinsulinemia *per se* is also a key contributor alongside hyperglycemia to diabetic myopathy. Humans and animals with T1DM that are not subjected to insulin treatment develop a catabolic state where the rate of protein catabolism exceeds the rate of protein synthesis, and in turn results in a net loss of protein that is especially evident in skeletal muscle [75]. Hence, the use of intensive and conventional insulin therapy treatment for sedentary and exercised diabetic animals in the present study was also likely a significant contributor to the maintenance of body and whole-muscle weight as well as fiber CSA that was observed in all groups. The effects of insulin treatment on skeletal muscle mass in T1DM have been determined to be due primarily to an anti-catabolic effect in the reduction of muscle protein breakdown as opposed to an increase in muscle protein synthesis [39,76]. Significant inhibition of muscle protein catabolism can be induced by even minute increases in circulating insulin concentration (i.e. to 104.2 pmol/l), whereas increases in insulin

within postprandial ranges does not exert an increase in muscle protein synthesis. The important role of insulin supplementation in the maintenance of skeletal muscle mass has been investigated, the results of which are especially pertinent to the present study. The investigators used both low multiple-dose (45 mg/kg x 5 days, IP) and single high-dose (180 mg/kg, IP) STZ injections for comparison [40]. Mice subjected to multiple-dose STZ exhibited a reduction in body weight (13%) and 1.88 fold increase in myostatin (MSTN) compared to controls, while high-dose mice exhibited a severe reduction in body weight (27%) and a 3.7 fold increase in MSTN, likely indicating a role of MSTN expression and severity of muscle atrophy [40]. As a growth and differentiation factor, MSTN is known as a potent negative regulator of muscle mass in humans and animals, and MSTN inhibition has been shown to increase muscle mass, fiber size, insulin sensitivity, p-Akt expression and GLUT4 expression, improve muscle regeneration and reduce atrophy [77–85]. Of particular significance is that treatment of the multiple-dose STZ animals with insulin after 2 weeks of diabetes (4IU/day x 3 days, subcutaneous injection) not only corrected hyperglycemia and loss in body weight but completely ameliorated the increased MSTN and atrogin-1 expression [40]. Not only does this suggest that diabetes in the absence of insulin, even using a multiple low-dose STZ protocol can significantly reduce body weight and upregulate MSTN and atrogin-1; but that insulin treatment can completely "reverse" these atrophic indices. Another recent rodent study has suggested the use of MSTN inhibition as an adjuvant therapy in treating T1DM, after finding that in addition to prevention of muscle loss, MSTN mRNA reduction led to increases in insulin sensitivity as well as increases in GLUT4 expression which led to significant improvement in blood glucose regulation [85]. Hence, it is possible that the maintenance of body weight, muscle mass and fiber CSA may be partially due to the role of insulin treatment in attenuating MSTN expression as well as preventing a net protein catabolism

of skeletal muscle from taking place.

Insulin treatment in DCT, DIT and exercised animals (DRE, DHE, DCE) was also essential for controlling blood glucose levels and thus reducing the negative effects of hyperglycemia on skeletal muscle mass. We had hypothesized that intensive insulin therapy (blood glucose 7-9 mmol//l) would result in superior maintenance of muscle fiber CSA compared to conventional therapy (9-15 mmol/l) in sedentary animals. We based this upon the known effects of hyperglycemia, including an acceleration of the process of glycation resulting in the formation of cross-linkages between proteins known as advanced glycation end products (AGE) [86,87]. AGE accumulation in both serum and muscle has been associated with reducing motor function and grip strength in non-diabetic elderly individuals [1,86,88–91]. In T1DM muscle, AGE accumulation is most evident in fast fibers which are also the most affected by diabetic myopathy [90,92,93]. In addition, hyperglycemia is also associated with elevated ROS and activation of the polyol pathway, both of which may contribute to diabetic myopathy The fact that both DCT and DIT were relatively unaffected by differences in hyperglycemia compared to CS further suggests that the presence of insulin may override the negative impact of hyperglycemia on skeletal muscle, at least in the short term. As expected, intensive insulin therapy permitted the same benefits that conventional insulin therapy conferred in maintaining body weight, whole-muscle weight and fiber CSA compared to healthy controls. However, the present data seem to indicate that treatment with an increased dose of insulin may have differential effects on specific MHC fiber types in sedentary rodents despite the reported role of insulin only increasing muscle protein synthesis in the presence of elevated amino acid levels [39,76]. Although maintained or improved CSA compared to DCT animals was expected in DIT animals, the observation that mean MHC IIa fiber CSA was significantly larger than all other conditions including exercise

was surprising. As food and water were provided *ad libitum*, it is possible that DIT animals may have engaged in hyperphagia, elevating circulating amino acid levels and thus increasing the rate of muscle protein synthesis in conjunction with reducing the rate of muscle protein breakdown. However, the finding that DIT MHC IIa/IIx fibers are also significantly larger than all other conditions and exhibit a frequency distribution that appears somewhat bimodal and is noticeably right-shifted seems to indicate that a transition from MHC IIx to IIa is responsible for the robust increase in DIT MHC IIa CSA and the slightly higher MHC IIa percent composition. Interestingly, DIT animals also exhibited far fewer hybrid fibers than any other group (Table 2.2). This could indicate that elevated insulin levels "lock in" single phenotype fibers or that any fiber type transitions resulting from insulin supplementation are complete by the end of 12 weeks.

The present study's investigation of fiber type percent composition in plantaris also provided insight into the fiber specific effects of diabetic myopathy. In general, all groups maintained similar percent composition of specific fiber types as has been previously observed in healthy rat plantaris muscle, where slow oxidative (MHCI) fibers occupy ~10% of the fiber population and fast oxidative glycolytic and fast glycolytic (MHC IIa, MHCIIx and MHC IIb) make up the remaining 90% of the fiber population [95].This is in agreement with previous studies [13,18]. However, both exercise and insulin supplementation appeared to differentially influence muscle fiber proportions particularly within the MHCIIx fibers (Table 2.2). The composition of the more oxidative MHCI and MHCIIa were largely unaltered by diabetes, with or without exercise, probably as a consequence of the presence of insulin and the fact that they are frequently recruited. In rodents, resistance training has been shown to result in increases in the expression of MHCIIx fibers, whereas aerobic endurance training generally increases the percent

composition of MHCIIa and MHCI fibers [96]. In the present study, all exercise conditions exhibited significantly elevated proportions of hybrid IIa/IIx fibers suggesting that both resistance and aerobic exercise tended to generate fiber type transitions. In fact, more than 20% of muscle fibers assessed in the DCE group were MHCIIa/IIx hybrids and DCE also had significantly fewer MHCIIx fibers than CS or DIT. These data suggest that both DHE and DCE groups may be driving less aerobic MHCIIx to more aerobic fibers expressing MHCIIa. The picture is less clear with the DRE resistance exercise condition but given a high proportion of MHCIIa/IIx (over 20%) and the observation of 2 distinct peaks of cross-sectional area in these hybrids (Fig 2.6) it also appears that the resistance training employed in the present study may also transition MHCIIx to MHCIIa fibers. The DIT group which experienced the greatest insulin supplementation in contrast had very few hybrid fibers. As noted above, the reason for this is uncertain but may be associated with the degree of insulin supplementation. In CS and DIT animals, the relative percent composition of MHCIIx fibers was significantly higher than that of DCE animals, MHCIIx/IIb percent composition was significantly higher in CS and DCT animals compared to DIT. This is expected in comparison to the exercise conditions due to a reduced neural influence of physical activity [23,96,97]. Furthermore, in humans, increases in the percent composition of glycolytic fibers has been observed in the skeletal muscle of men with well-controlled T1DM [9,11]. It has been proposed that periodic influxes of glucose in response to insulin treatment could result in the expression of glycolytic muscle fiber genes and drive this increased relative expression, which may explain the observations in DIT animals due to their intensive insulin therapy [11].

The effects of different intensities of insulin therapy and exercise modality on the CSA of the heterogeneous fiber population in the present study also provided several interesting insights.

Within groups and in agreement with previous research, muscle fibers showed a gradual increase in CSA moving from more oxidative fibers to more glycolytic fibers, where MHCIIb fibers had the largest CSA of all fiber types [13,50,95,98].

The effects of diabetes and exercise became more apparent when the CSA of different fiber types was compared between groups. Fibers expressing MHCI appeared to be unaffected by either diabetes or exercise. An expected finding, as MHCI has been consistently reported to be resistant to the atrophy induced by T1DM [11,18,23]. Surprisingly, diabetes did not result in any loss of fiber CSA when compared to control animals. Prior research had suggested that fast-twitch, particularly MHCIIx and MHCIIb fibers might be particularly sensitive to diabetes resulting in reduced CSA [8,11,13,15,16,74,99]. The fact that no changes were observed in the present study suggests that even a minimum amount of insulin is able to maintain fiber size and reduce atrophy. It was hypothesized that intensive insulin therapy (DIT) would provide superior maintenance of fiber CSA compared to conventional therapy (DCT). This hypothesis was supported but it was surprising that for faster fiber types, DIT CSA were actually greater than both the conventional diabetic group (DCT) and the control (CS). This was particularly evident for MHCIIa fibers, where the average CSA for DIT fibers was greater than any other group and these animals also exhibited some of the largest MHCIIa fibers (Fig 2.5). Although the n is small (33 fibers), an examination of MHCIIa/IIx hybrids also reveals higher average fiber sizes for DIT as well as two populations of fiber sizes which might suggest that transitions are occurring. As the anabolic effects of insulin treatment are known to primarily be limited to attenuating muscle protein breakdown, these increases in fiber hypertrophy beyond control and exercised animals is somewhat perplexing [39]. However, as elevated levels of amino acids in conjunction with insulin have been shown to increase the rate of muscle protein synthesis, it is possible that DIT animals

engaged in *hyperphagia*, thus driving the increased fiber CSA observed [39,100]. Perhaps fibers expressing MHCIIa are particularly sensitive to insulin, although other studies have reported that fibers expressing MHCI to have the greatest insulin responsiveness [101].The greatly reduced number of hybrid fibers co-expressing MHC's in DIT animals is another surprising finding, and in conjunction with DIT's effect on CSA warrant further investigation.

Exercise also induced significant and varied changes in CSA in the heterogeneous fiber population of diabetic animals. Overall, DHE animals essentially maintained muscle fiber CSA while DRE and DCE animals exhibited significant increases in the CSA of specific glycolytic fiber populations. Aerobic endurance exercise has typically been shown to induce increases in oxidative fibers in non-diabetic rodents and humans (MHCI, MHCIIa, respectively) and even result in MHCI and MHCIIa fiber hypertrophy [51,53,96]. Although hypertrophy of these fiber types was not evident in the present study, the reduced MHCIIa CSA when compared to other exercise conditions (Fig 2.5A), coupled with the trend toward higher MHCI/IIa hybrid fiber CSA (Fig 2.4A) and presence of some large MHCI fibers (Fig 2.3G) suggests that perhaps the intensive aerobic exercise of DHE is driving muscle fibers to a more oxidative phenotype. DHE animals also displayed an increase in MHCIIb fiber CSA compared to DCT animals which may have been due to recruitment of these larger glycolytic fibers during the intense aerobic exercise sufficient to induce fiber growth.

As expected, animals that underwent resistance training displayed pronounced increases in the CSA of glycolytic fibers (MHCIIa/IIx – MHCIIb – Figs 2.6-2.9), which is consistent with previous research on resistance training induced fiber hypertrophy adaptations [102]. These increases in fiber CSA occurred independently of whole-muscle hypertrophy. However, the lack of gross hypertrophy in the plantaris muscle of DRE and DCE rats in the present study is

supported by previous research using the same resistance training protocol, where only flexor hallucis longus (FHL) muscle showed signs of gross hypertrophy [61]. Both conditions which incorporated resistance training exhibited significantly larger type II fibers, with the exception of MHCIIb fibers, compared to DHE. This could be partially a function of the additional load placed on these fibers during weight lifting or perhaps the smaller MHCIIa-MHCIIx fibers observed in DHE may reflect an adaptation where a reduced diffusion distance is critical [103,104]. These differences were particularly evident in the MHCIIa fiber population as even the largest fibers in the DHE tended to exhibit a smaller CSA than in the other conditions (Fig 2.5). An examination of the range of CSA for MHCIIx fibers in the conditions which incorporated high intensity running (Fig 2.7G and H) reveals three peaks in fiber size. Although speculative, it is possible that this is further evidence of muscle fiber type transition. Another interesting observation was that the combined exercise group DCE exhibited the largest MHCIIb fibers (Fig 2.9), despite lifting weight only half as often as DRE. Indeed, as noted above, even the MHCIIb fibers in DHE were similar in size to those in DRE. Both DRE and DCE animals carried similar and progressively heavier loads over the course of the study. However, it is possible that this discrepancy may be due to the fact that DRE animals trained 5 days a week, whereas DCE animals alternated between daily aerobic endurance and resistance training. This may have permitted greater time for muscle recovery between bouts of resistance training. Previous research investigating the effect of "over-training" in resistance trained rats has reported findings that support this notion [105]. In that study, rats performed a progressive "jumping" resistance training protocol over the course of 12 weeks, increasing from 60% to 85% of body weight overload, which was reported to be about 15% greater than recommended for inducing muscle hypertrophy [105]. Resistance training was performed 5 days per week in order to limit recovery

time between bouts of exercise. Following 12 weeks of training, exercised rats showed decreased body weight and significantly reduced plantaris muscle fiber CSA. The maintenance and hypertrophy of muscle fibers in DCE animals seems to indicate that combined exercise training may be the superior exercise modality of choice for skeletal muscle benefits in T1DM. Not only does exercise potentially increase insulin sensitivity, improving glycemic control - reducing the risk of cardiovascular disease, it is possible that when performed concurrently with resistance training as in DCE animals endurance training may also increase muscle mass while reducing the risk of late onset hypoglycemia [44–46,59]. It should be noted, however, that as endurance and resistance exercise training drive the expression of diverse adaptive gene programs, the concept of an "interference effect" whereby combined training results in compromised adaptations has been postulated [42].In human studies of concurrent exercise training, evidence to support the notion of concurrent exercise training having superior effects on muscle fiber size has been reported. In one study, 5 weeks of combined resistance and endurance training in humans was shown to induce greater increases in vastus lateralis muscle fiber CSA (17% versus 9%) than resistance training alone, indicating that combined training does not appear to hinder but may actually improve the hypertrophic response of muscle fibers [54]. Ultimately, the fiber CSA results of the present study indicate that combined exercise training results in the greatest benefit in terms of muscle hypertrophy, and that the benefits of exercise of any modality in terms of fiber CSA are similar to those observed in intensively treated animals, despite exercised animals having moderate hyperglycemia. Furthermore, the varied effects of insulin treatment and exercise modality on the size of specific muscle fibers in the heterogeneous fiber population observed in the present study warrant further investigation, where identifying the molecular mechanisms underlying the myriad of fiber adaptations at present will help to further

characterize the means by which these fiber size differences occur.

2.5 Conclusion:

The deleterious effects of T1DM on skeletal muscle mass and function, collectively termed diabetic myopathy, has been reported to result in a preferential atrophy of glycolytic muscle fibers compared to oxidative fibers. As T1DM typically develops during adolescence, a time of significant skeletal muscle growth and development, early intervention in preventing or treating the development of these negative effects of the diabetic milieu on skeletal muscle may prove important in maintaining healthy skeletal muscle mass across the lifespan. However, the current body of research regarding diabetic myopathy's fiber-specific atrophic effects has been primarily conducted through the use of rodent models of T1DM that are poorly representative of the human T1DM population due to extreme levels of hyperglycemia and/or hypoinsulinemia as well as high doses of myotoxic STZ to induce β-cell destruction. Moreover, despite the known beneficial effects of exercise on skeletal muscle, few studies have been conducted on the effects of exercise on muscle fiber size in T1DM, and fewer still have compared the effects of different exercise modalities on the heterogeneous fiber population. The aim of the present study was to investigate the effects of conventional insulin therapy (DCT; blood glucose 9-15 mmol/l), which is more representative of the general T1DM population, compared to intensive insulin therapy (DIT; 7-9mmol/l) and different long-term exercise training modalities (DRE, DHE, DCE; resistance, aerobic endurance and combined, respectively) on the CSA of MHC-specific muscle fibers [36,70]. The findings reported presently have afforded insight into how muscle fibers are differentially affected by these conditions while utilizing a model STZ-induced T1DM that maintains translational capacity to humans with T1DM through the incorporation of subcutaneous insulin pellet treatment and specific blood glucoses ranges.

The maintenance of body weight and whole-muscle weight in diabetic animals (sedentary and exercised) compared to non-diabetic, sedentary animals showed that even conventional insulin treatment is capable of attenuating the reduced body and muscle weight reported in previous models of STZ-induced T1DM. As DCT animals, which received both conventional insulin treatment and sedentary lifestyles, did not show significant reductions in these measures, it is apparent that even moderate hyperglycemia is insufficient to induce severe reductions in body weight and muscle mass with mild insulin treatment. Moreover, DCT animals did not exhibit reduced fiber CSA compared to CS animals (except for MHCIIx/IIb fibers), further highlighting the utility of insulin treatment in maintaining fiber CSA.The only significant difference in body weight observed was between DIT and DRE animals, where DRE body weight was significantly lower ($P<0.05$) than DIT animals; however, as DIT and DRE muscle weights did not differ this discrepancy could be due to differences in lean versus fat mass accumulation. DIT animals displayed unexpected increases in muscle fiber CSA compared to both CS and exercised animals, where MHCIIa and MHCIIa/IIx CSA's were significantly larger than all other groups.

In accordance with previous research, exercise did not result in gross hypertrophy of plantaris muscles, although exercise modality did have significant and varied effects on the hypertrophy of specific muscle fiber populations [61]. In general, DHE animals exhibited maintenance of fiber CSA, where DRE and DCE animals displayed specific fiber populations with markedly increased CSA's. Although aerobic endurance training has been shown to be sufficient to induce hypertrophy in oxidative fibers (MHCI, MHCIIa), the lack of fiber CSA increase relative to sedentary control and diabetic animals in DHE animals seems to indicate that the effects of aerobic exercise were limited to protection from atrophy, possibly through

increased PGC-1α expression [23,106]. As expected, both DRE and DCE groups displayed significant but unique differences in glycolytic fiber CSA's. While DRE and DCE animals exhibited increases in MHCIIa, MHCIIa/IIx, MHCII, and MHCIIx/IIb, only DCE MHCIIb CSA was significantly greater than all other treatment groups. It is possible that the consecutive nature of training in DRE animals, where resistance training was performed 5 days per week, resulted in insufficient recovery time and thus inhibited the hypertrophy of MHCIIb fibers. Comparatively, DCE animals engaged in alternating aerobic endurance and resistance training, allowing greater time for muscle recovery while still carrying similar weight loads during resistance training. As these findings seem to indicate that combined exercise training may be superior in terms of maintaining muscle mass and increasing muscle fiber size across the spectrum of oxidative and glycolytic fibers, further studies investigating combined training in humans with T1DM are necessary. In general, further support for the notion of a preferential utilization of combined training in T1DM patients may be gleaned from the fact that high intensity anaerobic training performed prior to aerobic endurance training has been shown to reduce the risk of post-exercise hypoglycemia, which is the primary concern of many T1DM patients engaging in exercise [45,46,107]. In summary, the present study provided important insights into the nature of diabetic myopathy's fiber specific CSA effects in different insulin therapy regimens and exercise training modalities; however, identifying the molecular mechanisms responsible for these differential effects should be the subject of future studies in order to better characterize these adaptations or identify potential pharmaceutical targets.

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A1. Streptozotocin Induction

REVISION DATE: 13/06/2012

PURPOSE:
To induce Type I diabetes in rats

MATERIALS:
Gloves
Lab Coat
Streptozotocin (STZ)
5X Stock Citric Acid/Citrate Buffer
- Anhydrous Citric Acid
- Sodium Citrate Dihydrate
- MilliQ Deionized Water
13M HCl
3 Falcon Tubes
Sterile Filter

EQUIPMENT:
Biological Safety Cabinet
Weigh Scale
pH Meter

PROCEDURE:
Preparing 5X Citric Acid/Citrate Buffer
1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add:
i. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
ii. 23.8g Sodium Citrate Dihydrate (Sigma), Mix into…
iii. 175mL of MilliQ water
The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)
2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

Making up Streptozotocin (STZ) for Injection
**NOTE Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM

2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.
4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15 minute time frame.
Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg.

20mg/kg X 0.2kg = 4mg per animal

The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g)
5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2µm syringe filter. Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution 4mg ÷ 16mg/mL solution = 0.25mL
6. STZ is time dependent and must be used within 15 minutes

Injecting and Follow-Up of the Animals
1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 15 minutes after it has been dissolved in the sodium citrate buffer.
2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container.
3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection).
4. Repeat this procedure the following day.
5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of >18 mmol Diabetes should be achieved after 5-8 injections (i.p. 20mg/kg).

Reference: Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols.200

A2. Insulin Pellet Implant

REVISION DATE: 13/06/2012

MATERIALS:

LinShin LinPlant Insulin Pellet
Rat anesthetic – Isoflurane
Ampicillin
Sterile water
1ml syringe with 25 g needle
10% providone-iodine solution
gauze (or swab)
Tissue forceps
Scalpel handle and blades (or scissors)
Silk suture
Needle drivers

EQUIPMENT:

Isofluorane Anaesthetic Machine
Hair clippers
Heat lamp

Special Safety:
Must don lab coat and gloves before handling rodents. Any bite or scratch that breaks the skin must be thoroughly scrubbed with soap and water (report to Occupational Health and Safety).

PROCEDURE:

Pellet implantation (for a rat):

1. Anesthetize the animal using the isofluorane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O2 flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isofluorane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% providone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.
6. Cleanse a 12g trocar with 10% providone-iodine solution and insert it through the puncture site to a depth of at least 2 cm.

7. Using forceps, briefly immerse the pellet in 10% providone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for the first 350g of body weight.
9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % providone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:
1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% providone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets. 4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

A3. Immunohistochemistry Protocol for Myosin Heavy Chain Isoforms

Advance Preparation

1. Serial 10µm-thick plantaris cryosections, adhered to VWR Superfrost® Plus microslides, stored at -30°C
2. Ensure all slides are labelled with group, animal number, and slide identification with fine tipped pencil
3. Prepare blocking solution aliquots in 1.5 mL Eppendorf tubes and store at -30°C until use:
   BS-1: 10% (v/v) horse serum + 1% (w/v) BSA (ALB001.50, Bioshop) in Tween-PBS (pH 7.4)
   BS-2: 10% (v/v) goat serum + 1% (w/v) BSA (ALB001.50, Bioshop) in Tween-PBS (pH 7.4)
4. Ensure adequate supply of the following items and solutions:
   - Corning coverslips (#1, 22 x 55 mm)
   - Krystalon™ Harleco (EM Science) mounting medium
   - 10x PBS Stock: KCl 2.0g
     NaCL 80g
     Na_2HPO_4: 11.5g
     KH_2PO_4: 2.0g
     Bring to 1L with $_{dd}H_2O$
     Adjust pH to 7.4
   - 1 x PBS (re-adjusted to pH 7.4
   - 1 x Tween-PBS: 1 x PBS + 0.5 mL Tween-20 (Fisher Scientific) per 1L, re-adjust pH to 7.4
   - 30% H_2O_2 (Caledon Laboratory Chemicals), stored at -30°C
   - 100% ethanol
   - Xylenes
   - The following Vector Lab products: ImmunEdge hydrophobic barrier pen
     Avidin-Biotin Blocking Kit (SP-2001)
     DAB Substrate Kit (SK-4100)
   - ABC Peroxidase Staining Kit (#3202, Thermo Scientific)
   - Primary anti-bodies (see below)
   - Vector biotinylated secondary anti-bodies: horse anti-mouse IgG (H+L) (BA-2000)
     goat anti-mouse IgG (H+L) BA-9200)
5. Dehydration tanks, 150 mL each of: 2 x 70% EtOH, 2 x 95% EtOH, 2 x 100% EtOH, 2 x 100% xylene
   - Seal tanks with paraffin wax to prevent evaporation

6. Determine and record specific slide stain designations

Immunohistochemistry Protocol

Day 1

1. Remove slides from freezer.
2. Cover bottom of humidity chambers with $_{d}$H20 soaked paper towels – place slides on racks so that edges hang overhang slightly. Allow slides to reach RT in humidity chambers for ~10 min.
3. Double circle sections with ImmEdge hydrophobic barrier pen.
4. Wash 1 x 5 min in Tween-PBS, 2 x 5 mins PBS.
5. Incubate in 3% H_20_2 for 1 x 15 min at RT.
6. Wash 1 x 10 min in Tween-PBS, 1 x 5 min min in PBS.
7. Prepare the blocking solution/Avidin mixture by adding 4 drops of Avidin D to each ml of blocking solution (BS-1 or BS-2). Approx. 60 µl are required for each section. Incubate for 1 hr at RT. Thaw primary antibodies on ice.
8. Knock off excess blocking solution.
9. Apply approx. 60 µL of dilute primary antibody which has been diluted in the appropriate blocking solution/Biotin mixture (4d Biotin per ml of blocking solution) per section. Cover control sections with blocking solution/Biotin mixture without primary antibody. Cover humidity chamber and incubate at 4°C overnight.

Primary Antibody	**Blocking Solution**	**Dilution**	**Secondary Antibody**	**Dilution**
BA-D5	BS-1	1:20	biotinylated horse-anti-mouse IgG (H+L)	1:200
SC-71	BS-2	1:10	biotinylated horse-anti-mouse IgG (H+L)	1:200
6H1	BS-2	1:10	biotinylated goat-anti-mouse IgG (H+L)	1:200
BF-F3	BS-1	1:100	biotinylated goat-anti-mouse IgG (H+L)	1:400

Day 2

1. Wash 1 x 5 mins Tween-PBS, 1 x 5 mins PBS.

2. Prepare appropriate biotinylated 2° antibody at 1:400 or 1:200 in the corresponding species blocking solution (BS-1 or BS-2).
3. Apply 60 µL of biotinylated 2° Ab per section, and incubate 60 for 1 hour at RT.
4. Prepare ABC Peroxidase Staining Kit: ex. 2 drops of reagent A + 2 drops of reagent B to 10 mL of PBS. **Allow to stand for 30 min at RT before use.**
5. Wash 1 x 5 mins Tween-PBs, 1 x 5 mins PBS.
6. Apply 60 µL of ABC Peroxidase Stain solution and incubate for 45 min at RT.
7. Wash 1 x 5 mins Tween-PBS, 1 x 5 mins PBS.
8. Prepare DAB substrate: to **each 2.5 ml of $_{d}$H20 add**
   - 1 drop of buffer stock
   - 2 drops of DAB stock and mix well
   - 1 drop of H202 stock and mix well
   - 1 drop of Nickel stock and mix well
   **** 1 drop = ~ 50 µL****
9. Apply ample substrate solution (DAB/$NiCl_2$) to each section and incubate for 6-8 min at RT.
10. Rinse in $_{d}$H20 to stop the reaction, place slides in in carriage.
11. Dehydrate rapidly: 3 mins in 70% ethanol, 1 min in 95% ethanol x 2, 1 min in 100%. ethanol; clear in 100% xylene for 1 min.
12. Adhere coverslips with mounting medium.
13. Dispose of excess DAB/$NiCl_2$ solution in designated DAB waste bottle.

A4. Metachromatic Dye Myosin ATPase Histochemical Stain

Advance Preparation:

1. Acidic pre-incubation solution: 0.49% KCH_3COO (w/v)
   0.26% $CaCl_2 \cdot 2H_2O$ (w/v)
   - adjust pH to 4.4 with glacial acetic acid immediately prior to use

2. 0.1 Tris buffer: 12.10g Trizma base

   2.60g CaCl2·2H2O

   -makes 1L, bring pH to 7.8

3. 1% Calcium Chloride Dihydrate Solution: 10g CaCl2·2H2O

   -add to 1L ddH2O

4. 0.1% toluidine blue solution: 0.1% toluidine blue (w/v) for desired volume

   - ex. 50 mL of solution = 50 mg toluidine blue, mix well

5. 150 mL each of 95% ethanol, 2 x 100% ethanol, 1 x 100% xylene

6. ATP Incubation solution: 0.4% glycine (w/v)

   0.42% CaCl2·2H2O (w/v)

   0.32% NaCl (w/v)

   0.19% NaOH (w/v)

   0.3% ATP (w/v)

   -adjust pH to 9.4 with 5N HCl immediately prior to use

   - **prepare this solution immediately prior to staining**

Staining Protocol:

1) Incubate 8 mins at RT in pre-incubation solution
2) Wash 3 x 2 mins in Tris buffer
3) Incubate 30 mins at RT in incubation solution
4) 4 dips x 3 changes in CaCl2 2H2O solution
5) Incubate 90s in 0.1% toluidine blue solution
6) Rinse in running ddH2O for 30s (max.)
7) Dip 5 x in 95% ethanol, 5 times in 2 changes of 100% ethanol, incubate 1 min in xylene
8) Mount with toluene based mounting medium

B. Ethics Approval

Western

AUP Number: 2014-009
PI Name: Noble, Earl
AUP Title: Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes
Approval Date: 06/26/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Innovation To Reduce Muscular And Cardiovascular Complications Of Diabetes" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2014-009::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Curriculum Vitae

John Zachary Nickels

EDUCATION

Western University, JD Candidate – Law (expected graduation 2020)

Western University, MSc Candidate- Kinesiology –Integrative Physiology (2017)

University of Windsor, BHK – Hons. Movement Science (2012)

AWARDS

Western Graduate Research Scholarship, (WGRS; 2013-2015)

RESEARCH EXPERIENCE

Exercise Biochemistry and Molecular Biology Laboratory: Research Assistant

Advisors: Dr. Earl G. Noble, Dr. C.W.J. Melling, PhD (2013-2015)

Western University, Kinesiology

TEACHING EXPERIENCE

Graduate Teaching Assistant, Western University

2230: Introductory Exercise Physiology – Dr. G Belfry, Prof. M. Herbert (2013-2015)

PROFESSIONAL AND ADMINISTRATIVE EXPERIENCE

Kinesiology Graduate Students Board, Western University, KGSA board member (2014-2015)

Society of Graduate Students, Western University, Kinesiology Dept. SOGS representative (2014-205)

Exercise is Medicine on Campus, Western University, newsletter team member (2015 - 2017)

Scholar's Elective Undergraduate Student Research, Western University, student mentor/aide (2015-2016)

Honor's Thesis Undergraduate Student Research, Western University, student mentor/aide (2013-2014)

K1N Peer Mentoring, University of Windsor, Human Kinetics, Vice President and student mentor (2010-2012)