Proteomic Analysis of Diurnal Variation in Human Skeletal Muscle Performance

By

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Abstract

Phenotyping of human muscle based on its profile of myosin heavy chain isoforms is commonly used to help understand changes in muscle function. However, in many instances, measureable changes in force output or contractility occur in the absence of any change in myosin heavy chain profile. Therefore, more sophisticated analysis is required. Proteomic techniques including 2-dimensional gel electrophoresis, high-performance liquid chromatography and peptide mass spectrometry can be used to investigate changes in the abundance of hundreds of proteins simultaneously. To date, such techniques have not been used to specifically characterise the human myofibrillar proteome, or study how the myofibrillar proteome relates to muscle outputs such as peak isometric force or the velocity of contraction. This thesis presents a series of studies that develop proteomic techniques for the analysis of myofibrillar proteins as well as optimisation of techniques for measuring the range of muscle output from isometric through to velocity maximum of the human knee extensor muscles *in vivo*. After optimisation, the proteomic and muscle function measurement were employed to study diurnal variation. Time-of-day differences in sports performance and muscle function are widely reported, and typically, performance is ~10 % greater in the evening compared to the morning. This is consistent with our result in Chapter 3; we investigated this chapter by conducting a battery of muscle performance tests in a population of well-familiarised participants. Our data show that RFD exhibits the greatest diurnal variation (18 %) followed by isometric force (10.2 %). The diurnal variation in IKD data was less robust (range 8.1 - 9.8 %), which may have been due to the lesser precision of this technique compared to MVC and RFD. Therefore MVC and RFD were used in the final study. In final study, this thesis reports significantly ($P<0.05$) greater peak isometric force (11 %) and rate of force development (16 %) of knee extensor muscles of young strength-trained males in the evening compared to morning. Proteomic analysis of biopsy samples of the vastus lateralis profiled more than 100 myofibrillar protein species and detected 8 significant differences in protein abundance between morning and evening samples. The greatest difference was in the abundance of the slow isoform of myosin binding protein C (MyBPC1), which is known to modulate the activity of actin-bound myosin ATPases. MyBPC1 was resolved to 6 species; therefore the difference in abundance of one species reported here likely represents a change in post-translational modification. Therefore, this thesis provides associational evidence that post-translational modification of MyBPC1 contributes to the diurnal variation in muscle function.

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List of Abbreviations, Acronyms and Symbols

°.S^{-1}	Degree per second
2DGE	Two dimensional gel electrophoresis
ACN	Acetonitrile
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AV	Average power
BMAL1	Brain and muscle ARNT-like 1
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK	Creatine kinase
CLOCK	Circadian locomotor output cycles kaput
CO^2	Carbon dioxide
CRH	Corticotrophn releasing hormone
CSA	Cross sectional area
CV	Coefficient variation
Da	Dalton
ddH^2O	Distilled-deionised water
dH^2O	Distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDL	Extensor digitorium longus
EDTA	Ethylenediaminetetraacetic acid
EM	Electron multiplier
EMG	Electromyography
ESI	Electrospray ionization
FA	Formic acid
FDR	False discovery rate
FG	Fast glycolytic
FOG	Fast oxidative glycolytic
FTICR	Fourier transform ion cyclotron resonance
f-v	Force-velocity
HCR	High capacity runner
HFABP	Heart-type fatty acid binding protein
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IEF	Isoelectric focusing
IKD	Isokinetic dynamometry

IPG	Immobilized pH gradient
kDa	Kilo Dalton
LC	Liquid chromatography
LCR	Low capacity runner
LIT	Linear ion trap
LMW	Low molecular weight
m/z	mass-over-charge
MALDI	Matrix-assisted laser desorption/ionization
MOWSE	Molecular weight search
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
MS	Mass spectrometry
MVC	Maximal voluntary contractions
MW	Molecular weight
MyHC	Myosin heavy chain
MyLC	Myosin light chain
N:NIH	Normalized/National Institutes of Health
PAGE	Polyacrylamide gel electrophoresis
PAR-Q	Physical readiness questionnaire
PDF	Portable Document Format (Adobe Acrobat)
pI	Isoelectric point
Pi	Inorganic phosphate
POMS	Profile of mood states
PT	Peak torque
PTM	Post translation modifications
QTM	Qualisys track manager
Rad	Radius
RFD	Rate of force development
RPE	Rate of perceived exertion
RT	Room temperature
RTU	Ready to use
SCN	Suprachiasmatic nuclei
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SO	Slow oxidative
SPSS	Statistical package for social sciences
SRM	Selective reaction monitoring
TC	Thermal comfort
TFA	Trifluoroaceic
T_m	Muscle temperature
TOF	Time of flight
T_{rec}	Rectal temperature
TRIS	Tris(Hydroxymethyl)aminomethane
TSH	Thyroid stimulating hormone

T_{sk}	Skin temperature
v/v	volume/volume
w/v	weight/volume
xg	*gravity*
YOTU	Young old trained untrained
ω	Angular speed

General Introduction

INTRODUCTION TO THESIS

This thesis aims to combine new techniques in muscle proteomics with optimised measures of human muscle force production *in vivo* and use diurnal variation as a model. In doing so, we hope to bring new understanding regarding the role of muscle proteins in underpinning muscle force production.

Proteomics is the study of proteins using high-throughput techniques and relies on a combination of genomics, mass spectrometry and protein biochemistry. The human genome contains of approximately 20,000 genes that are transcribed into mRNA and then can be translated in to proteins. Researchers can test hypotheses regarding individual mRNA or proteins using techniques such as Northern blots (for mRNA expression) or Western blots (for protein abundance) (Wackerhage, 2014). This 'reductionist' approach, where biology is reduced to individual questions, has been the mainstay of biological research. However, data arising from hypothesis-led studies clearly indicates that biology is not organised or controlled by isolated events. Rather, biological systems are organised as complex networks and multiple interactions occur to bring about physiological changes. Therefore, more comprehensive (e.g. '-omic') analysis techniques are required in order to advance our understanding of biological systems.

The proteome is cell-specific and dynamic, responding on a minute-by-minute basis to changes in cell environment. Consequently, the proteome reflects the particular stage of development and current environmental condition the cell finds itself

experiencing. With regard exercise proteomics, Burniston and Hoffman, (2011) report proteomic studies have mostly focused on striated muscle responses to endurance training, which are associated with health benefits underpinned by improvements in aerobic capacity. However, few studies have specifically used proteomic techniques to investigate the human myofibrillar sub-proteome, which will be the main focus of the work conducted in this thesis. Although limited in number of proteins, the myofibrillar sub-proteome is complex because each myofibrillar protein can be expressed as different isoforms and splice-variants, and many also undergo post-translational modifications, such as phosphorylation. Therefore, this thesis investigates different proteomic approaches for capturing this complex information so that it can be correlated with changes in muscle function.

Traditionally, the functional characteristics of a muscle have been related to the size and composition of its myofibres. Human muscle consists of 3 principal fibre types, type I, type IIa and type IIx and studies on the contractile properties of single human muscle fibres in vitro demonstrate each fibre type exhibits a different force-velocity profile (Larsson et al., 1997). While the peak isometric force produced by each fibre type is relatively similar, the maximal velocity of shortening differs markedly; the maximum velocity of shortening of IIa fibres is ~4-times greater than slow type I fibres, and the maximal velocity of shortening of IIx fibres is approximately double that of fibres that express myosin heavy chain IIa. Therefore, differences in the relative proportions of each fibre type can alter the gross force-velocity profile of the muscle. This has direct consequences on power output and athletic performance, but many fibres are hybrids of two fibre types and so attempts to explain changes in

muscle function based on changes to the proportion of type I, type IIa and type IIx fibres becomes difficult.

Muscle fibres are designated as begin type I, IIa or IIx based on their whether the express myosin heavy chain (MyHC) isoform I (protein code MYH7), MyHC IIa (protein code MYH2) or MyHC IIx (protein code MYH1). Each myofibre nucleus (i.e. myonucleus) contains the entire genetic information for Type I, IIa and IIx as well as all other related proteins, but the myofibrillar genes are selectively expressed based on the activity pattern of muscle use. Typically muscle of untrained individuals exhibits relative proportions of 40 % type I, 45 % type IIa and 15 % type IIx (e.g. Holloway et al., 2009). Resistance exercise training is associated with hypertrophy of each myofibre type and, in addition, results in a shift toward greater number of IIa fibres such that the contribution of type IIa fibres to gross muscle performance becomes greater (Widrick et al., 2002). In contrast, endurance training is associated with selective hypertrophy of type I fibres (Gollnick et al., 1973).

Phenotyping of muscle based on its relative expression of MyHC isoforms has provided important insights regarding muscle function and plasticity, but such characterisation is relatively simplistic. Skeletal muscle peak force is proportional to physiological cross sectional area (CSA) and myosin heavy chain isoform (Pearson et al., 2006), but muscle power output is a complex variable that may be influenced by numerous qualitative changes in other myofibrillar proteins. It is important to appreciate the diverse heterogeneity of human muscle fibres, which comprise different myosin heavy chains, as well as many other ancillary proteins (Schiaffino

& Reggiani, 1996). Indeed, studies (Cristea et al., 2008; McGuigan et al., 2003) report significant changes in muscle function induced by squat jumping (Malisoux et al., 2006) and sprint training (Holloway et al., 2009) in the absence of changes in MyHC content. Changes in isoform expression, splice variation or post-translational modification (PTM) of MyHC and other myofibrillar proteins, such as troponins and myosin light chains (MyLC), can alter contractile characteristics in the absence of changes in MyHC. Therefore, it is important to investigate the entire myofibrillar sub-proteome in order to gain further understanding of the proteins that dictate muscle function.

Recent technological developments including robust two-dimensional gel electrophoresis (2DGE), high-performance liquid chromatography (HPLC) and mass spectrometry (MS) enable comprehensive investigations and inductive 'discovery science' approaches at the protein level and have recently been applied to questions of muscle adaptation. This thesis begins by investigating the type of proteomic technique that is best suited to analysis of myofibrillar proteins. Specifically our aim was to advance the study of changes in myofibrillar proteins beyond MyHC in order to more accurately understand the mechanisms that underpin the force-velocity (f-v) characteristics of human muscle. After investigating different proteomic techniques our next aim was to establish techniques for robust measurement of human muscle performance *in vivo*. Finally, we attempted to bring these two areas of research (i.e. muscle proteomics and muscle function) together to investigate proteins that correlate with diurnal changes in muscle force production.

The power output of human muscle and human sports performance exhibit diurnal variation (Drust et al., 2005; Reilly and Waterhouse, 2009). Diurnal variation in muscle force and sports performance in young healthy individuals is widely reported but the mechanism underpinning this phenomenon are not yet understood. Muscle force production is least between the hours of 06.00 – 08.00 in the morning and greatest from 16.00 – 18.00 in the afternoon. Table 1 summaries research studies that have investigated the effects of diurnal variation on muscle performance (see Table 1). Regardless of the dependant variable measured (e.g. maximum isometric force, peak torque etc.) muscle performance is consistently better during the evening as opposed to the morning. The magnitude of the change in muscle output from morning to evening is reported to range between 5-20%. The underlying cause of this variation has been attributed to variations in metabolism, circulating levels of hormones, and differences in core or local muscle temperature, all of which have shown patterns of circadian rhythms and may therefore affect performance (Araujo et al., 2011; Racinais & Oksa, 2010).

Table 1. Studies reporting diurnal variation in muscle output

Test	Author	Difference (%)
MVC		
	Martin et.al (1999)	8.9%
	Racinais et al. (2005)	9.2%
	Reilly (2007).	9.0%
	Edwards et al. (2013)	12.6%
IKD		
60	Reilly (2007).	6.2%
90		4.6%
180		8.2%
60	Edwards et al. (2013)	9.6%
240		10.6%
Anaerobic		
Broad jump	Reilly (2007).	3.4%
Stair run		2.1%
Flight time		2.4%

Fluctuations in force production co-occur alongside biological rhythms in core temperature but we have found diurnal variation in muscle performance is not entirely explained by differences in muscle temperature. For example, passive heating in the morning to stimulate the warmer afternoon muscle temperature did not bring about similar elevation in muscle performance. Similarly, passive cooling in the afternoon to replicate the cooler muscle temperature of the morning was not associated with the decrease in performance. Thus intrinsic differences occur in the ability of skeletal muscle to produce force during the course of the day. The parameters of muscle performance (i.e. maximum force and maximum speed) are determined by collections of contractile proteins. Differences in genetic background, habitual activity or training status affect the relative amounts of each muscle protein and are one of the main reasons for the broadly different physique and physical performance of sprinters compared to marathon runners. The same contractile proteins can also be rapidly modified to cope with short-term changes in demand. This latter mechanism contributes to the positive effect of warming up prior to strenuous exercise, and similar mechanisms could able responsible for the greater output of skeletal muscle in the afternoon compared to the morning. Therefore further investigation involving proteomic analysis of muscle biopsies needs to be done.

Aim and objectives of the thesis

The overall aim of this thesis was to combine proteomic analysis of muscle biopsy samples with measurements of muscle performance in humans. Because the proteome is what defines a cell (or tissue) and dictates its functional properties we reasoned that changes in muscle function must be underpinned by changes in the muscle proteome. More specifically, changes in muscle force production will be underpinned by changes to the myofibrillar proteins and we used diurnal variation as a model. To achieve our overall aim we conducted a series of experiments to establish and optimise techniques for proteomic analysis of muscle biopsy samples and techniques for the analysis of human muscle function in vivo. The purpose of this chapter is to summarise the findings from each of the foregoing experimental chapters and detail how the main aims of this thesis were met. A secondary purpose of this chapter is to provide recommendations for further research based upon this body of work.

The specific aims of this thesis were:

1. To establish new proteomic techniques for the analysis of the myofibrillar sub-proteome of human muscle

2. i) To investigate whether measurements of isolated knee extension can be used to predict performance during more complex sport-related movements (i.e. vertical jump performance)

ii) To investigate the reliability of isolated knee extension measurements encompassing the entire range of the human force-velocity relationship in vivo

4. To determine which method of measurement or which feature of knee extensor performance exhibits the greatest diurnal variation

5. To discover diurnal differences in the myofibrillar sub-proteome of human vastus lateralis and investigate whether these correlate with differences in muscle function.

Chapter 1

Analysis of Animal and Human Skeletal Muscle Using Traditional and Proteomic Analysis

1.1 INTRODUCTION

Chapter background

Skeletal muscle

Skeletal muscle represents ~40 % of body weight in healthy adults and is important in metabolism, thermogenesis and locomotion. The ability of muscle to produce force underpins all sporting performance. Individual skeletal muscle fibres have two main protein structures that make up the myofilament and incorporate actin (thin filament) and myosin (thick filament). These proteins are assembled in a specific manner to form the basic repeating functional unit of a myofibril – the sarcomere. The sarcomere consists of thick myosin filaments that are anchored to a protein sheet (the M-band) and overlap with the thin actin filaments. This arrangement gives the skeletal muscle its characteristic striated appearance. The sarcomeres (Figure 1) are arranged in sequence along the myofibrils, and it is the interaction between actin and myosin within each sarcomere that enables a muscle to contract.

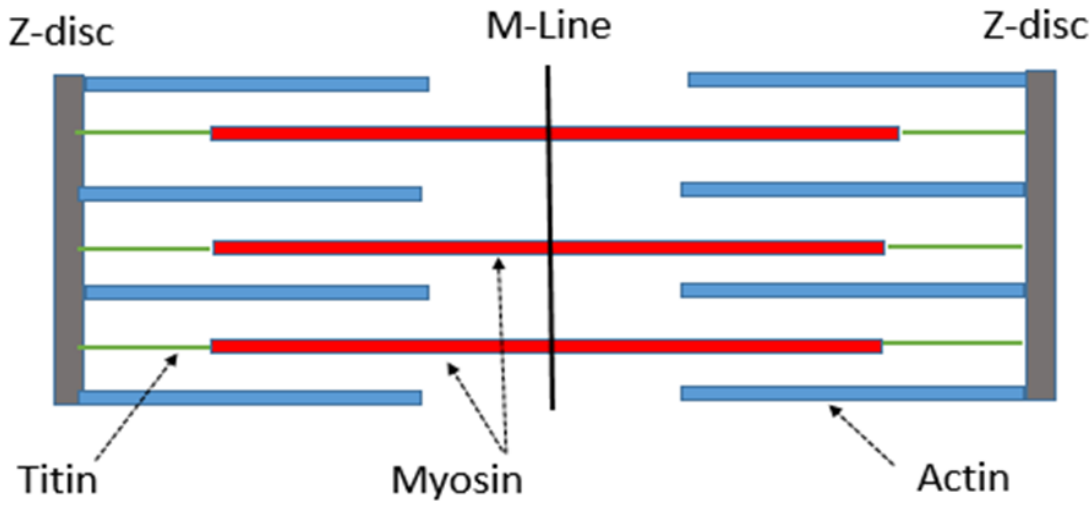


Figure 1. Figure of sarcomere component.

The myosin molecule is made up of 6 polypeptides – 2 heavy chains and 4 light chains. The myosin heavy chain contains a myosin head that binds to the actin molecule and forms the basis for muscle contraction. The myosin head region serves as the binding site for adenosine triphospate (ATP) and contains the enzyme adenosine triphosphatase (ATPase), which enables the hydrolysis of ATP into adenosine diphosphate (ADP) and inorganic phosphate (Pi), thus providing the energy for muscle contraction (Scott et al. 2001). The thin actin filament consists of 2 regulatory proteins – troponin and tropomyosin. Under resting conditions, tropomyosin binds to the active sites on actin, and prevents actin and myosin from binding together.

The stimulus for muscle contraction is an action potential, which is conducted along the sarcolemma. Depolarisation of the fibre leads to the release of calcium from the sarcoplasmic reticulum and the activation of the cross bridge cycle that results is muscle contraction and force generation. Cross-bridge cycling continues as long as calcium and ATP are available, although the speed at which this occurs is mainly determined by the rate at which the myosin head ATPase can hydrolyse ATP.

Skeletal muscle comprises a heterogeneous mixture of myofibres, in human muscle 3 fibre subtypes are recognized based on their contractile and metabolic properties (Schiaffino, 2010). Fast-twitch fatigable fibres rely predominantly on glycolytic metabolism and are designated FG (fast glycolytic), whereas, fast-twitch fatigue-resistant and slow-twitch fibres have relatively greater mitochondrial content and are designated FOG (fast oxidative glycolytic) and SO (slow oxidative), respectively.

The fibretype-specific differences in contractile function are due to differential expression of a diverse range of isoforms of each myofibrillar protein (Schiaffino & Reggiani, 1996). Myosin heavy chain (MyHC) isoforms are intimately associated with myofibre contractile and energetic properties and are commonly used molecular markers: FG fibres express MyHC IIx/d, FOG fibres express MyHC IIa and SO fibres express MyHC I, which is also the predominant isoform in adult human myocardium. Phenotyping of muscle based on its profile of FG, FOG and SO fibres or relative expression of MyHC isoforms has provided important insights regarding muscle function and plasticity, but such characterization is relatively simplistic. For example, changes in isoform expression splice variation or post-translational modification of other myofibrillar proteins, such as troponins and myosin light chains, can alter contractile characteristics in the absence of changes in MyHC. Moreover, each myofibre type is metabolically heterogeneous and can exhibit a broad spectrum of different metabolic enzyme concentrations that determine substrate utilisation and resistance to fatigue (Pette 1985). Proteomics affords the capability to analyse large numbers of contractile and metabolic proteins simultaneously and, therefore, could be used to develop a more sophisticated understanding of muscle diversity and plasticity.

Proteomics

The terms ‘proteomic’ and ‘proteome’ were coined by Mark Wilkin at the Siena Conference in 1994 (Wasinger, 1995). These ‘-omic’ terms symbolize a redefinition of how we think about biology and the workings of living systems. The proteome is the cell-specific protein complement of the genome, and the proteins expressed

within a cell are what define that cell and dictate its functional characteristics. Anderson & Anderson (2002) stated that a proteome contains a wide diversity of unique proteins ranging in physical and chemical properties and are present over a large dynamic range in abundance. In muscle, the top 10 myofibrillar proteins (e.g myosin, actin, troponin, tropomyosin etc.) account for ~50 % of the total protein content (Geiger et al., 2013). An important characteristic of the muscle proteome is that each myofibrillar protein can be expressed as different isoforms and splice-variants. The high abundance of these proteins means that subtle changes in isoform expression have overt effects on muscle function. However, presently the majority of our knowledge relates to the effects of different myosin heavy chain (MyHC) isoforms only. Sub-proteome analysis of myofibrillar proteins could greatly advance muscle phenotyping by enabling measurement of changes in isoform expression, splice variation or post-translational modification of myofibrillar proteins, and correlate these to changes in muscle function. For example, in adult skeletal muscle, troponin T is expressed as fast and slow isoforms and each isoform can undergo numerous splice variations and post-translational modifications. Proteomic techniques can study each of these different troponin T species (Holloway et al., 2009), whereas immunological techniques differentiate fast and slow isoforms only (e.g. Malisoux et al., 2006).

Proteomics studies include the characterization of the proteome in terms of protein structure, function and expression level under particular biological conditions (James, 1997). Recent technological developments including robust of two-dimensional (2-D) gel electrophoresis, high-performance liquid chromatography (HPLC) and mass spectrometry (MS) enable comprehensive investigations and

inductive ‘discovery science’ approaches at the protein level. Proteomics can provide useful information about the mechanism of human diseases and developing improved methods for disease diagnosis and treatment. Central to this ability is the ‘open’ or non-targeted nature of proteomic experiments. The areas of study are fairly diverse in scope, for example, considering protein structure, function, protein-protein interactions, in addition to the role of proteins in metabolic or signal pathways and their contribution to biological processes (Marcotte et al., 1999; Schwikowski et al., 2000). Furthermore, biomarker discovery can be achieved by using expression proteomics (differential profiling) to identify change in protein abundance that correlates with the physiological state of the organism as tissue.

The proteome presents a difficult analytical challenge because not all proteins are amenable to separation or analysis using a single technique (e.g. hydrophobic vs hydrophilic proteins). Santoni et al., (2000) report hydrophobic proteins are critical components of cellular membranes, and are more difficult to manipulate in solution due to their poor solubility in water. To date, attempts to study changes in the skeletal myofibrillar proteome beyond MyHC (e.g. Malisoux et al., 2006) have been largely unsuccessful due to the complexity of different protein species and limitations of immunological techniques, i.e. their in ability to distinguish between isoforms or variants. Therefore, it is necessary to establish the most suitable techniques for analysis of the myofibrillar sub-proteome.

Proteomic techniques

Considering the complexity of the proteome, improving the methods of analysis is an important objective. The methodologies employed in proteomics vary depending on the objectives of the experiment. Proteome mining experiments aim to catalogue all detectable proteins within a sample. This is important work because unlike of levels of analysis such as genomics or transcriptomics, the entire complement of protein species within a sample is not known and cannot be predicted. Expression proteomics, or differential protein profiling, entails the determination of relative protein abundance in a comparative scheme for example comparing the proteome of a tissue under two physiological conditions. Regardless of the overall objective (i.e. mining or profiling) proteomic analysis schemes incorporate at least two stages: (i) protein separation and (ii) protein identification by mass spectrometry.

Protein separation

The analysis scheme used to study the proteome is referred to as the proteomics workflow. Several workflows have been devised for conducting differential expression analysis or proteomic profiling. Usually the workflow uses a ‘Top-down’ gel-based approach or bottom-up solution-based approach. The gel-based approach involves multidimensional separation of proteins through two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), which is often abbreviated to 2D gel electrophoresis (2DGE). Bottom-up, in-solution analysis, typically uses high-performance liquid chromatography (HPLC) of tryptic peptide digests of the protein samples.

The majority of previous studies have used 2DGE as it affords high-resolution separation of proteins (Ofarrell, 1975; Gorg et al., 2000). Frequently sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used as stand-alone method of protein separation as known as 1D-PAGE (de Godoy et al., 2008) but the 2D-PAGE technique combines isoelectric focusing (IEF), as a first dimension separation, and SDS-PAGE as a second dimension. The IEF first dimension involves the extraction and solubilisation of proteins using a buffer containing a variety of protease inhibitors non-ionic detergent and solubilizing agents (Ofarrell, 1975; Gorg et al., 2000). Proteins are then loaded onto a polyacrylamide or agrose gel strip, containing an immobilized pH gradient (IPG) (Bjellqvist et al., 1982; Gorg et al., 1988). After the sample has been absorbed on to the strip, an electric field is applied across the gel strip causing proteins to migrate in a direction based on their charge until they reach their isoelectric point (pI) on the pH gradient. Prior to the SDS-PAGE second dimension, proteins are denatured using the anionic surfactant sodium dodecyl sulfate (SDS). In 2D-PAGE, the focused IPG strip is first equilibrated with an SDS solution, using dithiothreitol (DTT) and iodoacetamide (IAA), respectively, which cleave disulfide bonds and prevent disulfide bond reformation. This improves digestion of cysteine-containing proteins and detection of cysteine-containing peptides. Then the strip is placed onto an SDS gel slab. An electric field is applied with the positive electrode at the bottom and the negative at the top and SDS bound-proteins (i.e. negatively charged) migrate through the gel dependent upon their relative molecular mass. The larger proteins travelling a much smaller distance than the smaller proteins. After that, the gels are digitised scanned and saved in grayscale tiff format to capture both the area and density of each spot. Digitised scanned

images of stained gels are then measured for the concentration of each band or spot by densitometry and can be used to interpret relative protein abundance.

Importantly, at this stage differences in abundance of proteins between the study groups is known but the identity of these proteins is not known. This is fundamental to the non-targeted/unbiased nature of proteomics experiments and enables new discoveries to be made.

In contrast to gel-based workflow, the other workflow employs a solution-based, peptide level analysis which involves the enzymatic digestion of the proteome prior to multidimensional liquid chromatography separations and MS peptide sequencing. High-Performance Liquid Chromatography (HPLC) is highly applicable as an initial step to fractionate protein mixtures and the advantages of HPLC is the diversity of separation modes available (Lieber, 2002), but typically reverse phase is used to separate peptides based on their hydrophobicity. Peptides can be separated by running a linear gradient of the organic solvent. Molecules are bound to the hydrophobic matrix in an aqueous buffer (polar) and eluted from the matrix using a gradient of organic solvent (non-polar). The matrix usually consists of spherical silica beads (3-5 micron) which have linear octadecane groups (C_{18}) attached to the surface via co-valent bonds. These beads are usually porous in order to increase the surface area of the beads available for binding. There are three common stationary phase chain lengths, C_4, C_8, and C_{18}. C_4 is generally used for intact proteins and C_{18} is generally used to capture peptides or small molecules. The idea here is that the larger protein molecule will likely have more hydrophobic moieties to interact with

the column and thus a shorter chain length is more appropriate. Peptides are smaller and need the more hydrophobic longer chain lengths to be captured, so C_8 and C_{18} are used for peptides or small molecules.

Mass Spectrometry

Non-targeted proteomic analyses are made possible by mass spectrometry (MS) which is used to identify proteins against genome databases. MS relies on separating charged peptide ions by their mass-to-charge ratio (m/z). It became an ideal tool for high-throughput analysis of large numbers of proteins from complex mixtures when its speed and sensitivity were capable of analysing proteins on HPLC timescale (i.e. peptides elute within a 1 min window). Generally a mass spectrometer can be broken down into three parts: an ion source, an m/z analyser and a detector.

Ion Sources

The first stage of the MS instrument is the ion source and it serves two main purposes. First, it brings molecules into the gas phase and secondly the molecules are ionized so that they can be manipulated by electric fields within the mass analyser. The development of the soft ionization methods for MS analyses of biological macromolecules were contributed by Fenn et al. (1989) with the technique of electrospray ionization (ESI) and the other technique of matrix-assisted laser desorption/ionization (MALDI) by Karas & Hillenkamp (1988), which was subsequently applied to whole protein analysis by Tanaka et.al (1988). Fenn's contributions specifically related to the development of electrospray ionization and

earned a share of the Nobel Prize in Chemistry in 2002. In MALDI source, the sample to be analysed is mixed with a chemical matrix, which contains organic molecules that donate protons to the sample when energised by the laser. The admixture of sample and matrix is then spotted onto a small plate or slide and then allowed to evaporate in air. The co-crystallization of small organic molecules with the protein sample from a solid allows ionization through the use of a laser, resulting in desorption and ionization of the protein or peptide. However, one of the disadvantages of MALDI compared to LC-ESI is it considered to be more difficult to couple MALDI to separation platforms such as LC, although this is possible, i.e LC-MALDI (Burniston & Connolly, 2010; Holloway et al., 2009). LC-MALDI is considered as a very powerful technique but it is very time consuming because it is not 'online'.

Meanwhile ESI which was used in the current thesis is a relatively simple ion source which permits the direct coupling of liquid samples to MS. Fragmentation of peptide ions is favoured by positive charges on the peptide ions and the HPLC chromatographic characteristic of peptides are improved at acidic pH values. Therefore analysis is performed against a background of 0.1% formic acid (FA). The mechanics of ESI source are relatively simple. The sample enters the source through a flow stream and passes through a fine bore fused silica capillary held at high voltage. As the flow exits the tip of capillary, it sprays out in a fine mist of droplets which contain peptide ions. Peptide ions are separated from the solvent components and transferred to the mass analyser through a process of evaporation.

The Mass Analyser

The mass analyser is the heart of a mass spectrometer and is responsible for resolving peptides ions. Ion separation is achieved by taking advantage of the differences in behaviour between ions of different mass to charge ratio (m/z) within an electric and/or magnetic field. The four analysers in common use on MS nowadays are time of flight (TOF), quadrupole, ion trap and Fourier transform ion cyclotron resonance (FTICR) analysers. Time-of-flight (TOF), quadrupole and ion trap analysers use electric fields while the latter group comprises magnetic sector and ion cyclotron analysers. The analysers used in the work will be discussed in detail.

The instrument employed in studies presented in this thesis was the quadrupole-high capacity ion-trap (HCT Ultra ETD II; Bruker Daltonics, Bremen, Germany). The ion trap mass analyser is a popular instrument for proteomic analysis (March, 1996; Jonscher, 1997). It comes in two different varieties: the 3D trap / Paul traps, and the 2D trap / quadrupole or linear ion trap (LIT). In each design, ions are retained, or trapped, within the mass analyser by use of alternating electric field with radio frequency and a direct current electric field. An ion trap consists of three electrodes: two capping electrodes at each end of the ion trap and a ring electrode that surrounds the trap chamber. The ion trap mass analyser is particularly popular for proteomic analysis due to its improved sensitivity over quadrupole instruments and its ability to perform several stages of tandem MS. That is measurement of peptide ions (i.e. MS) fragment ions (i.e. MS/MS) and selected ions from peptide fragments (i.e. MS/MS/MS; or MS^n)

The Detector

The last part is the detector, which detects the ions resolved by the mass analyser. The detector used in the current work was an electron multiplier (EM) design consisting of a series of dynodes held at a high negative potential. When the positively charged peptide/fragment ions hit the detector secondary particles are released, including electrons. The electrons are amplified through a series of dynodes to create a current that is equivalent to number of peptide/fragment ions that entered the detector. In this way, the intensity or abundance of each ion is recorded.

In short, the MS converts components of a mixture to ions and then separates them on the basis of their mass-to-charge ratio (*m/z*). The data are automatically recorded by the instrument and can then be retrieved for manual or computer-assisted interpretation.

A short history of muscle phenotyping

Muscle fibres contain the entire genetic complement of the organism but only specific genes and isoforms are selectively expressed based on the activity pattern and muscle use. As early as the 1800s, Ranvier observed that the gross appearance of muscle fiber ranged from white to deep red, reflecting the amount of myoglobin content (Kernell, 2006). In the early 1970's, studies using animal models reported white muscles had relatively high peak tension and contractile velocities but were easily fatigued, whereas red muscle exhibited slow contractile properties and a high resistance to fatigue (Burke et al., 1973). In 1985, Pette & Vrbova used microscopy

and histochemical techniques in rat and human muscle; these techniques rely on chemical staining using the myosin ATPase within each muscle fibre after selectively denaturing myosin isoform using different pH pre-incubations. They discovered the importance of activity in the control of gene expression of the mammalian muscle fibre. Much later, Kosek et al., (2006) used sophisticated co-immunohistochemistry of MyHC isoforms to investigate the effects of resistance training on myofibres in the muscle of young and elderly individuals. Muscle phenotype can also be determined biochemically using analysis of relative abundances of each MyHC isoform. One dimensional denaturing sodium dodecyl sulphate (SDS) - PAGE is a simple and inexpensive method for resolving proteins in complex mixtures. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). Betto et al., (1986) first reported the separation distinct polypeptides corresponding to type I, IIa and IIb MyHC isoforms using SDS-PAGE. Following these pioneering studies, many studies (e.g. Hori et al., 1989; Kobayashi et al., 1990) focused on describing MyHC isoform profile in muscular dystrophies compared to normal controls using this technique. Baumann et al., (1987) used the SDS-PAGE in their study on exercise training and report changes in protein expression of the contractile apparatus associated with fibre type inter conversion within the established sequence of types IIb > IIa > IIc > I. These and many other studies conducted during this period represent muscle protein biochemistry. The advance of muscle proteomics was made possible by the introduction of mass spectrometry protein identification, which enables non-targeted analysis of proteins and the generation of new hypotheses.

A short history of muscle proteomics

In theory, proteomics techniques should be able to characterise and measure the relative abundance of each myofibrillar protein species (i.e. encompassing isoforms, splice-variants and post-translational states) simultaneously. This more comprehensive analysis circumvents the need to reduce the description of muscle to its relative proportion of 3 fibre types and we hope our work will demonstrate that whole muscle function can be more thoroughly understood by relating changes in muscle performance to the entire myofibrillar proteome.

The first study to report proteomic analysis of skeletal muscle was Isfort et al., 2000 and reported the effects of atrophy in rat slow twitch muscle. Since this initial publication there have been many studies in skeletal muscle – a summary of proteomic studies investigating muscle of laboratory animals is presented in this chapter (Table 2), whereas a more specific list of studies reporting proteomic analysis of human muscle is presented in Chapter 4 (Table 21). In particular 2DGE has been used to catalogue the proteins expressed in striated muscle. Early investigations produced 2D gel maps of mouse mixed-fibre gastrocnemius muscle (Sanchez et al., 2001), and compared archetypal muscles such as rat extensor digitorum longus (EDL) and soleus that comprise predominantly fast- or slow-twitch fibres, respectively (eg. Bozzo et al., 2005). Meanwhile Hojlund et al., (2003) used 2DGE of human vastus lateralis to observe proteins with potential roles in type 2 diabetes. Furthermore 2DGE of human samples has been used to provide more detailed information regarding proteome changes due to aging (Gelfi et al., 2006), obesity (Hittel et al, 2005) and exercise training (Holloway et al., 2009; Egan et al,

2011). Gelfi et al. (2003) in their study of 2D gel map of human vastus lateralis muscle provide a valuable resource for the definition of functional properties of muscle fibres. These and other proteome mining works provide important catalogues of accessible muscle proteins and extend earlier biochemical and histochemical studies describing muscle diversity.

Table 2. Summary of proteomic studies on animal skeletal muscle

Author, Year	Sample	Technique
Isfort et al., 2000	Rat soleus denervation	2DGE, MALDI-TOF
Sanchez et al., 2001	Mouse skeletal muscle	2DGE, MALDI-TOF
Yan et al., 2001	Rat skeletal muscle	2DGE, MALDI-TOF
Le Bihan et al., 2004	Mouse skeletal muscle	SELDI-TOF
Donoghue et al., 2005	Rabbit skeletal muscle electro-stimulation	2DGE, MALDI-TOF
Piec et al., 2005	Rat skeletal muscle ageing	2DGE, MALDI-TOF
Okumura et al., 2005	Rat skeletal muscle	SDS-PAGE, 2DGE, MALDI-TOF
Gelfi et al., 2006	Rat soleus & gastrocnemius ageing	2-DGE, LC-ESI-MS/MS.
Guelfi et al., 2006	Rat skeletal muscle acute exercise	2DGE, LC-ESI-MS/MS
O'Connell et al., 2007	Rat skeletal muscle ageing	2DGE, MALDI-TOF
De Palma et al, 2007	Rat skeletal muscle hypoxia	2D DIGE, LC-ESI-MS/MS
Donoghue et al. 2007	Rabbit tibialis anterior electro-stimulation	2DGE, MALDI-TOF
Burniston 2008	Rat Plantaris exercise	2DGE, MALDI-TOF/TOF
Doran et al., 2008	Rat skeletal muscle ageing	2D DIGE, MALDI-TOF
O'Connell et al., 2008	Rat gastrocnemius ageing	2DGE, MALDI-TOF
Moriggi et al. 2008	Rat skeletal muscle dytrophy	2D DIGE, MALDI-TOF
Gannon et al. 2008	Rat gastrocnemius ageing	2DGE, MALDI-TOF
Gannon et al., 2009	Rat gastrocnemius ageing	2DGE, MALDI-TOF
Donoghue et al. (2010)	Rat skeletal muscle ageing	2DGE, MALDI-TOF, LC-ESI-MS/MS

Lewis & Ohlendieck 2010	Mouse skeletal muscle dystrophy	2DGE , LC-ESI-MS/MS
Mullen & Ohlendieck 2010	Rats gastrocnemius diabetes	2DGE, LC-ESI-MS/MS
Mullen & Ohlendieck 2011	Rat gastrocnemius diabetes	2DGE, LC-ESI-MS/MS
Macedo et al. 2012	Mouse skeletal muscle	MALDI-TOF/TOF
Malik et al., 2013	Rats soleus & Human vastus lateralis	LC-ESI-MS profiling, LC-ESI-MS/MS, SRM
Burniston et al., 2014	Rat soleus, artificial selection	2D DIGE, LC-ESI-MS/MS

Two-dimensional gel electrophoresis (2DGE), Matrix-assisted laser desorption ionisation (MALDI), Surface-enhanced laser desorption ionisation (SELDI), Time of flight (TOF), Tandem time of flight (TOF/TOF), Liquid chromatography (LC), Electrospray ionisation (ESI), Mass spectrometry (MS, Tandem mass spectrometry (MS/MS), Difference in-gel electrophoresis (DIGE), Selective reaction monitoring (SRM).

The use of 2DGE offers robust comparative analysis of protein species, which represent different post-translational states of a protein (Jungblut et al., 2008). However, 2DGE requires a significant level of skill is laborious and has a number of technical limitations, including difficulties in resolving proteins at the extremes of the mass and pH scales, and a limited dynamic range. Therefore, there is currently a drive to move away from 2DGE and develop simpler (SDS-PAGE) or more automated HPLC workflows. In muscle proteomics, the time efficiency of orthogonal SDS-PAGE and HPLC separations has been investigated (Parker et al., 2009). The combination of SDS-PAGE with HPLC coupled directly to electrospray ionisation (ESI) tandem mass spectrometry (MS/MS), known as GeLC-MS/MS is of particular utility in skeletal muscle protein identification (Hojlund et al., 2008). Several studies have applied the GeLC-MS/MS technique in proteomic research, Yi et al., (2008) used GeLC-MS/MS and reported combination of proteomic and transcriptomic analyses. In 2008, Hojlund and colleagues catalogued almost 1,000 proteins in the human skeletal muscle proteome using GeLC-MS/MS and Norheim

et al., (2011) used this method for identification of proteins secreted by skeletal muscle cells *in vitro*.

Work in our laboratory has pioneered the application of proteomic techniques to investigate cardiac and skeletal muscle responses to endurance exercise (summarised in Burniston & Hoffman, 2011). Further to standard proteomic techniques using 2DGE (e.g. Burniston, 2008), we have developed specific methods for analysing muscle proteins (e.g. Burniston & Hoffman, 2011; Burniston & Connolly, 2010; Holloway et al., 2009) using sub-fractionation and solution-based protein separations (i.e. 1D HPLC). The current thesis further develops this workflow by specifically investigating the myofibrillar sub-proteome using liquid chromatography separation of proteins with real-time tandem mass spectrometry. Our aim is to create a more robust analysis platform for measuring differences in the abundance of dozens of important myofibrillar proteins based on quantitative label-free mass spectrometry techniques. The main proteins of interest (in addition to myosin heavy chain {MyHC} isoforms) will be the isoform, splice variant and post-translational sub-species of regulatory and essential myosin light chains, tropomyosins and troponins, which may also modulate muscle contractile characteristics.

Rational of this study

Proteomics is at the heart of our understanding of the effects of exercise in skeletal muscle. Recent developments in the field of proteomics have led to a renewed interest in muscle fibre phenotyping. The percentage of different types of muscle fibres affects muscle performance. The myofibrillar proteome determines the muscle function and there are more proteins than just MyHC for example troponins, MyLC etc. that contribute to the contractile properties of muscle. Therefore proteomic techniques are needed to capture more comprehensive information. The principal separation approaches used with intact proteins are 1D- and 2D-SDSPAGE, preparative isoelectric focusing (IEF) and HPLC (Liebler, 2002). In most of the current literature two-dimensional gel electrophoresis is used to resolve proteins, with mass-spectrometry employed to identify the protein for example, muscle proteins were successful analysed using 2-DE and MyHC isoforms using 1-DE (Holloway et al., 2009). However, 2D gel electrophoresis is laborious and presents challenges to resolve proteins at larger mass and pH ranges. Therefore, differential analysis using high-performance liquid chromatography online mass-spectrometry is used to overcome this resolution problem and as this approach is mostly an automated analysis, it should have less human error associated.

Skeletal muscle may be ideal for LC-MS profiling because the abundance of metabolic enzymes and myofibrillar proteins is high. Most analytical proteomics problems begin with a protein mixture that contains varying molecular weights, solubility and modifications. In order to prepare sample for MS analysis, the complex mixtures of proteins or peptides must be separate into somewhat less

complex mixture and the intact proteins must be cleaved to peptides. Consequently, we used different type of skeletal muscle such as rat muscle and human muscle on various analytical techniques investigate whether LC-MS could provide a rapid automated method for profiling the major metabolic proteins in skeletal muscle and therefore lead to a step increment in level of sophistication of current muscle phenotyping techniques. In addition, the advanced technique of selective reaction monitoring (SRM) was used to verify differences in the abundance of protein detected by LC-MS profiling. Selective/multiple reaction monitoring (S)/MRM assays are an attractive option for biomarker development because they enhance the sensitivity, selectivity and speed of the assay (Han & Higgs, 2008) but as yet these approaches have not been applied to the skeletal myofibrillar proteome.

In summary, the literature clearly shows that most differential proteomic experiments have used 2DGE for expression profiling. Secondly, proteome mining studies have commonly used GeLC-MS/MS. Therefore, our first task was to investigate whether direct LC-MS profiling would work on skeletal muscle. This cannot be taken for granted because muscle is made of a small number of myofibrillar protein isoforms, which have large amounts of sequence homology. Therefore, we first performed LC-MS of soluble proteins because this could provide profiling based on metabolic properties. Secondly, we used LC-MS profiling of human myofibrillar proteins, which is more challenging than the soluble protein fraction. And third, we used SRM/MRM assays in an attempt to overcome the problems due to the large amount of sequence homology the myofibrillar proteome. To do this, we used SRM/ MRM assays to target peptides that are isoform specific, similar to previous work cardiac muscle reported in Burniston & Connolly (2010).

Aim of this study

In order to develop a robust and automated technique it is necessary to perform measurements using several methods and utilising different analytical approaches. To achieve the aims of this chapter we used existing rat and human muscle samples from experiments where it was reasonable to expect that overt difference in the muscle proteome exist. Specifically, we tested label-free LC-MS profiling on soleus muscle from rats artificially selected as either high- or low-capacity runners (HCR LCR, respectively) that exhibit a greater than 5-fold difference in aerobic capacity. Our hypothesis being that LC-MS analysis of soluble protein provides 'metabolic profiling' that explain the difference in aerobic capacity. Secondly, techniques for the analysis of the human myofibrillar proteome were investigated using samples were provided from a recent study (Cobley et al., 2012) from vastus lateralis of young and elderly participants that were designated as being either trained or untrained.

1.2 METHODS

Analysis of soluble muscle proteins

1.2.1 Animal model of high- and low-capacity runners

The inception of high-capacity runner (HCR) low-capacity runner (LCR) strains from a founder population of genetically heterogeneous N:NIH rats has been described in detail (Koch & Britton, 2001). Ten male HCR/LCR rats (n = 5, in each group) from generation 25 (12-13 weeks old) were imported from the University of Michigan. The transfer of animals to the UK and subsequent procedures were conducted under the British Home Office Animals (Scientific Procedures) Act 1986 and according to UK Home Office Guidelines. Rats were housed in a conventional facility and the environmental conditions controlled at 20 ± 2 ºC, 45-50 % relative humidity with a 12 h light (0600-1800) and dark cycle. Food and water were available ad libitum during a 14-day acclimatization period. After an overnight fast (~10 h), animals were asphyxiated with CO2 and killed by cervical dislocation. Skeletal muscles and other organs were isolated and cleaned of fat and connective tissue before being weighed and frozen in liquid nitrogen. This part of the experiment was performed by my supervisor, Dr Jatin Burniston.

1.2.2 Muscle processing

Soleus muscles were pulverised in liquid nitrogen then homogenised on ice in 8 volumes of 1 % (v/v) Triton X-100, 50 mM Tris pH 7.4 containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK). Samples were incubated on ice for 15 min then centrifuged at 1,000 xg, 4 ºC for 5 min. Supernates were precipitated

in acetone and resuspended in Lysis Buffer: 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 30 mM Tris, containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK). After clearing by centrifugation (12,000 g, 4 ºC for 45 min) protein concentrations were measured using the Bradford assay (Sigma, Poole, Dorset, UK) and each sample adjusted to 5 μg μl^{-1}. Aliquots containing 100 μg proteins were precipitated in 5 volumes of acetone for 1 h at -20 ºC. Pellets were resuspended in 0.1 % (w/v) Rapigest SF (Waters; Milford, MA) in 50 mM ammonium bicarbonate and incubated at 80 ºC for 15 min. Dithiothreitol (DTT) was added (final concentration 1 mM) and incubated at 60 ºC for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide (IAA) at 4 ºC. Sequencing grade trypsin (Promega; Madison, WI) was added at a protein ratio of 1:50 and digestion allowed to proceed at 37 ºC overnight. Digestion was terminated by the addition of 2 μL concentrated trifluoroacetic acid (TFA) and peptide solutions were cleared by centrifugation at 13 000 g for 5 min.

1.2.3 Liquid chromatography-mass spectrometry

Label-free liquid chromatography-mass spectrometry (LC-MS) analysis was performed using a quadrupole-high capacity ion-trap (HCT Ultra ETD II; Bruker Daltonics, Bremen, Germany) coupled online via an electrospray ionisation source to a nano-flow HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA). Tryptic digests (0.8 μg/ μl) were diluted 1:10 with aqueous 0.1 % FA and 5 μl loaded via a Zorbax 300SB C_{18} 5 μm, 5 x 300 μm pre-column (Agilent Technologies Ltd). Peptides were separated using a Zorbax 300SB C18 3.5 μm, 15 cm x 75 μm analytical reverse phase column (Agilent Technologies Ltd) at a flow rate of 300 ml min^{-1} using a non-

linear gradient rising to 40 % acetonitrile 0.1 % FA over 160 min. Mass spectra for LC-MS profiling were recorded between 200 m/z and 2500 m/z using Standard Enhanced mode (8100 (m/z)/s). In addition, equivalent data-dependent tandem mass spectrometry (MS/MS) spectra were collected from triplicate analysis of a pooled standard comprising each HCR and LCR sample. MS/MS spectra of collision-induced dissociation fragment ions were recorded for the 5 most abundant precursors from each survey scan (350 m/z to 1600 m/z).

1.2.4 Progenesis LC-MS protein profiling

Both MS and MS/MS spectra were aligned using Progenesis LC-MS software (Nonlinear Dynamics, Newcastle, UK). Prominent ion features (mean ± SD per chromatogram: 455 ± 42) were used as vectors to warp each dataset to a common reference chromatogram. An analysis window of 15 min - 145 min and 200 m/z - 1800 m/z was selected, which encompassed a total of 32,824 features with charge states of +2, +3 or +4. Log transformed MS data were normalised by intersample abundance ratio and used to investigate differences in expression between LCR and HCR groups by one-way analysis of variance. MS/MS spectra (16, 872 queries) were exported in Mascot generic format and searched against the Swiss-Prot database (2011.6) restricted to 'Rattus' (7617 proteins) using a locally implemented Mascot (www.matrixscience.com) server (version 2.2.03). The enzyme specificity was trypsin allowing 1 missed cleavage, carbamidomethyl modification of cysteine (fixed), deamidation of asparagine and glutamine (variable), oxidation of methionine (variable) and an m/z error of ± 0.5 Da. The Mascot output (xml format), restricted to nonhomologous protein identifications was recombined with MS profile data in

Progenesis LC-MS. Peptide features with MOWSE scores <10 (MudPIT scoring) were excluded and potential conflicts in peptide assignments were resolved manually. Functional annotation was conducted using the Database for Annotation, Visualization and Integrated Discovery [DAVID; (Huang et al., 2009)]. Over-representation of gene ontology (GO) classes: cellular component (CC), biological process (BP) and molecular function (MF) was investigated. Association of proteins with pathways of the Kyoto Encyclopedia of Genes and Genomes [KEGG; http:// www.genome.jp/kegg/, (Kanehisa & Goto, 2000)] was also assessed.

1.2.5 Selective reaction monitoring (SRM) of heart-type fatty acid binding protein

Selective reaction monitoring (SRM) was used to verify differences in the abundance of heart-type fatty acid binding protein (HFABP) detected by LC-MS profiling. HFABP exhibited the greatest fold difference between HCR and LCR muscles, and SRM of serum HFABP levels has previously been reported (Zhen et al., 2007) SRM was performed using similar instrument settings described in section 1.2.4 but samples were separated using a linear chromatographic gradient rising to 40 % acetonitrile 0.1 % FA in 15 min, rather than 160 min used during LC-MS profiling. Samples were analysed in duplicate and in a randomised order. A single transition 454-551 was monitored, which represents the y5 ion (551.21 m/z) of the doubly charged peptide SLGVGFATR (2+ 454.19 m/z) of residues 23-31 of HFABP.

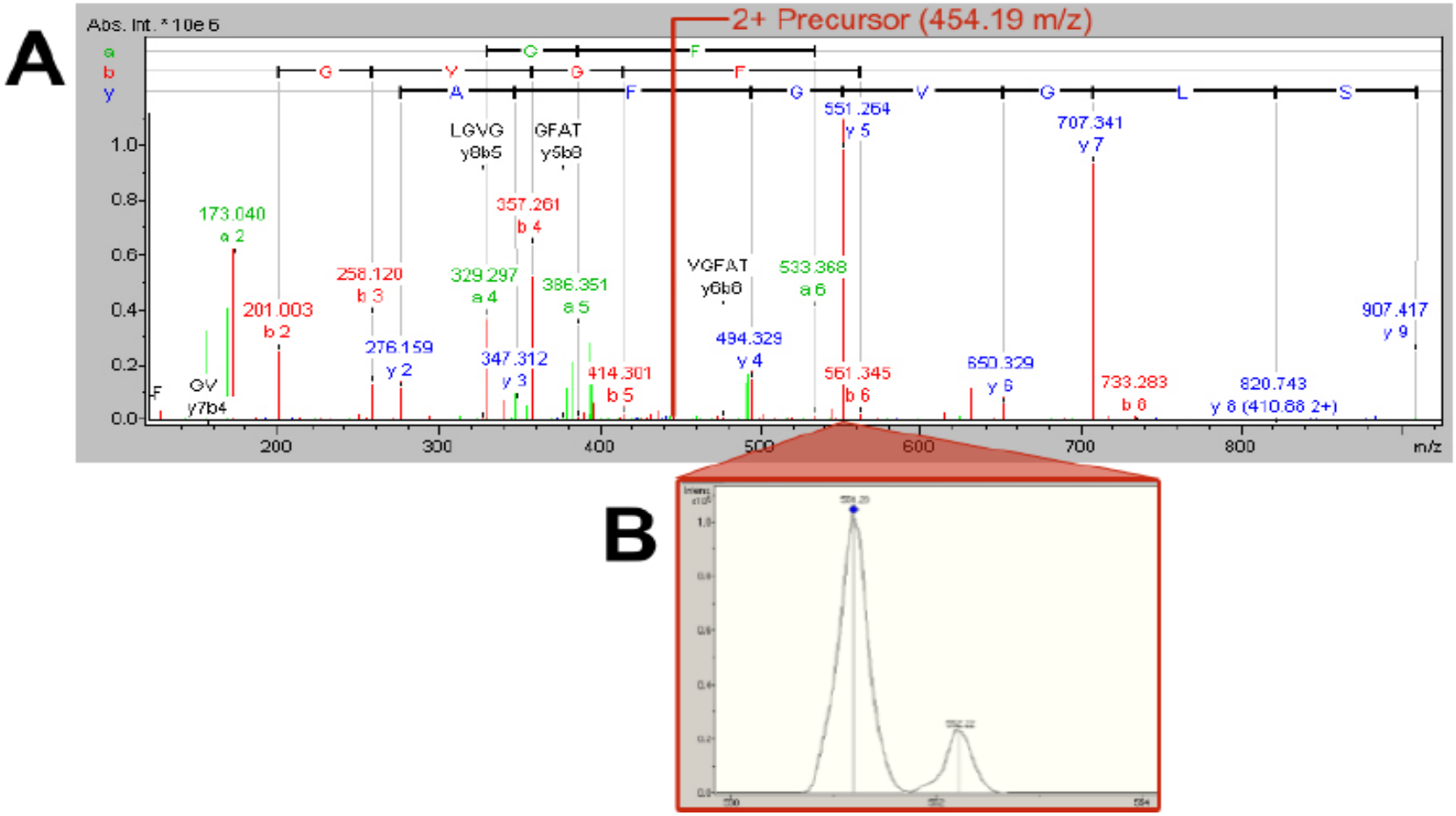


Figure 2. Selective reaction monitoring of FABPH. Annotated MS/MS spectra of the doubly-charged precursor, 454.19 m/z (A) of residues 23–31 (SLGVGFATR) of FABPH and (B) monitoring of the selected fragment ion (551.21 m/z).

Traditional biochemical analysis of muscle fibre type (Human sample)

The profile myosin heavy chain (MyHC) isoform in muscle is an important determinant its contractile and metabolic properties. Research in this area typically uses traditional biochemical analysis of muscle fibre type by 1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1DE; SDS-PAGE). SDS-PAGE separates proteins according to their size and no other physical feature, the difference in mass of the 3 human MyHC isoforms is type I (223,097 Da), type IIa (223,044 Da) and type IIx (223,145 Da) which is relatively minor and so resolving the protein isoforms can be challenging. Nonetheless we used this traditional analysis methods as a basis from which to develop more sophisticated analytical techniques.

1.2.6 Human samples

In the current work samples were provided from a recent study (Cobley et al., 2012) that collected biopsy sample from vastus lateralis of young and elderly participants that were designated as being either trained or untrained (n = 24). Hence, there were 4 groups young trained, young untrained, old trained and old untrained. Trained participants were competitive amateur cyclists that habitually completed at least five endurance sessions per week. Older trained participants were required to have adopted such a regimen for minimum ten years. Untrained participants did not partake in endurance-based training and completed < 3 exercise sessions per week. These individuals exhibited marked difference in age and training status which are expected to affect muscle phenotype. Therefore these samples provide ideal substrate for establishing the biochemical analysis techniques.

1.2.7 Muscle processing

Muscle samples were ground using a pestle and mortar, under liquid nitrogen. The powder was accurately weighed (20 mg) and homogenised on ice in 10 volumes of lysis buffer, using a glass-teflon homogeniser. The Lysis and solubilising buffer consisted of 1 % (v/v) Triton X-100, 40 mM Tris, phosphatase inhibitor and complete protease inhibitor cocktail (Roche, Indianapolis, USA). Homogenates were centrifuged at 1,000 xg, 4 °C for 10 min and the supernatant was decanted and stored in 200 µl aliquots at -80 °C. The current thesis reports analysis of the pellet that contains myofibrillar protein was resuspensed in 1 % SDS buffer and boiled (95°C) for 5 minutes in preparation for one-dimensional gel electrophoresis.

A Bradford Assay (Bradford 1976) was performed to ascertain the protein concentration in each sample. Bovine serum albumin (BSA) standards (from 0.2 mg ml^{-1} to 1.0 $mg.ml^{-1}$ in 0.2 $mg.ml^{-1}$ increments) were prepared from a commercial stock solution (1.0 $mg.ml^{-1}$; Sigma-Aldrich, Poole, Dorset) and pipetted in triplicate (5 µl per well) onto microtitre plate. Muscle supernatants were diluted 1:10 to bring them within the standard range and pipetted (5 µg per well) in duplicate. Bradford reagent (Sigma) was added (250 µl per well) and the mixtures incubated at room temperature for 5 minutes, before reading at a wavelength of 595 nm (Tecan GmbH, Austria). Interpolation from the BSA standard curve was used to determine the protein concentration in each muscle supernatant sample. For subsequent loading onto the gels, samples were normalised to 5 µg or 0.5 µg/µl in Laemmili buffer (Laemmli, 1970) and boiled for 5 min. For experiments using a ‘pooled standard’ a sample was created by combining an aliquot of 10 µg protein from each individual.

1.2.8 1DE SDS-PAGE (8 % for MyHC)

MyHC isoforms were separated according to molecular weight by 8 % denaturing SDS-PAGE as described previously (Talmadge & Roy, 1993). Mini-slab gels of dimensions 11 x 8.5 x 0.75 cm were prepared according to the manufacturer's instruction (Mini-VE system, GE Healthcare, Sweden). Glass plates were cleaned with 70 % ethanol to ensure all traces of protein were removed. Cassettes were formed (8 x 10.5 cm) by two glass plates separated by spacers of the required thickness (1.0 mm) placed at the sides. The stacking gels consisted of (final concentration): 30 % (v/v) glycerol, 4 % (v/v) acrylamide-N,N'–methylene–bis-acrylamide bis (50:1), 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4 % (w/v) SDS. The separating gels were composed of 30 % (v/v) glycerol, 8 % (v/v) acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, 0.4 % (w/v) SDS polymerisation was initiated with 0.05 % (v/v) N, N, N',N' –tetramethylethylenediamine (TEMED) and 0.1 % (v/v) ammonium persulfate (APS). A 10-well comb was inserted during polymerisation (RT for 1 h) of the stacking gel. Combs were removed and the wells washed with running buffer: 0.3 % (w/v) SDS, 3.6 % (w/v) Tris and 3.4 % (w/v) glycine to remove unpolymerised acrylamide. According to Bamman et al. (1999), the lower electrode buffer for human muscle was a dilution of 250 ml upper electrode buffer diluted with 1250 ml dH_2O. Ten microliters of each sample equal to 5 µg of protein were loaded equivalently into each lane and electrophoresed on ice through the stacking gel at 280 V for 15 hours. Following the process, the gel was removed from the glass plates and washed 3 times in ddH_2O, with each wash taking ~5 minutes. The ddH_2O was poured off and then the gels were stained in 100 ml of colloidal Coomassie blue (Bio-safe, Bio-rad laboratories, Hercules, USA) for 1 hour before being de-stained overnight in ddH_2O.

1.2.9 1DE SDS-PAGE (15% for low molecular weight (LMW) myofibrillar proteins)

Low molecular weight (i.e 50 - 15 kDa) myofibrillar proteins were separated by 15 % denaturing SDS-PAGE. Mini-slab gels of dimensions 11 x 8.5 x 0.75 cm were prepared as described in previous section. The stacking gels consisted of (final concentration): 5 % RTU bis-acrylamide solution, 1.0 M Tris-HCl (pH 6.8), and 0.4 % (w/v) SDS, 0.1 % bromophenol blue, ddH_2O polymerisation was initiated with TEMED and 10 % APS. The separating gels were composed of 15% RTU Bis-acrylamide solution, 1.5 M Tris-HCl (pH 8.8), 0.4 % (w/v) SDS, ddH_2O polymerisation was initiated with TEMED and 10 % APS. A 10-well comb was inserted during polymerisation (RT for 1 h) of the stacking gel. Combs were removed and the wells washed with running buffer: 0.1 % (w/v) SDS, 0.3 % (w/v) Tris and 1.4 % (w/v) glycine to remove unpolymerised acrylamide. Ten microlitres of each sample equal to 50 µg of protein of muscle homogenates were loaded equivalently into each lane and electrophoresed through the stacking gel at 100 V for 30 min then increased to 200V and continue until tracking dye reached the bottom edge of the resolving gel (~1 hour 30 min). Following the process, the gel was removed from the glass plates and washed and stained as described previously in 1.2.8.

1.2.10 Image Analysis using Progenesis

Digitalised scanned images (16-bit greyscale, 300 dpi, ~85 µm pixel size) of stained gels were analysed using T120 software (Total Lab, Newcastle UK). Gels were scanned and saved in tiff format. T120 software enables the measurement of the

protein content with each band by densitometry and can be used to interpret the percentage distribution of MyHC isoforms. Lanes were detected on an image by selecting the 'Create Lanes' icon from the Lane Creation Toolbar. There are several different algorithms for determining the background values. The algorithms were tested by clicking on the 'Subtract Background', and the rolling ball method was chosen (Figure 3). Quality was checked using Profile Deconvolution, which is the process of separating a lane profile into a series of individual curves, each representing a single band. The bands were detected using automatic or fixed width edge detection and band volume measurement occurs automatically when the 'Detect Bands' icon is pressed. Following the process, the bands volume data was exported from the measurement window for data analysis.

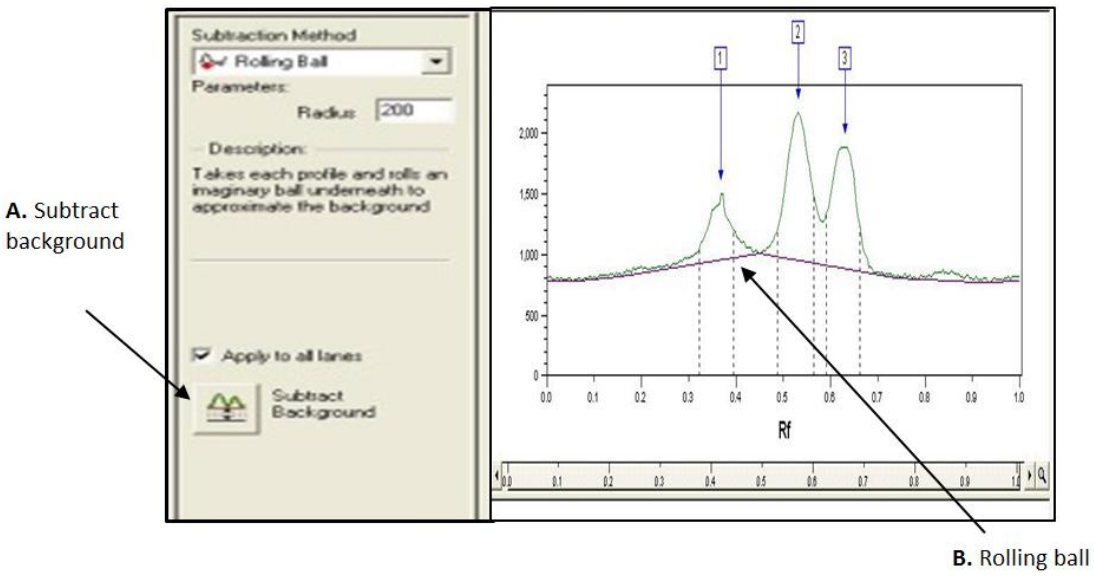


Figure 3: The process of subtracting the background using the rolling ball method. Green line represents the staining density of each protein band (1, 2, 3) of myosin heavy chain isoforms and the red line represents the gel background.

Data for ‘pooled standard’ and individual samples were extracted by T120 software that enables measurement of the concentration of each band by densitometry and can be used to calculate the relative percentage of each MyHC isoform to investigate differences in expression between groups. The data for grouping were analysed using Statistical Package for Social Sciences (SPSS) version 17.0 software (SPSS v.17, SPSS Inc, Chicago). Volume measurements from each detected band (Total lab TL 120) were exported into Microsoft Excel and entered manually in SPSS. A two-way between participants ANOVA was employed to analyse the influence of training status (e.g. trained vs. untrained) and age (e.g. young vs. old). Significance level was set at <0.05, and all data were presented as mean ±SD.

Two-Dimension Gel Electrophoresis of Myofibrillar fraction

It is likely each 1DE band contains several proteins, therefore two dimension gel electrophoresis (2DGE) was used to discover which myofibrillar proteins were resolved in to multiple species. 2DGE was performed using similar sample and muscle processing described in sections 1.2.7. However, the samples need to be diluted in non-ionic lysis buffer (7 M urea, 2 M thiourea and 4 % CHAPS) in order to maintain protein change states for isoelectric focusing (IEF). A pooled sample was made and precipitated with 5 volumes of acetone. Buffer was prepared by adding 2.8 mg DTT, 5µl IPG buffer pH 3-10 to 1 ml of rehydration buffer containing 8 M urea, 2 % (w/v) CHAPS, 20 mM dithiothreitol, and 0.5% (v/v) ampholytes.

1.2.11 2DGE

Muscle homogenates were prepared for 2D electrophoresis by centrifugation at 8,000 rpm for 2 min. An aliquot of the pooled myofibrillar fraction, containing 125 µg of protein was loaded onto 7-cm immobilized pH gradient (IPG) strips (pH 3–10; GE Healthcare, Little Chalfont, UK) overnight. Isoelectric focusing was conducted as follows: 50 V for 15 min, 250 V for 15 min, linear gradient to 4,000 V over 2 h and 4,000 for 2 h. The IPG strips were then equilibrated in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) sodium dodecylsulfate, and a trace of bromophenol blue. Proteins were electrophoresed through a linear 12 % polyacrylamide gel at 20 °C; initially at a constant current of 15 mA per gel for 30 min and then at 30 mA per gel. After electrophoresis, the gel was removed from the glass plates and washed 3 times in ddH_2O, with each wash taking ~5 minutes. The ddH_2O was poured off and then the gels were stained in 100ml of colloidal Coomassie blue (Bio-safe, Bio-rad laboratories, Hercules, USA) for 1 hour before being de-stained overnight in ddH_2O.

1.2.12 Image Analysis using Progenesis

Digitalised scanned images (16-bit greyscale, 300 dpi, ~85 µm pixel size) of stained gels were scanned and saved in tiff format. Clear spots were selected and numbered to identify the related proteins.

1.2.13 In-gel digestion and LC-MS

In-gel digestion was performed to prepare proteins separated by gel electrophoresis for identification by mass spectrometry. Gel spots were cut using a glass pipette and dispensed into clean labelled plastic tubes. One hundred microliters of 50:50 acetonitrile (ACN)/50 mM Ambic was added to each tube and the gel plugs incubated for 10 min at 37º C. This step is done to de-stain the gel plug and remove the Colloidal Coomassie Blue. Then the de-stain solution was removed using a gel-loading tip. This de-staining process was repeated at least 3 times or until no more blue colour could be seen. Samples were incubated in 50 µl absolute ACN for 10 mins at 37 °C. Then ACN was removed by using a gel-loading tip and the gel dried by incubating for 15 mins at 37 °C. Sequence grade trypsin was prepared and 20 µl of diluted trypsin stock was added to the dehydrated gel plug and incubated at 37 °C for 1 hour. After 1 h 10 µl of 50mM Ambic was added to each tube and incubated overnight at 37 °C. The reaction was stopped by addition of 2 µl of 2.6 M formic acid. This method was modified slightly for 1D gel pieces. After de-staining, DTT was added (150 µl, 9.8 mM in 50 mM Ambic) and incubated at 37 ºC for 30 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide in 50 mM Ambic at 4 ºC for 30 min. Then the solution was removed using a gel-loading tip. 75 µl absolute ACN was added and incubated at 37ºC for 10 min until the gel plug dehydrated and became white. Sequencing grade trypsin (Promega; V5113) was added at a protein and digestion allowed to proceed at 37 ºC overnight. Digestion was terminated by the addition of 3 µL concentrated TFA and peptide solutions were cleared by centrifugation at 13400 xg for 1 min.

Protein identification

Label-free liquid chromatography-mass spectrometry (LC-MS) analysis was performed using a quadrupole-high capacity ion-trap (HCT Ultra ETD II; Bruker Daltonics, Bremen, Germany) coupled online via an electrospray ionisation source to a nano-flow HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA). MS/MS spectra were exported in Mascot generic format and searched against the Swiss-Prot database (2011.6) restricted to 'Rattus' (7921 entires) using a locally implemented Mascot (www.matrixscience.com) server (version 2.2.03). The enzyme specificity was trypsin allowing 1 missed cleavage, carbamidomethyl modification of cysteine (fixed), deamidation of asparagine and glutamine (variable), oxidation of methionine (variable) and an m/z error of ± 0.5 Da.

Solution-based analysis of human myofibrillar proteins (Human)

1.2.14 Sample and muscle processing

This technique was performed using muscle samples from Cobley et al., 2012, processed in section 1.2.7. An aliquot of the myofibrillar fraction of human muscle was prepared for in-solution digest, by precipitating supernates in 5 volumes of acetone and resuspended in Lysis Buffer: 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 30 mM Tris, containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK). After clearing by centrifugation (12,000 xg, 4 ºC for 45 min) protein concentrations were measured using the Bradford assay (Sigma, Poole, Dorset, UK) and each sample adjusted to 5 µg μl^{-1}. Aliquots containing 100 µg protein were precipitated in 5 volumes of acetone for 1 h at -20 ºC. Pellets were resuspended in

0.1% (w/v) Rapigest SF (Waters; Milford, MA) in 50 mM ammonium bicarbonate and incubated at 80 ºC for 15 min. DTT was added (final concentration 1 mM) and incubated at 60 ºC for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide at 4 ºC. Sequencing grade trypsin (Promega; Madison, WI) was added at a protein ratio of 1:50 and digestion allowed to proceed at 37 ºC overnight. Digestion was terminated by the addition of 2 μL concentrated TFA and peptide solutions were cleared by centrifugation at 13 000 xg for 5 min.

1.2.15 Liquid chromatography-mass spectrometry

Label-free liquid chromatography-mass spectrometry (LC-MS) analysis for human sample was performed similar process described in section 1.2.3, which includes MS for profiling and MS/MS for protein identification. The MS only data were exported to Profile Analysis (Bruker Daltonics, version 2.0) for quantitative analyses. The group was defined into two attributes (age and training status) and the Profile Analysis project was saved. Then peptides were assigned to 'buckets' which represent time and MS space, e.g. elution time 10 min and the mass-to-charge 600 m/z, then the peptides regulation ratios were calculated and peptides that were significantly different between samples with different group attributes were determined. The results of the calculation were saved and inspected in the Bucket Table and the T-Test result Table; then the quantitative results were exported to SPSS. A two-way between participants ANOVA was employed to analyse the influence of training status (e.g. trained vs. untrained) and age (e.g. young vs. old). Significance level was set at <0.05, and all data were presented as mean ±SD.

After MS/MS data were imported into ProteinScape (Bruker Daltonics, version 2.0), all LC-MS/MS were combined in to a single database search. After that a Mascot protein database search was performed on the combined MS/MS data set. Peptides identifications were linked to the peptide regulation ratios determined in Profile Analysis and protein regulation ratios were calculated from peptide regulation ratios. Finally a PDF report was generated by selecting the Mascot search result entry.

1.2.16 Multiple reaction monitoring (MRM) of myosin light chain protein

Multiple reaction monitoring (MRM) was used to verify differences in the abundance of myosin light chain protein (MyLC) detected by LC-MS profiling. MyLC exhibited the greatest fold difference between either significant main effect for age or training status or significant interaction. MRM was performed using similar instrument settings described in section 1.2.4 but samples were separated using a linear chromatographic gradient rising to 40 % acetonitrile 0.1 % FA in 15 min, rather than 160 min used during LC-MS profiling. Samples were analysed in duplicate and in a randomised order. A transition 498-759 was monitored for MYL3, 771-835 and 600-658 for MYL1, 660-735 and 762-780 for MLRS. Abundance data (MRM Transition) were exported to SPSS for 2-way ANOVA (Table 12 and Table 13).

1.3 RESULTS

Analysis of soluble muscle proteins from HCR/LCR rats

1.3.1 Physical and Physiological Characteristics of HCR and LCR Rats

The body weight of LCR (460 ± 40 g) was 1.38-fold greater ($p < 0.0001$) than HCR (334 ± 36 g) and there was a 6.44-fold difference ($p < 0.0001$) difference in running capacity (1,625 ± 112 m vs. 252 ± 43 m) between HCR and LCR strains (Figure 4).

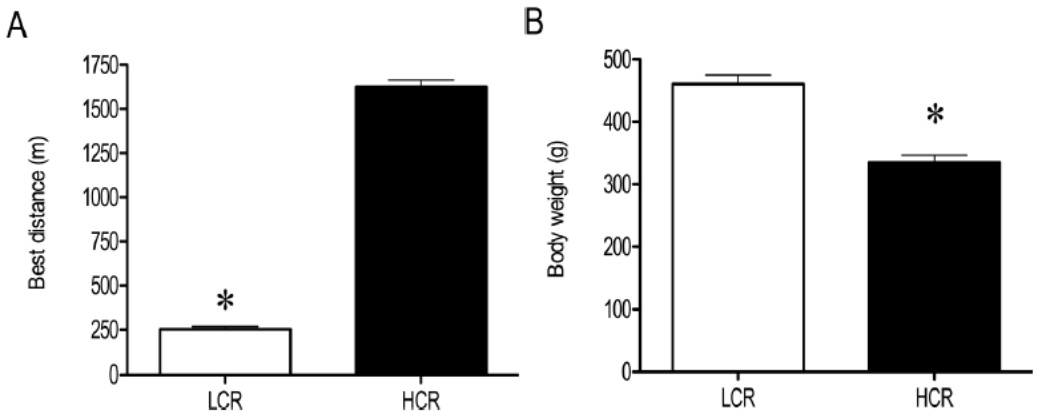


Figure 4. Physical characteristics of high-(HCR) and low-capacity runner (LCR) rats. Furthest distance (m) completed during a standardised treadmill test of LCR and HCR rats at approximately 10 weeks of age (A). Body weight of LCR and HCR rats prior to tissue harvesting at approximately 14 weeks of age (B). Data are expressed as Mean ± SD (n = 5, in each group), * $p < 0.05$.

In total 207 protein entries (2,999 peptides) mapped to features in progenesis LC-MS and were used for quantitative analysis. Log transformed MS data were normalised by inter-sample abundance ratio and used to investigate differences in expression between LCR and HCR groups by one-way analysis of variance. Proteins more abundant in HCR muscle were associated with aerobic metabolism and hierarchal clustering highlighted two prominent groups of proteins (respiratory chain and fatty acid metabolism) that shared patterns of expression across the biological replicates.

1.3.2 Liquid chromatography-mass spectrometry (Rat soleus muscle)

Differential analysis of HCR and LCR samples highlighted 16 proteins (Table 3) that were more abundant ($p < 0.05$) in HCR soleus. No proteins were found to be significantly more abundant in LCR muscle.

Table 3. Differences in protein abundance between LCR and HCR soleus muscle.

Description	Database ID	MOWSE (peptides)	Fold Diff.	p value
Cytochrome b-c1 complex subunit 1	QCR1	322 (11)	1.10	0.0274
Cytochrome b-c1 complex subunit 2	QCR2	459 (15)	1.09	0.0290
Cytochrome c oxidase subunit 2	COX2	111 (4)	1.14	0.0458
Cytochrome c oxidase subunit 4 isoform 1	COX41	159 (5)	1.15	0.0206
ATP synthase subunit beta	ATPB	1185 (21)	1.14	0.0326
ATP synthase subunit d	ATP5H	64 (2)	1.16	0.0081
ADP/ATP translocase 1	ADT1	562 (10)	1.16	0.0232
Voltage-dependent anion-selective channel protein 1	VDAC1	412 (12)	1.12	0.0111
Voltage-dependent anion selective channel protein 3	VDAC3	53 (4)	1.16	0.0137
Electron transfer flavoprotein subunit beta	ETFB	279 (8)	1.13	1.13
Fatty acid-binding protein, heart	FABPH	349 (7)	1.54	0.0064
Fatty acid-binding protein, adipocyte	FABP4	95 (4)	1.15	0.0426
Methylmalonate-semialdehyde dehydrogenase	MMSA	115 (5)	1.05	0.0235
Dihydrolipoyl dehydrogenase	DLDH	184 (6)	1.15	0.0077
3-Ketoacyl-CoA thiolase	THIM	396 (7)	1.25	0.0071
Enoyl-CoA hydratase	ECHM	76 (3)	1.19	0.0245

Description and Database ID relate to the protein name and accession number identified from Mascot searches of the UniProt Rattus database. Protein abundance relative differences (fold difference) in HCR compared to LCR are reported for proteins exhibiting significant ($p < 0.05$) differences in abundance at a FDR of <10%.

1.3.3 SRM Verification of FABPH

LC-MS profiling found FABPH exhibited the greatest difference (1.54-fold, p = 0.0064) in abundance between HCR and LCR muscle. SRM verified this finding and measured a 2.84-fold greater (p = 0.0095) abundance in HCR soleus (Figure 5). The reproducibility of the SRM method was assessed using technical replicates of HCR and LCR samples. The assay exhibited no significant systematic bias in retention time or area under the curve of the ion chromatogram, which was used to estimate abundance. The average retention time was 21 ± 0.2 min (coefficient of variation 0.4%) and the coefficient of variation of measures of FABPH abundance was 20%.

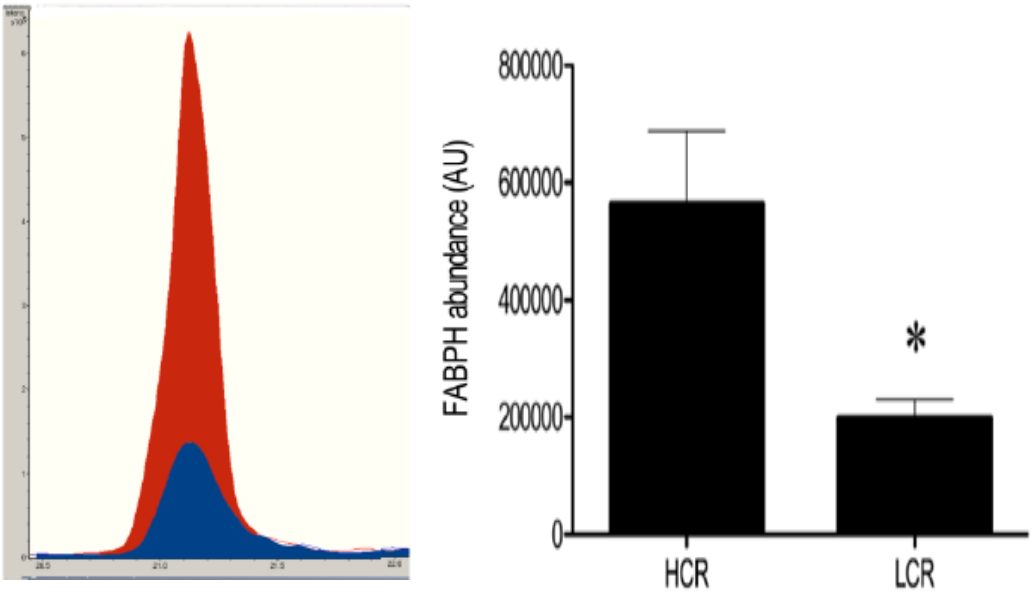


Figure 5. Selective reaction monitoring chromatograms of the 454.19 m/z to 551.21 m/z transition in HCR and LCR soleus. The red shaded area represents a HCR sample and the blue area represents a LCR sample. The histograms represent the ion abundance of the areas under the chromatogram curves for the respective groups. Data are presented as means ± SD (n = 5, in each group), * $p < 0.05$.

Gel-based analysis of human myofibrillar proteins

1.3.4 Characteristics of human muscles samples

Analysis was performed on human muscles samples collected by percutaneous needle biopsy during a previous study (Cobley et al., 2012). Samples were collected from vastus lateralis of 12 young and 12 older participants that had different habitual training status: Young trained (YT n = 6), young untrained (YU n = 6), old trained (OT n = 6) and old untrained (OU n = 6). Baseline participant characteristics are displayed in table 4.

Table 4. Baseline participant characteristics, values presented as mean ± SD

Parameter	YT	YU	OT	OU
Age (years)	24.2 ± 3.6*	22.1 ± 3.4*	64.5 ± 5.5$^{\#}$	63.8 ± 3.0$^{\#}$
Weight (kg)	66.2 ± 10.0	84.5 ± 10.8	71.8 ± 7.8	95.5 ± 24.8*$^{\#}$
Height (cm)	176.6 ± 8.6	181.6 ± 6.5	174.6 ± 6.8	174.8 ± 9.3

Data are from Cobley et al., (2012) are presented as mean ± SD for young trained (YT), young untrained (YU), old trained (OT) and old untrained (OU). * $P<0.05$ significantly different from OT. $^{\#}$ $P<0.05$ significantly different from YT.

1.3.5 Traditional muscle phenotyping: Analysis of Myosin heavy chain isoforms by SDS-PAGE.

Analysis of 'pooled standard'

The electrophoresis conditions were optimised using a 'pooled standard' that was created by combining an aliquot (10 µg protein from each individual). The running conditions were optimised until the MyHC isoforms were clearly resolved mid – way through the minigel (Figure 6). Figure 6 shows representative images and analysis of MyHC band volumes. Using this method we were able to separate MyHC

isoforms into 2 distinct band (IIa/x, I). The average percentages of MyHC I (52.4%) and MyHC IIa/x (47.6%), found in the 'pooled standard' of vastus lateralis homogenates by this minigel protocol (Table 5).

Individual samples from each group

Individual samples from young trained (YT; n = 6), young untrained (YU; n = 6), old trained (OT; n = 6), and old untrained (OU; n = 6) were separated using the optimised method (Figure 7). MyHC IIx was less than 10 % from samples of young trained, untrained and trained elderly individuals whereas young untrained exhibited the highest abundance of the MyHC IIa/x isoform. The elderly trained result also found the MyHC I (78.1 %) was significantly ($P<0.05$) greater than MyHC IIa (21.7 %) (Table 6).

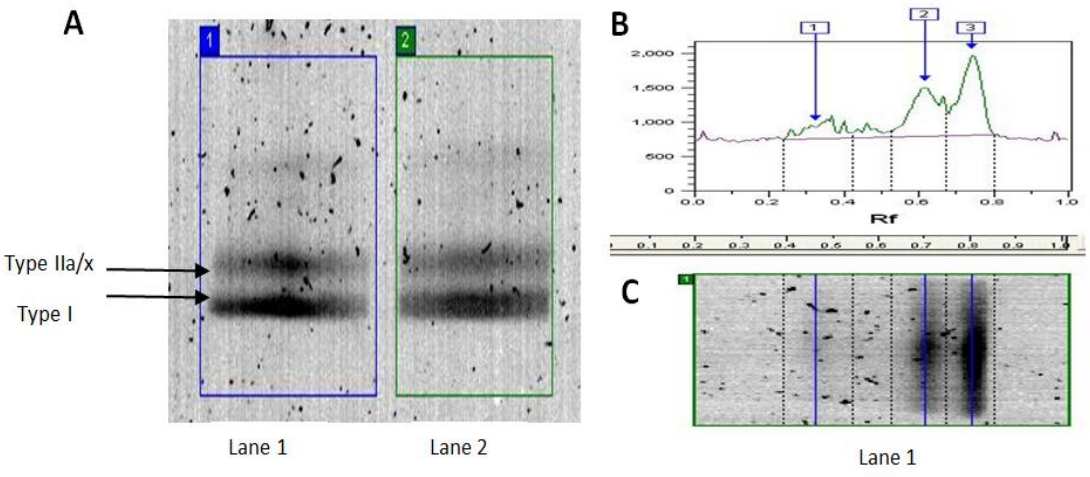


Figure 6. Separation of MyHC isoforms by SDS-PAGE. (A) Representative image of gel lanes showing separation of MyHC isoforms. (B) Density plot showing area of interest, into two distinct bands (IIa/x and I) based in differences in migration (Pooled standard sample from all participants). (C) Images of lanes aligned with the area of interest.

Table 5. Duplicate analysis of 'pooled standard'. Volume and percentage for each band from pooled standard sample in two different lanes.

Band	Lane 1 (Volume)	%	Lane 2 (Volume)	%	Volume mean ± SD	% mean± SD	% CV
IIa/x	1323	46.3	1603	48.8	1429 ± 241.1	47.6 ± 3.9	3.6
I	1528	53.7	1690	51.2	1609 ± 114.6	52.4 ± 1.7	1.2

Data represented 'pooled standard' proteins were identified by T120 software which enables the concentration of each band to be measured by densitometry and can be used to calculate the relative percentage of each MyHC isoform.

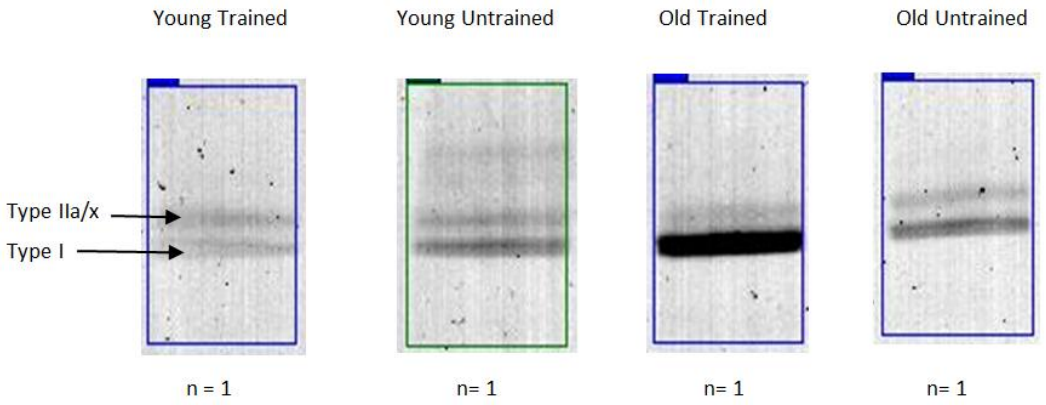


Figure 7. Separation of myofibrillar isoforms by SDS-PAGE. Representative image of gel lanes from young trained (YT), young untrained (YU), old trained (OT) and old untrained (OU) individuals showing separation of MyHC isoforms into distinct bands (IIa/x, I) based in differences in migration of four different group samples.

Table 6. Volume and percentage for each band from four different group sample.

Band	YT	YU	OT	OU
IIa/x(%)	44.6 ± 14.2	64 ± 15.1	21.9 ± 13.8*	39.5 ± 20.7
I(%)	55.4 ± 27.8	36 ± 12.1	78.1 ± 14.2*	60.5 ± 30.1

Data are from Cobley et al., (2012) are presented as mean ± SD for young trained (YT), young untrained (YU), old trained (OT) and old untrained (OU). Each group proteins were identified by T120 software which enables to measure the concentration of each band by densitometry and can be used to interpret percentage of MyHC isoforms. * $P<0.05$ significantly different from YU.

1.3.6 Analysis of low molecular weight (LMW) myofibrillar proteins

In total 12 bands could be reliably separated in human muscle (Figure 8). Band densities spanned one order of magnitude i.e. ranged from 6900 to 71000 (Table 7). The molecular weight range of proteins was separated using 15% gels was (6- to 90-kDa). LC-MS/MS of in gel tryplic digests was used to identify the proteins present in each band. Protein identification focused on a range from 45 to 15 kDa consistent with optimum resolution for 15% gels. Figure 8 represent the bands detected using automatic fixed width edge detection and band volume measurement.

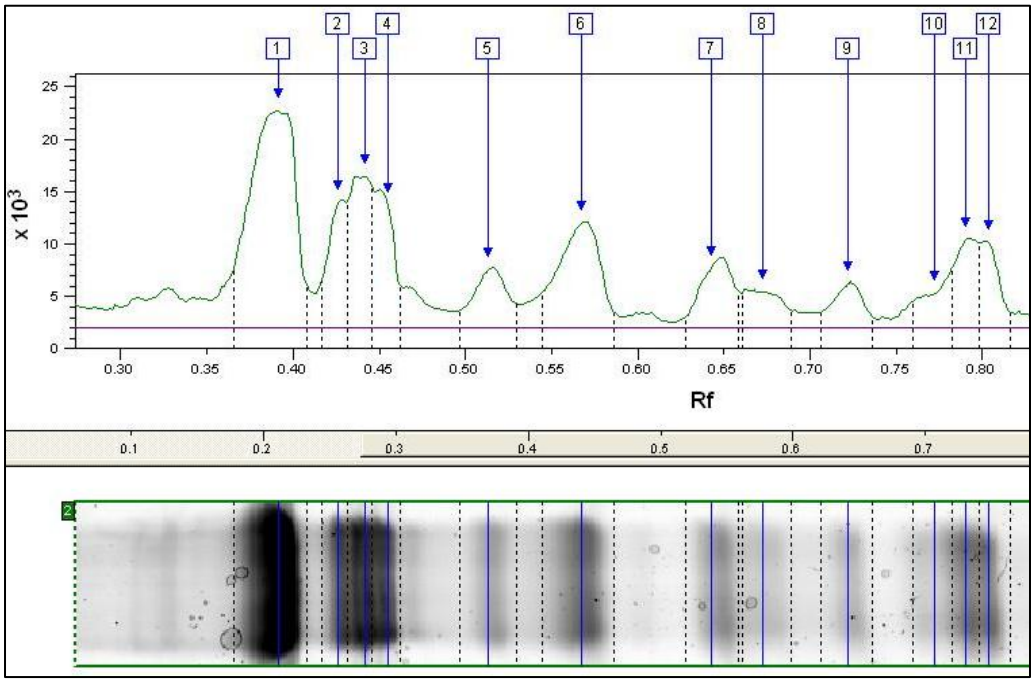


Figure 8. Optical density plot of myofibrillar bands in human muscle vastus lateralis. (A) Peaks are labelled corresponding to band number (1-12) and density plot showing area of interest. (B) Images of lanes aligned with the area of interest.

Individual samples from four different groups (n = 24) were separated using the optimised method (Figure 8) and the bands identified by T120 software which enables to measure the concentration of each band by densitometry and investigate differences in expression between groups by one-way analysis of variance. One

statistically significant difference was detected. Band 8 was 42 % more abundant in the young untrained, interaction was $p = 0.029$. Proteins identified within each band by mass spectrometry and database searches, were categorised further and ranked by MOWSE score to determine the primary protein typically found in each band (Table 8).

Myofibrillar Proteins

Table 7. Protein expression of 1-DE gel bands (bands 1-12 in Figure 8).

Band	YT	YU	OT	OU
1	67243±8735	64735±11595	70248±16665	61158±14620
2	15637±6576	18445±8069	17286±5878	14137±6637
3	18437±2410	21044±9215	26920±12653	21739±7969
4	18435±5130	18798±5023	15944±2935	19584±5843
5	9912±2583	9866±5523	10023±4387	8980±7092
6	27235±2157	32333±9400	33644±9901	29128±8493
7	10191±2942	9229±4860	15489±6931	9836±2700
8*	11807±4674	21005±5631	10785±3137	10997±3835
9	11005±3463	12372±7708	13493±10147	8946±7833
10	10098±6137	6942±3458	6942±3473	7382±6326
11	14910±6682	10236±6370	14137±8278	14346±14554
12	16432±7402	13009±7210	15100±9291	15146±13225

Data is presented as mean ± SEM. * Significant difference between training status and age. Data represented each group proteins were identified by T120 software which enables to measure the concentration of each band by densitometry. There is only significant difference was evident in band 8 ($p < 0.05$) where the volume of MyLC2/Troponin C protein was higher in trained and young participants.

Table 8. Proteins detected in each band (bands 1 to 12) of myofibrillar bands in human muscle vastus lateralis.

Band	Protein description	MW (kDa)	Score
1	Actin, alpha skeletal muscle	42	789.2
	Creatine kinase M-type	43.1	705.5
	Fructose-bisphosphate aldolase	39.4	124.1
	PDZ and LIM domain protein 3	39.2	53.8
2	Tropomyosin beta chain	32.8	879
	Tropomyosin alpha-1 chain	32.7	683.8
	Tropomyosin alpha-3 chain	32.8	553.4
	Glyceraldehyde-3-phosphate dehydrogenase	36	403.8
	Troponin T, fast skeletal muscle	31.8	225.6
	Troponin T, slow skeletal muscle	32.9	67.9
	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	37.5	52.2
3	Tropomyosin alpha-3 chain	32.8	764.3
	Tropomyosin beta chain	32.8	731.7
	Troponin T, slow skeletal muscle	32.9	649
	Tropomyosin alpha-1 chain	32.7	644.3
	Troponin T, fast skeletal muscle	31.8	354
4	Tropomyosin alpha-1 chain	32.7	962.9
	Tropomyosin alpha-3 chain	32.8	767
	Tropomyosin beta chain	32.8	465.5
	Myozenin	31.7	484.8
	Troponin T, fast skeletal muscle	31.8	279.6
	Troponin T, slow skeletal muscle	32.9	129.5
5	Trypsin-1	26.5	57.5
6	Myosin light chain 1/3, skeletal muscle isoform	21.1	700
	Myosin light chain 3	21.9	683.4
	Troponin I, slow skeletal muscle	21.7	238.3
	Troponin I, fast skeletal muscle	21.3	218.3
7	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	18.8	710
	Myosin light chain 1/3, skeletal muscle isoform	21.1	218.3
8	Myosin regulatory light chain 2, skeletal muscle isoform	19	619
	Troponin C, skeletal muscle	18.1	383.1

9	Troponin C, slow skeletal and cardiac muscles	18.4	502
	Myoglobin	17.2	411.5
	Hemoglobin subunit beta	16	85.5
10	Hemoglobin subunit beta	16	425
	Myoglobin	17.2	137.4
	Hemoglobin subunit alpha	15.2	96.3
11	Hemoglobin subunit beta	16	537.5
	Hemoglobin subunit delta	16	535.2
	Cytochrome c	11.7	174.1
	Fatty acid-binding protein, heart	14.8	129
12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	13.5	66.1

Data represent proteins were identified by mass spectrometry and database searches, these were categorised further and ranked by score to determine the primary protein typically found in each band. Proteins are ranked by score, highest to lowest.

Band 8 showed significant differences contain Myosin regulatory light chain and troponin C. The protein here similar weight (i.e. 18, 19 kDa) but the predict pH are different. So 2D gel is required to determine which of these proteins is different in this group.

1.3.7 Two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was used to resolve proteins species within each 1DE and mass-spectrometry was employed to identify the proteins within each spot. Small format 2D gel was used because sample was limited and the myofibrillar fraction was not expected to be too complex. To ascertain the resolution of this technique a pooled sample was used. All samples were combined to create a consistent 'pooled standard' sample and the molecules were separated according to their isoelectric point using isoelectric focusing (IEF) and molecular weight (SDS-PAGE). Selected gel spots were cut and from the gel and processed by manual in-gel tryplic digestion in preparation for LC-MS/MS analysis. Figure 8 indicated that using this method we were able to separate the myofibrillar isoforms by SDS-PAGE into more diverse proteins and 91 spots were resolved, cut and digested. Only 59 spots were successfully identified (Table 8). Each spot identified by protein name, score, number of peptides, sequence percentage, MW and pI. From 59 spots there were 26 unique proteins.

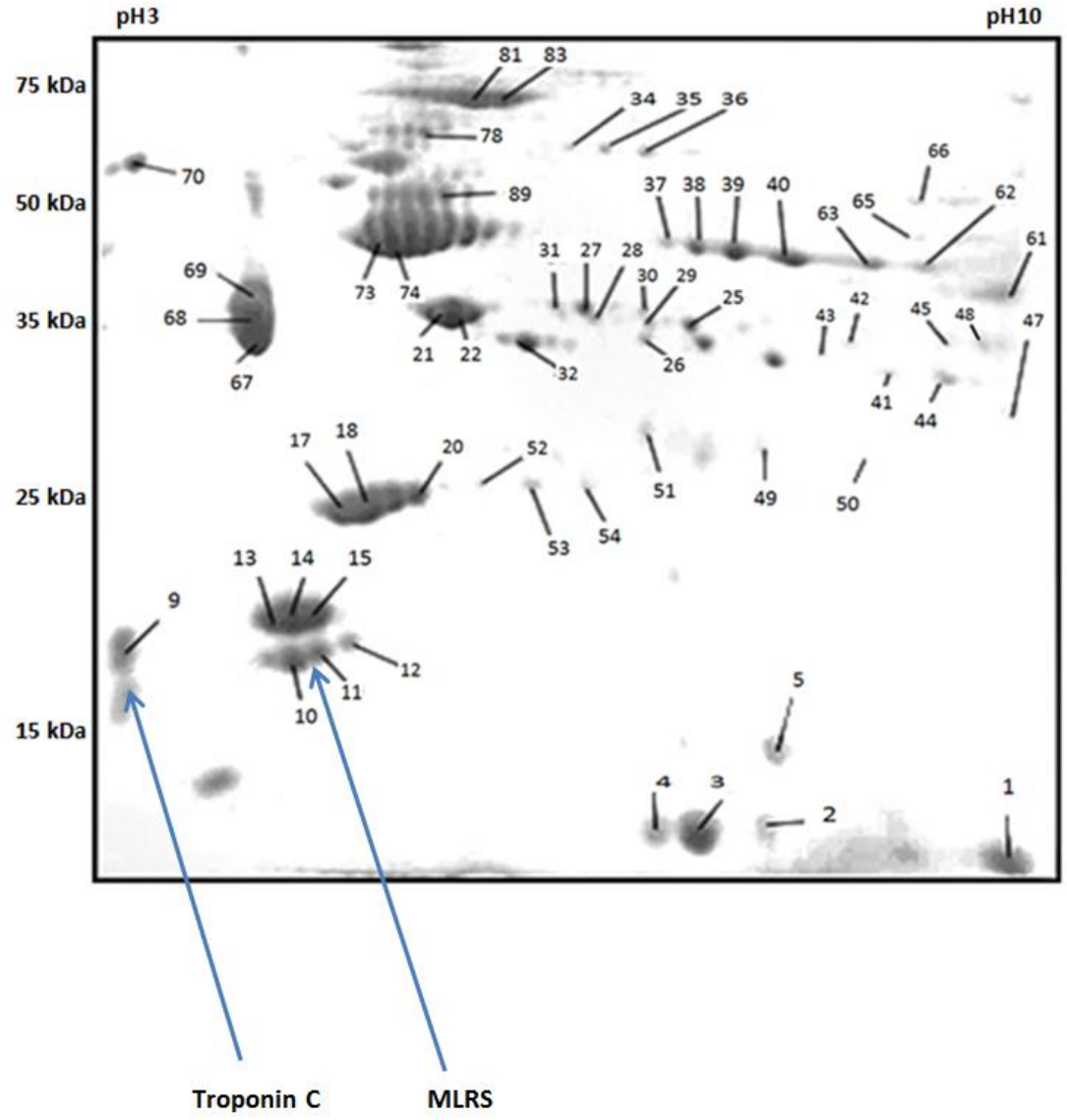


Figure 9. Separation of myofibrillar isoforms by 2DE SDS-PAGE. Distinct spots separated based in differences in pH and migration (Pooled standard sample from all participants). 26 proteins were successfully identified.

Table 9. Proteins detected in 2D gel spots.

#	Accession	Protein name	Score	SC (%)	Peptides	MW	pI
48	LDB3	LIM domain-binding protein 3	438	18	8	77.1	9.4
81	ALBU	Serum albumin precursor	923	37	23	69	5.9
83	ALBU	Serum albumin precursor	1160	50	25	69	5.9
69	K2C1	Keratin, type II cytoskeletal 1	310	11	6	66	8.8
70	K2C1	Keratin, type II cytoskeletal 1	319	12	7	66	8.8
66	ATPA	ATP synthase subunit alpha, mitochondrial precursor	610	32	16	59.7	9.6
34	FIBB	Fibrinogen beta chain	475	34	11	55.9	9.3
35	FIBB	Fibrinogen beta chain	909	54	19	55.9	9.3
36	FIBB	Fibrinogen beta chain	886	55	21	55.9	9.3
78	FIBG	Fibrinogen gamma chain precursor	384	40	14	51.5	5.3
65	ENOB	Beta-enolase	504	35	10	46.9	8.6
37	KCRM	Creatine kinase M-type	631	38	12	43.1	6.9
38	KCRM	Creatine kinase M-type	454	38	12	43.1	6.9
39	KCRM	Creatine kinase M-type	715	38	15	43.1	6.9
63	KCRM	Creatine kinase M-type	465	38	10	43.1	6.9
40	KCRM	Creatine kinase M-type	674	46	18	43.1	6.9
73	ACTS	Actin, alpha skeletal muscle	1381	61	22	42	5.1
89	ACTS	Actin, alpha skeletal muscle	713	50	17	42	5.1
74	ACTS	Actin, alpha skeletal muscle	1149	54	20	42	5.1
61	ALDOA	Fructose-biphosphate aldolase	284	29	7	39.4	9.2
62	ALDOA	Fructose-biphosphate aldolase	392	31	9	39.4	9.2
45	G3P	Glyceraldehyde-3-phosphate dehydrogenase	325	31	6	36	9.3
21	TNNT1	Troponin T, slow skeletal muscle	520	29	15	32.9	5.8
22	TNNT1	Troponin T, slow skeletal muscle	727	35	15	32.9	5.8
68	TPM2	Tropomyosin beta chain	1598	38	16	32.8	4.5

20	TPM3	Tropomyosin alpha-3 chain	186	21	7	32.8	4.5
52	TPM3	Tropomyosin alpha-3 chain	1880	53	22	32.8	4.5
67	TPM1	Tropomyosin alpha-1 chain	1488	49	19	32.7	4.5
25	TNNT3	Troponin T, fast skeletal muscle	244	25	6	31.8	5.6
26	TNNT3	Troponin T, fast skeletal muscle	209	25	7	31.8	5.6
42	TNNT3	Troponin T, fast skeletal muscle	183	14	3	31.8	5.6
27	TNNT3	Troponin T, fast skeletal muscle	268	30	8	31.8	5.6
43	TNNT3	Troponin T, fast skeletal muscle	191	19	5	31.8	5.6
28	TNNT3	Troponin T, fast skeletal muscle	290	25	8	31.8	5.6
29	TNNT3	Troponin T, fast skeletal muscle	146	25	7	31.8	5.6
30	TNNT3	Troponin T, fast skeletal muscle	254	26	7	31.8	5.6
31	TNNT3	Troponin T, fast skeletal muscle	201	25	5	31.8	5.6
32	TNNT3	Troponin T, fast skeletal muscle	246	25	8	31.8	5.6
41	MYOZ1	Myozenin-1	342	38	7	31.7	9.3
44	MYOZ1	Myozenin-1	334	48	10	31.7	9.3
49	CAH3	Carbonic anhydrase 3	260	32	7	29.5	7.1
59	CAH3	Carbonic anhydrase 3	352	39	8	29.5	7.1
51	CAH1	Carbonic anhydrase 1	516	45	9	28.9	6.7
53	MYL6B	Myosin light chain 6B	364	47	11	22.7	5.5
54	MYL6B	Myosin light chain 6B	380	51	12	22.7	5.5
17	MYL1	Myosin light chain 1/3, skeletal muscle	1451	89	18	21.1	4.8
18	MYL1	Myosin light chain 1/3, skeletal muscle	1689	74	17	21.1	4.8
10	MLRS	Myosin regulatory light chain 2	833	88	17	19	4.7
11	MLRS	Myosin regulatory light chain 2	804	78	12	19	4.7
12	MLRS	Myosin regulatory light chain 2	608	54	7	19	4.7

13	MLRV	Myosin regulatory light chain 2, venticular/cardiac muscle	655	74	17	18.8	4.7
14	MLRV	Myosin regulatory light chain 2, venticular/cardiac muscle	642	72	15	18.8	4.7
15	MLRV	Myosin regulatory light chain 2, venticular/cardiac muscle	1098	90	20	18.8	4.7
9	TNNC2	Troponin C, skeletal muscle	777	54	11	18.1	3.9
5	MYG	Myoglobin	624	55	9	17.2	7.9
2	HBB	Haemoglobin subunit beta	686	75	11	16	6.9
3	HBB	Haemoglobin subunit beta	3235	83	12	16	6.9
4	HBB	Haemoglobin subunit beta	1321	82	11	16	6.9
1	HBA	Haemoglobin subunit alpha	846	70	8	15.2	9.4

Data represent proteins which were identified by mass spectrometry and database searches, these were categorised further and ranked by score to determine the primary protein typically found in each spot.

In-solution analysis of human myofibrillar proteins

1.3.8 Liquid chromatography-mass spectrometry (Human - YOTU)

Two-dimensional gel electrophoresis resolved Band 8 but it did not resolve all expected abundant myofibrillar proteins for example MyHC, Myosin binding protein C, alpha actinin-2 were missing from table 8. Therefore, differential analysis using high-performance liquid chromatography online mass-spectrometry was investigated to see if it could overcome this resolution problem. In total 49 protein entries (484 peptides) were consistently detected and used for label-free quantitative analysis. This number of identified proteins is more than the number identified by 2D gel. Table 10 details the entire list of Mascot protein identifications and normalised abundance of each protein. Based on MOWSE score the most robust identification were type IIa myosin heavy chain (MYH2) and type I myosin heavy chain (MYH7).

The lowest scoring proteins detected were myomesin-2, keratin and fructose-biphosphate aldolase.

Table 10. List of Mascot protein identifications.

Accession	Protein name	Score	SC (%)	Peptides	Obs. MW	Obs. pI
MYH3	Myosin-3	12997	20	1	223.8	5.5
MYH13	Myosin-13	5824	14	1	223.5	5.4
MYH7	Myosin-7	31073	52	71	223	5.5
MYH1	Myosin-1	27777	41	11	223	5.5
MYH2	Myosin-2	36676	51	94	222.9	5.5
MYH4	Myosin-4	18379	28	1	222.9	5.6
MYOM2	Myomesin-2	116	9	5	164.8	5.8
MYPC1	Myosin-binding protein C, slow type	763	17	14	128.2	5.7
ACTN2	Alpha-actinin-2	5705	45	31	103.8	5.2
LDB3	LIM domain-binding protein 3	1259	18	9	77.1	9.4
ALBU	Serum albumin precursor	1689	41	20	69.3	5.9
K2C1	Keratin, type II cytoskeletal 1	171	10	6	66	8.8
PDLI5	PDZ and LIM domain protein 5	135	15	4	63.9	9.6
K1C10	Keratin, type I cytoskeletal	116	6	3	58.8	5
ATPB	ATP synthase subunit beta, mitochondrial	261	21	6	56.5	5.1
DESM	Desmin	1778	39	15	53.5	5.1
VIME	Vimentin	424	14	2	52.6	4.9
ENOB	Beta-enolase	324	47	4	46.9	8.6
CASQ1	Calsequestrin-1	1718	11	2	44.5	3.9
KCRM	Creatine kinase M-type	5182	43	1	43.1	6.9
ACTS	Actin, alpha skeletal muscle	12390	63	23	42	5.1
ACTB	Actin, cytoplasmic 1	6353	30	1	41.7	5.2
ALDOA	Fructose-bisphosphate	1159	41.8	9	39.4	9.2

	aldolase A OS					
ALDOC	Fructose-biphosphate aldolase C	102	18	1	39.4	6.5
FHL1	Four and a half LIM domains protein 1	398	14	3	36.2	10.5
G3P	Glyceraldehyde-3-phosphate dehydrogenase	599	38	7	36	9.3
TNNT1	Troponin T, slow skeletal muscle	1119	26	6	32.9	5.8
TPM2	Tropomyosin beta- chain	7878	38	13	32.8	4.5
TPM3	Tropomyosin alpha-3 chain	6338	44	7	32.8	4.5
TPM1	Tropomyosin alpha- 1 chain	7298	44	11	32.7	4.5
TNNT3	Troponin T, fast skeletal muscle	1262	24	6	31.8	5.6
MYOZ1	Myozenin-1	599	51	7	31.7	9.3
CAH3	Carbonic anhydrase 3	760	38	6	29.5	7.1
MYL6B	Myosin light chain 6B	152	15	1	22.7	5.5
MYL3	Myosin light chain 3	4390	68	10	21.9	4.9
TNNI1	Troponin I, slow skeletal muscle	1043	29	5	21.7	10.3
TNNI2	Troponin I, fast skeletal muscle	901	14	3	21.3	9.6
MYL1	Myosin light chain 1/3, skeletal muscle isoform	8270	8	15	21.1	4.8
MLRS	Myosin regulatory light chain 2, skeletal muscle isoform	7121	72	2	19	4.7
MLRV	Myosin regulatory light chain 2, venticular/cardiac muscle	3186	72	11	18.8	4.7
TNNC1	Troponin C, slow skeletal and cardiac muscles	261	9	2	18.4	3.9
TNNC2	Troponin C, skeletal muscle	2162	33	4	18.1	3.9
MYG	Myoglobin	3163	55	8	17.2	7.9
MYL6	Myosin light polypeptide 6	1402	30	2	16.9	4.4
HBB	Haemoglobin subunit beta	11403	88	12	16	6.9
HBD	Haemaglobin subunit delta	4206	69	2	16	9.1
HBA	Haemoglobin subunit alpha	2586	53	6	15.2	9.4

Data represent proteins were identified by mass spectrometry and database searches, these were categorised further and ranked by score to determine the primary protein typically found in each spot.

Profile Analysis

Table 10 reports Profile Analysis data aligned with mascot protein ID i.e. label-free profiling. Using profile analysis 265 buckets were resolved and exported to SPSS for two-way ANOVA. Forty buckets had significant differences but only 19 identified when aligned with mascot protein identification (Table 11).

Table 11. Differences in protein abundance in vastus lateralis muscle by profile analysis aligned with mascot protein identification. Red highlight represent myosin light chain group were selected for MRM analysis.

Accession & Protein name	min : bucket	p value Age	Training	Age * Training	Sequence
ACTS_HUMAN Actin	40.1min : 644.31m/z	0.552	0.289	0.02	GILTLK
	98.9min : 895.86m/z	0.024	0.315	0.336	SYELPDGQVITIGNER
	44.7min : 1162.47m/z	0.043	0.537	0.678	EITALAPSTMK
MLRS_HUMAN Myosin regulatory light chain 2, skeletal muscle	83.4min : 652.64m/z	0.159	0.029	0.05	VAPEEHPTLLTEAPLNPK
	103.8min : 1524.61m/z	0.117	0.03	0.2	FLEELLTTQCDR
MYH_HUMAN Myosin	32.1min : 1173.43m/z	0.06	0.026	0.399	DGIIDKEDLR
	104.4min : 1319.51m/z	0.045	0.046	0.465	GADPEDVITGAFK
	117.6min : 895.91m/z	0.026	0.096	0.211	NAYEESLDQLETLK
	21.9min : 1004.37m/z	0.268	0.348	0.041	ELEEISER
	144.6min : 1893.88m/z	0.004	0.051	0.051	LLSTLFANYAGADAPIEK
MYL1_HUMAN Myosin light chain 1/3, skeletal muscle	67.1min : 1507.59m/z	0.015	0.275	0.302	LTYTQQLEDLKR
MYL3_HUMAN Myosin light chain 3	21.5min : 1272.36m/z	0.015	0.51	0.87	TLEDQMNEHR
TPM_HUMAN Tropomyosin	20.0min : 1411.48m/z	0.876	0.832	0.027	ELTYQTEEDRK
	17.8min : 1482.51m/z	0.108	0.555	0.033	KVQHELDEAEER
	119.4min : 1010.42m/z	0.508	0.022	0.444	EAFLLFDR
	116.4min : 1542.60m/z	0.001	0.841	0.798	DQATYEDFVEGLR
	22.9min : 1524.60m/z	0.03	0.917	0.662	AAPAPAPPPEPERPK
	68.8min : 1243.50m/z	0.04	0.574	0.63	IQLVEEELDR

Differentially abundant protein included α-actin, MyHC, MyLC and tropomyosin. The majority of these proteins had multiple peptides assigned to them but cases (e.g. α-actin and MyHC). The peptides assigned to a protein each exhibited different patterns of differences in abundance, i.e. demonstrating either significant main effect for age or training status or significant interaction. This is likely due to high degree of homology across isoforms of these proteins. Therefore, α-actin, MyHC and tropomyosin were excluded from the follow up analyses. Tropomyosin was also excluded because this was based on single peptide. Proteins taken forward for confirmatory analysis were MLRS, MyLC 1 and 3.

Label-free profiling is a non-targeted discovery technique and is susceptible errors in the alignment of MS spectra. Therefore best practice is to perform confirmatory analysis using a targeted technique such as western blotting or selective reaction monitoring. In this case selective reaction monitoring (SRM) was used to verify differences in the abundance of proteins detected by LC-MS profiling.

1.3.9 Selective reaction monitoring (SRM)

SRM involves programming the mass spectrometer to detect pairs of peptide ions and fragment ions that are specific to the protein of interest. In skeletal muscle it is particularly important to select targets that use isoform-specific peptides. This removes ambiguity but the number of isoform-specific peptides represents a small proportion of the overall protein sequence, which makes this analysis challenging. Samples were analysed in duplicate and in a randomised order. Figure 10 show an

example of proteins identified in human samples, a single transition 600–658 was monitored, which represents the y5 ion (600.82 m/z) of the doubly charged peptide K.ITLSQVGDVLR.A of MYL1_HUMAN.

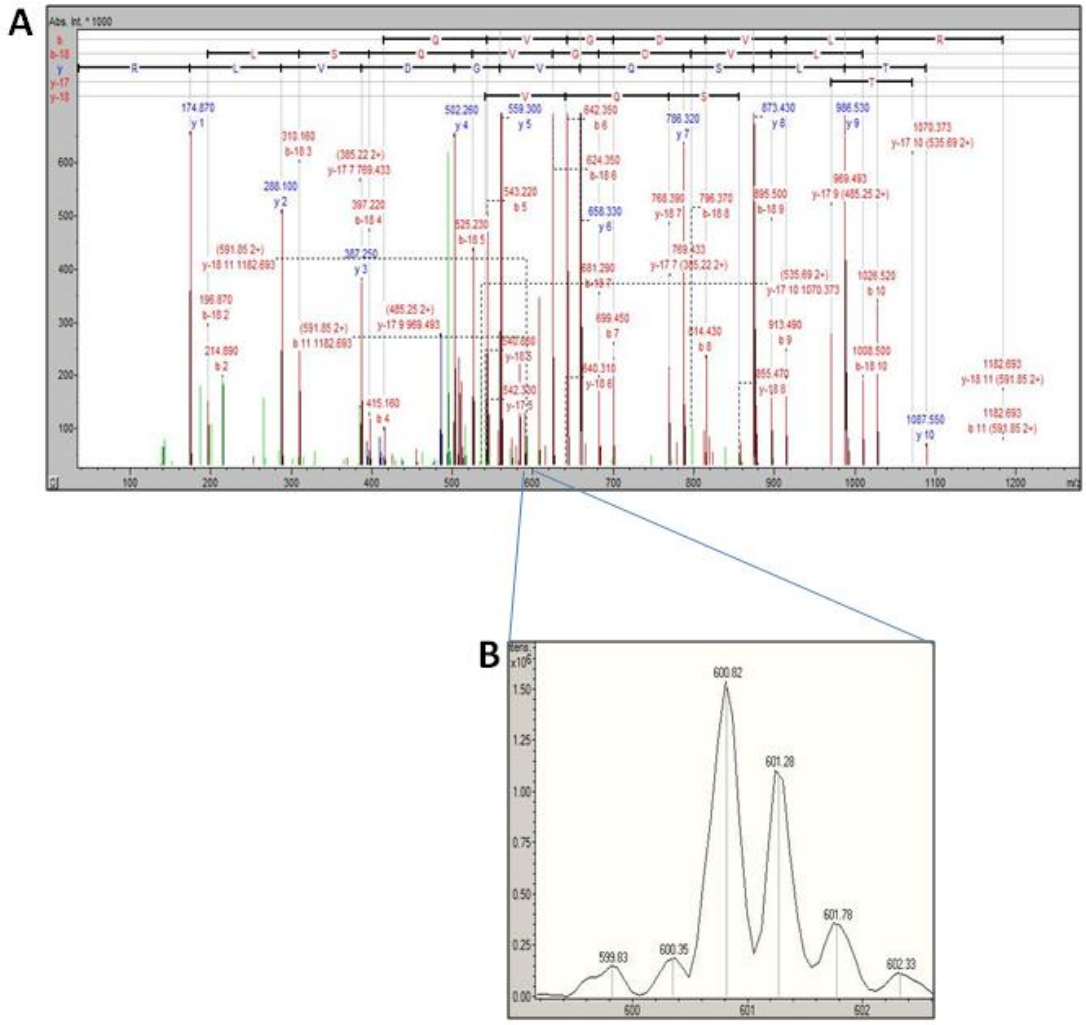


Figure 10. Selective reaction monitoring of MYL1. Annotated MS/MS spectra of the doubly-charged precursor, (A) of residues K.ITLSQVGDVLR.A of MYL1_HUMAN and (B) monitoring of the selected fragment ion (600.82 m/z).

1.3.10 Multiple reaction monitoring (MRM) Verification

Multiple reaction monitoring (MRM) using mass spectrometry is a highly sensitive highly selective (targeted), allowing us to fine tune an instrument to specifically look for peptides, or protein fragments, of interest. The MRM platform technology allows for targeted analysis of multiple selective reactions for proteins of interest for example like listed in table 12 and table 13.

MRM based on the result from age group on Profile Analysis

Table 12. Area coverage in protein between elderly and young vastus lateralis muscle.

	YT	YU	OT	OU
MYL1 (771.76 – 835.42)	1034397.9 ±482407.6	1779223.6 ±672895.4	703544.3* ±181593.3	907659.8* ±356365.7
MYL1 (600.8 – 658.3)	5777961.7 ±2532524.8	5707742.4 ±7583675.2	5457556.3* ±5430854.3	8436602.7 ±8908408.7
MYL3 (498.21 – 759.44)	4529903.6 ±1478327.4	2438270.8 ±1166681.2	7233900* ±3889691.5	5296113.1 ±2556299.8
MYL1 (Average)	1568154.8 ±801499.4	2698360.8 ±845963.9	1062304.8* ±186342.6	1451987.4* ±600787.3

Data is presented as mean ± SD. * Significant difference from YU group. Data represented each group proteins were identified by LC-MS which enables to measure the area coverage of each protein. There is significant difference was evident between YU and OT in all listed protein and between YU and OU in MYL1 (771.76 – 835.42) transition and MYL1 average.

MRM based on the result from training group on Profile Analysis

Table 13. Area coverage in protein between trained and untrained vastus lateralis muscle.

	YT	YU	OT	OU
MLRS (660.27 – 735.42)	829855.6 ±450898.4	1229365.2 ±345497.1	573957# ±273417.4	939341.5 ±463042.3
MLRS (762.81 – 780.27)	1632432.8 ±1160794.7	2571437.4 ±980082.3	869758.7* ±321700.3	1675950.5 ±1081564.5
MYL1 (505.7 – 550.22)	2829422.5 ±1822148.7	4829979.3 ±2072181.3	1199865.5* ±373670.5	2441917.1 ±1515769.9
MLRS (Average)	1231144.2 ±794307.8	1900401.3 ±658755.7	721857.8* ±295366.2	1307646 ±730069.8

Data is presented as mean ± SD. * ($p<0.05$) Significant difference from YU group and # ($p=0.05$) with YU group. Data represented each group proteins were identified by LC-MS which enables to measure the area coverage of each protein. There is significant difference was evident between YU and OT in MLRS (762.81 – 780.27) transition, MYL1 (505.7 – 550.22) transition and MLRS average. Meanwhile MLRS (660.27 – 735.42) transition the p-value equal to 0.05 between YU and OT group.

1.4 DISCUSSION

Label-free profiling of soluble proteins from HCR/LCR rats

The current work reports a time- and sample-efficient method of profiling the expression of the major metabolic enzymes in skeletal muscle. Using a simple LC-MS approach we surveyed more than 200 proteins, including almost all enzymes of the major metabolic pathways (i.e., glycolysis, fatty acid β-oxidation, the tricarboxylic acid cycle and some components of the respiratory chain). Recently (Hussey et al., 2012), spectral counting was successfully combined with GeLC-MS/MS to perform differential analysis of 438 proteins in human skeletal muscle. However, that relatively broad coverage of the muscle proteome was achieved at the expense of substantially greater machine time and sample usage. Hussey et al. (2012) separated 60 μg aliquots of muscle homogenate in to 20 molecular weight fractions, which were analysed by LC-MS/MS over 90 min periods. This equates to roughly 1,800 min machine time per sample (biological replicate). In the current work, approximately 50 ng of tryptic peptides from each biological replicate was resolved by LC-MS of 180 min duration. This represents a 10-fold improvement in time efficiency and a greater than 100-fold increase in sample efficiency; albeit at the expense of a 2-fold decrease in the number of proteins detected. We believe the current approach represents a useful compromise that may lead to more widespread application of LC-MS profiling as a semi-automated and efficient means of phenotyping skeletal muscle.

Here, we applied LC-MS profiling to muscle of HCR and LCR rats that exhibit differences in exercise capacity, cardiometabolic disease risk and life expectancy

(Koch et al, 2011). Deterioration in the metabolic flexibility of skeletal muscle is associated with insulin resistance, which is a cardinal feature of human metabolic syndrome, and also accompanies natural ageing. Metabolic dysfunction and insensitivity to insulin are also evident as correlated traits of selection on low running capacity. For example, in the basal (fasted) state LCR muscle has lesser uptake and oxidation of fatty acids and, when stimulated by insulin, glucose uptake of LCR hindlimb muscles is significantly less than HCR (Rivas et al., 2011). Transcriptome profiling (Kivela et al., 2010) reports enrichment of gene-sets, including oxidative phosphorylation, fatty acid metabolism and PPAR signalling, correlate with the differences in running activity of LCR and HCR animals. However, such differences between HCR and LCR are generally more pronounced in predominantly fast- compared to slow-twitch muscles. The proportion of type IIa MyHC (42 ± 16% versus 22 ± 14%) was significantly greater in HCR gastrocnemius used for transcriptome analysis, which may have contributed to some of the differences observed in metabolic markers. To obviate effects due to myofibre profile, we performed proteomic analysis of slow-twitch soleus muscle that has consistent myofibre proportions (~80% type I, ~20% type IIa) in HCR and LCR rats (Kivela et al., 2010).

LC-MS profiling detected 16 significant differences between HCR and LCR soleus muscles. Consistent with the selection paradigm and earlier reported by Kivela et al. (2010), the differences in soleus mitochondrial content, the majority of proteins were more abundant in HCR muscle (Table 3) and were associated with the mitochondrial respiratory chain. Specifically, HCR soleus had greater abundance of cytochrome b-c1 complex subunit 1 and cytochrome c oxidase subunit 4, isoform 1, which were

included in the oxidative phosphorylation gene-set enriched in HCR gastrocnemius muscle. In addition, 3-ketoacyl-CoA thiolase (THIM) and enoyl-CoA hydratase (ECHM) included in the HCR gastrocnemius gene-set "Fatty acid oxidation" were also more abundant in HCR soleus (Table 3).

FABPH exhibited the most prominent difference between HCR and LCR muscles, and we used selective reaction monitoring (SRM) to verify this initial discovery. SRM assays afford greater selectivity and sensitivity than LC-MS profiling and can often be performed more rapidly. During LC-MS profiling relative quantification is achieved by monitoring the intensity of parent-ion masses but this may be susceptible to interference from unrelated peptides of similar mass-to-charge ratio. In contrast, SRM analysis monitors the intensity of a known product-ion mass during fragmentation of a selected parent ion (known as a transition) and this additional level of filtering affords greater selectivity and sensitivity. SRM analysis of FABPH in rat serum has previously been reported by Zhen et al., (2007) and we report an identical parent/fragment ion transition can be used in rat skeletal muscle with good reliability (i.e., 20% coefficient of variation). Our method development was also aided by use of the same MS platform for both LC-MS profiling and SRM (Han & Higgs, 2008) and we were able to shorten analysis time per sample by a factor of 10. SRM confirmed our discovery by LC-MS profiling that FABPH is significantly more abundant in HCR muscle (Figure 6). FABPH binds long-chain fatty acids in the sarcoplasm and the abundance of this protein is tightly associated with the capacity of muscle to take up fatty acids (Binas & Erol, 2007). Thus the lesser FABPH content of LCR muscle is likely to be a key factor contributing to the lesser uptake of FA in LCR muscle (e.g., Rivas et al., 2011) under basal conditions. In

addition FABPH is also known to have reciprocal effects on the utilisation of glucose and long-chain fatty acids during exercise (Shearer et al., 2005). Therefore the lesser abundance of FABPH in LCR muscle may also play a role in the relative poor running capacity of these animals. Paradoxically, FABPH is less abundant in muscle of normal-weight individuals compared to obese and morbidly obese patients (Hittel et al., 2005), which may be expected to have relatively poorer exercise capacity. This phenomenon is likely to be connected with the elevated storage of intramuscular triacylglycerides, which occurs in both obese individuals and endurance-trained athletes (Shaw et al., 2010).

Proteomic analysis of human myofibrillar proteins

In the present study the results show the separation of human MyHC isoforms in mini-format SDS-PAGE stained with Coomassie colloidal blue. The average percentages were MyHC I (52.4%), MyHC IIa (38.4%), and MyHC IIx (9.2%) in the vastus lateralis homogenates (Figure 6). This demonstrates that we were able to use the traditional biochemical analysis as a point of comparison for developing proteomic techniques in human muscle samples. Consistent with previous studies relating to aging, we report differences in MyHC isoform profile exhibiting a loss of type IIx fibres and increase in the abundance of type I and type IIa fibres in older compared to young adults. Similarly, Andersen et al., (1999) reported that MyHC I and MyHC IIa were significantly greater in elderly participants compared to younger participants. The effect of increasing age on the human skeletal muscle may also influence fibre size and fibre number, mostly of type 2 fibres has also been reported (Lexxel et al., 1988) but our biochemical techniques are not able report data on these

variables. Many studies regarding MyHC report that participants undergoing resistance training have the ability to produce a shift from MyHC IIx to IIa (Adams et al., 1993; Carroll et al., 1998; Jurimae et al., 1996). Andersen et al., (1999) reported that there were small percentages of the MyHC IIx isoform in young trained participants but MyHC I and MyHC IIa were significantly greater in elderly participants compared to younger participants. In the present study (Figure 6) the result showed a similar outcome where the young and elderly trained had less MyHC IIx and a greater abundance of MyHC IIa. Williamson et al., (2000), reported there were significantly increase in the expression of MyHC I (pre 50.4 ± 6.7 and post 51.9 ± 7.9 %) as a result from low intensity training. In the study reported here that both untrained and trained elderly showed 0 % of MyHC IIx. This is supported by Sharman et al., (2001) reporting that MyHC expression with aging is not associated with increases in MyHC IIb/x isoform. The elderly trained had the greatest abundance MyHC I (89.2 %), which may be due to the combined effects of ageing and endurance training.

The results using SDS-PAGE 15% for low molecular weight (LMW) myofibrillar proteins also suggests training status alters the expression of the myofibrillar proteome. However the only significant difference was evident in band 8 where the volume of MLRS protein was highest in YU group and lowest at OT group. MLRS is the skeletal muscle isoform of myosin regulatory light chain 2 and is predominantly found in fast-twitch skeletal muscle. This finding is consistent with our preliminary results of myosin heavy chain profile that show in YU group they have 47.6% type II fibre and in OT group they have only 10.8% type II fibre. This is consistent with the selective atrophy in type II fibres with increasing age (Lexell &

Taylor, 1991). Proteomic analyses of human muscle samples also provided further information on ageing induced decline in myofibrillar isoforms, with young males showing higher band volume than older. However, 1-DE combined with MS analyses identified multiple proteins within each band making it difficult to determine specific changes in individual proteins, supporting a need for more thorough protocol such as 2-DE.

There were many studies conducted using the two dimension electrophoresis method in proteomics using human muscle samples (Hojlund et al., 2003; Capitanio et al., 2005; Holloway et al., 2009; Hody et al., 2011; Hartwig et al., 2014; Conti et al., 2014). Previous work in our lab (Holloway et al., 2009) used large format 2D (13cm) gel and resolved 256 spots, and paired t-tests identified 20 significant differences in expression (false discovery rate less than 10%). The differences in spot expression represent changes in post-transcriptional or post-translational processing. Furthermore studies using larger format 2D (18cm) gel, resolved more than 500 protein spots on each gel were detected by silver staining, of which 150 were excised, digested in-gel with trypsin and characterized by matrix assisted laser desorption ionization-mass spectrometry and tandem electrospray mass spectrometry (MALDI-MS/MS). Using these techniques, 124 spots including contractile proteins and metabolic enzymes were identified. Our current work used small format 2D gel because the myofibrillar fraction was expected to be less complex and using this method we were able to separate the myofibrillar isoforms into 91 spots (Figure 9). Gel spots were cut and processed by manual in-gel tryplic digestion in preparation for LC-MS/MS analysis. In total, 59 spots were successfully identified (Table 9).

From 59 spots there were 26 unique proteins were discovered in multiple spots, which may represent different splice variants or post-translational modification.

2D gel electrophoresis did not resolve all of the expected abundant myofibrillar proteins for example MyHC, Myosin binding protein C, alpha actinin-2 were missing. Therefore, differential analysis using high-performance liquid chromatography online mass-spectrometry was used to overcome this resolution problem. Yi et al., (2008) used HPLC-ESI-MS/MS analysis and managed to identify 507 proteins and the majority of the proteins identified by mass spectrometry also had their corresponding transcripts detected by microarray analysis, although 73 proteins were only identified in the proteomic analysis. In addition, the combination of 1D gel electrophoresis and HPLC electrospray ionization tandem mass spectrometry, managed to identify 954 different proteins in human vastus lateralis muscle obtained from three healthy participants (Hojlund et al., 2008). In the current work a total of 49 unique protein entries (484 peptides) mapped to features in progenesis LC-MS and were used for quantitative analysis. This number of proteins is greater than number of protein by 2D gels, and includes protein such as MyHC, Myosin binding protein C and alpha actinin-2 not detected by 2D gel electrophoresis.

LC-MS profiling was conducted to investigate whether this proteomic technique could provide a more automated method for muscle phenotyping. Myofibrillar proteins were identified by mass spectrometry and database searches were checked for the existence of expected myofibrillar proteins listed by Schiaffino & Reggiani (2011) (Table 14). The listed proteins were grouped according to the main structural

components of the sarcomere (Figure 11). All proteins of the thin filament and most of the thick filament were detected. However, relatively few proteins of the Z-Disk and M-Band were detected, which may be due to their relatively low abundance compared to the main myofibrillar proteins.

Table 14. List of Mascot protein identifications with the main components of the striated muscle.

Schiaffino (2011)	Accession Proteinscape	Protein name	# of spots
Z-DISC			
α-actinin-slow			
α-actinin-fast	ACTN2_HUMAN	Alpha-actinin-2	73
FATZ-2 (calsarcin-1)			
FATZ-1 (calsarcin-2)			
FATZ-3 (calsarcin-3)			
ZASP			
Myotilin, palladin, myopalladin			
FHL1 (SLIM1)			
αB-crystallin			
Muscle LIM protein	LDB3_HUMAN	LIM domain-binding protein 3	48
THIN FILAMENT			
Actin	ACTS_HUMAN	Actin, alpha skeletal muscle	74
Tropomyosin-α-fast	TPM3_HUMAN	Tropomyosin alpha-3 chain	20
Tropomyosin-α-slow	TPM1_HUMAN	Tropomyosin alpha- 1 chain	67
Tropomyosin-β	TPM2_HUMAN	Tropomyosin beta- chain	68
TnC-fast	TNNC2_HUMAN	Troponin C, skeletal muscle	
TnC-slow	TNNC1_HUMAN	Troponin C, slow skeletal and cardiac muscles	
TnI-fast	TNNI2_HUMAN	Troponin I, fast skeletal muscle	
TnI-slow	TNNI1_HUMAN	Troponin I, slow skeletal muscle	
TnT-fast (splicing	TNNT3_HUMAN	Troponin T, fast skeletal muscle	43

var)			
TnT-slow (splicing var)	TNNT1_HUMAN	Troponin T, slow skeletal muscle	21
M-BAND			
Myomesin-1			
Myomesin-1 EH variant			
Myomesin-2 (Mprotein)	MYOM2_HUMAN	Myomesin-2	
Myomesin-3			
THICK FILAMENT			
MyHC-1 or β/slow	MYH7_HUMAN	Myosin-7	
MyHC-2A	MYH2_HUMAN	Myosin-2	
MyHC-2X	MYH1_HUMAN	Myosin-1	
MyHC-2B	MYH4_HUMAN	Myosin-4	
MyHC-eo	MYH13_HUMAN	Myosin-13	
MyHC-emb	MYH3_HUMAN	Myosin-3	
MyLC-1s	MYL1_HUMAN	Myosin light chain 1/3, skeletal muscle isoform	17
MyLC-1f/3f	MYL3_HUMAN	Myosin light chain 3	
MyLC-2f	MLRS_HUMAN	Myosin regulatory light chain 2, skeletal muscle isoform	11
MyLC-2s	MLRV_HUMAN	Myosin regulatory light chain 2, venticular/cardiac muscle	13
MBP-C-slow	MYPC1_HUMAN	Myosin binding protein C, slow type	
MBP-C-fast			
Titin long isoforms			
Titin short isoforms			

The list showing the main components of the striated muscle sarcomere (Schiaffino & Reggiani, 2011) and the mutual list of proteins identified in Mascot proteinscape. All proteins detected as Schiaffino list in thin filament and most in thick filament.

Difference in protein abundance were calculated by linking the regulation ratios determined in Profile Analysis for each of the identified peptides from the parent protein. In total, the current study resolved 265 peptide features (termed 'buckets'), which were exported to SPSS for statistical analysis. Two-way ANOVA revealed 40 of these features exhibited statistically significant differences but just 19 of these were identified when aligned with the Mascot protein identifications. There were significant differences in the abundance of peptides from myofibrillar proteins such as MyHC, actin, MyLC and troponin. However, while we saw numerous peptides identified as MyHC it was not possible to determine the specific isoform (e.g. MYH7, MYH1 etc.) because these peptides originated from regions of the protein that are identical across all isoforms. In contrast, several isoform-specific peptides were identified for the essential and regulatory MyLC. The profiling data suggested that the fast skeletal muscle isoform of regulatory myosin light chain (MLRS) was more abundant in the muscle of young untrained people, whereas the fast isoform of myosin essential light chain (MYL1) was significantly less abundant in muscle of elderly compared to young adults.

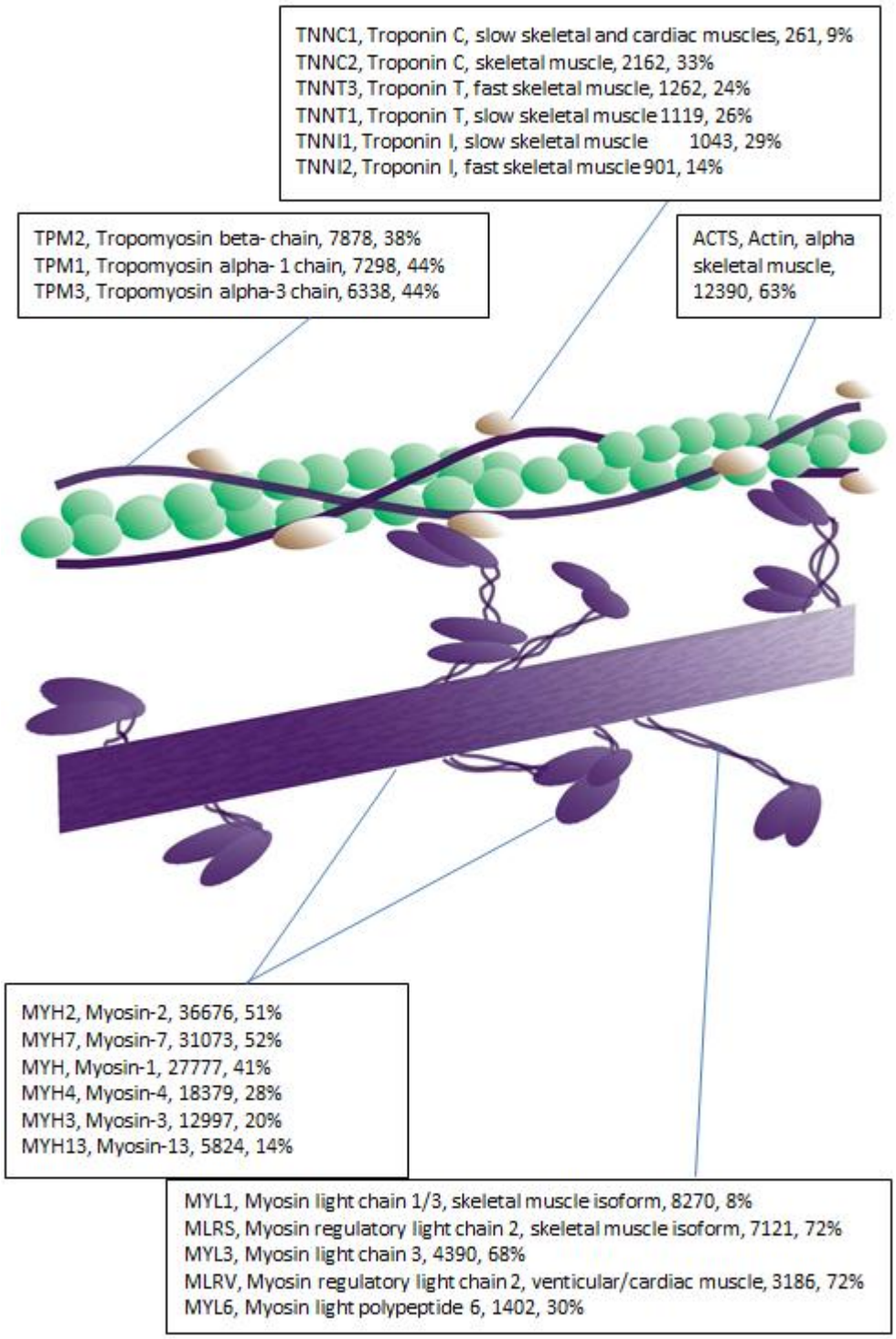


Figure 11. Illustration of the structure of thin filament and thick filament; and its main protein components that are known to exist as multiple isoforms. Protein identifiers and descriptions are from the Swiss-Prot database. The MOWSE score and sequence coverage for each protein are also reported from the database searches.

During LC-MS profiling relative quantification is achieved by monitoring the intensity of parent-ion masses but this may be susceptible to interference from unrelated peptides of similar mass-to-charge ratio. Therefore we checked our data by performing MRM assays for MYL1, MYL3 and MLRS peptides. SRM assays afford greater selectivity and sensitivity than LC-MS profiling because the analysis monitors the intensity of a known product-ion mass during fragmentation of a selected parent ion (known as a transition). This additional level of filtering affords greater selectivity and sensitivity and using this approach we were able to independently confirm our profiling data.

Our findings are consistent with other biochemical and proteomic studies of muscle exercise and ageing. A study conducted by Larsson et al. (1997) reports the effects of aging on shortening velocity of single human muscle fibres and used SDS-PAGE to measure the composition of MyHC and MyLC. Similar to our experience, they were unable to clearly resolve myosin light chains because of the uncertainties due to co-migration of other myofibrillar proteins such as troponins. Nonetheless, Baumman et al. (1987) reports high-intensity endurance training increases the abundance of the slow ventricular isoform of regulatory myosin light chain. Similarly, a more recent study by Reidy et al., (2014) reports the fast regulator isoform (MLRS) was less abundant in competitive compared to recreational runners. Gelfi et al., (2006) reports 2DGE analysis of age-related differences in human muscle proteins, including a relatively lesser abundance of the fast skeletal muscle regulatory light chain in muscle of elders compared to young individuals. Because Gelfi et al., (2006) and our current work are each cross-sectional studies, it is important to note the difference in our findings may be related to differences in the myofibre profiles of either the

young or elderly populations. Because Gelfi et al., (2006) did not report physiological data regarding their participants it is not possible to compare their exercise capacity with the participants used in Cobley et al., (2012), which were the substrate for the current work.

1.5 CONCLUSION

This study investigated whether it was feasible to use proteomic techniques to perform muscle phenotyping. Both traditional biochemical analysis (e.g. SDS-PAGE) and advanced proteome techniques (e.g. multiple reaction monitoring) were investigated. We report SDS-PAGE can be used to analyse MyHC isoforms but this technique does not offer sufficient resolution of low molecular-weight myofibrillar proteins. Indeed, MS analysis and 2DGE separation each showed that the majority of SDS-PAGE protein bands contain numerous myofibrillar proteins. Simple LC-MS profiling was able to identify almost all expected myofibrillar proteins and detect some significant differences between samples from young and elderly, trained and untrained individuals that supported the differences in muscle MyHC fibre profile. Nonetheless, relatively few of the identified proteins could be quantified. On balance, we find 'bottom-up' analysis is best suited to mining of the myofibrillar proteome, whereas 'top-down' analysis using 2DGE is better suited to differential profiling of the myofibrillar proteome.

The proteomic techniques development for automated analysis of myofibrillar proteins enhanced the level of sophistication of human myofibre phenotyping. To date attempts to study the myofibrillar sub-proteome in human muscle have been largely incomplete, therefore study of changes in myofibrillar proteins beyond MyHC are needed to more accurately understand the mechanisms that underpin the f-v characteristics of human muscle that will be discussed in next chapter.

Chapter 2

Measurement of the Force-Velocity Profile of Human Skeletal Muscle

Chart to contextualise Chapter 2

In this chapter, the study progression is quite complex so Figure 12 is included to guide the reader through this multi-component chapter.

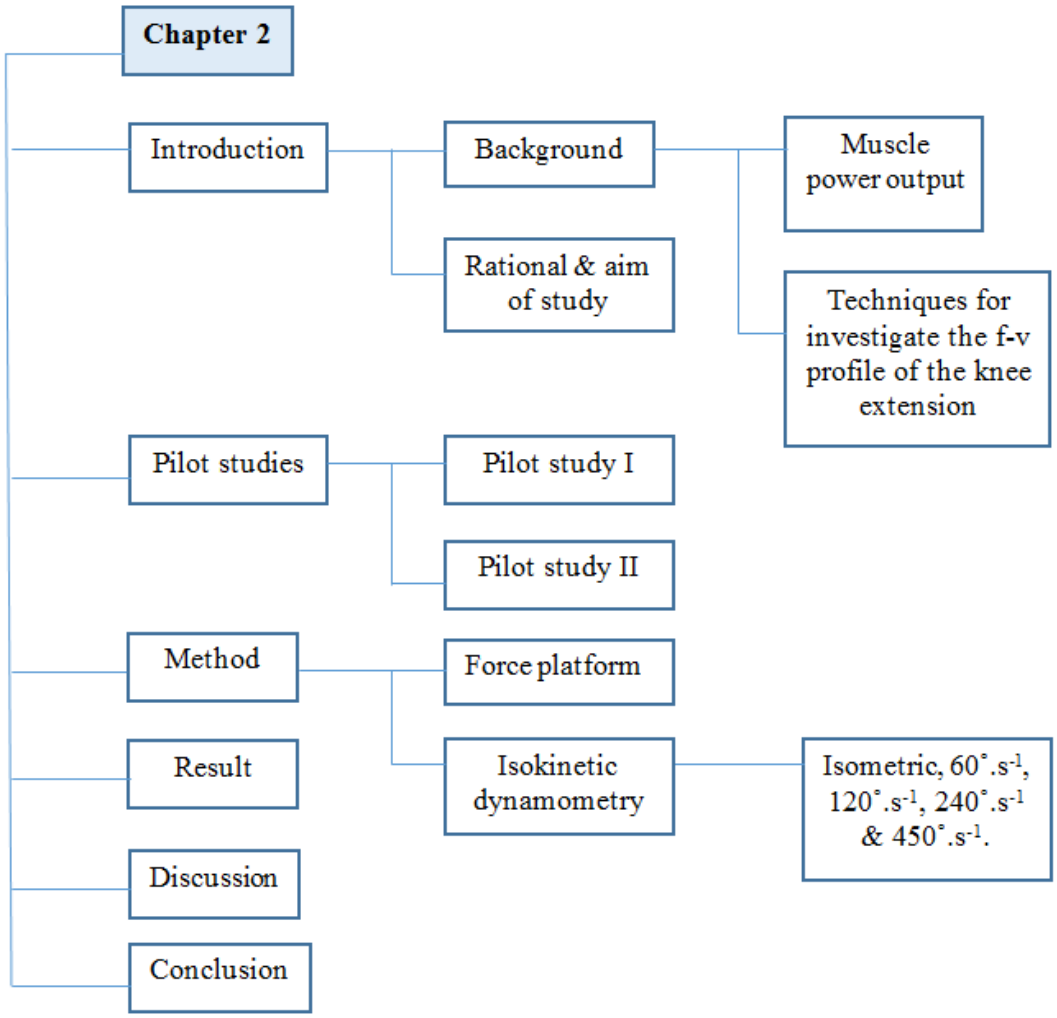


Figure 12. Chart to contextualise the chapter and help to see more clear progression and development of Chapter 2.

2.1 INTRODUCTION

Chapter background

Muscle power output

Muscle power output is a key determinate of success in many sports, in particular those involving jumping, sprinting and rapid changes in direction. The power output of a muscle is a function of its ability to produce force while shortening rapidly (power being the product of force*velocity). Many sports require forceful high-velocity movements but the force-velocity relationship of skeletal muscle (Figure 13) dictates that the force produced by a muscle decreases as the velocity of the contraction increases. The force-velocity relationship was first described by Hill (1938) and Katz (1939) and later summarized by Hill in 1970 as a rectangular hyperbolic equation.

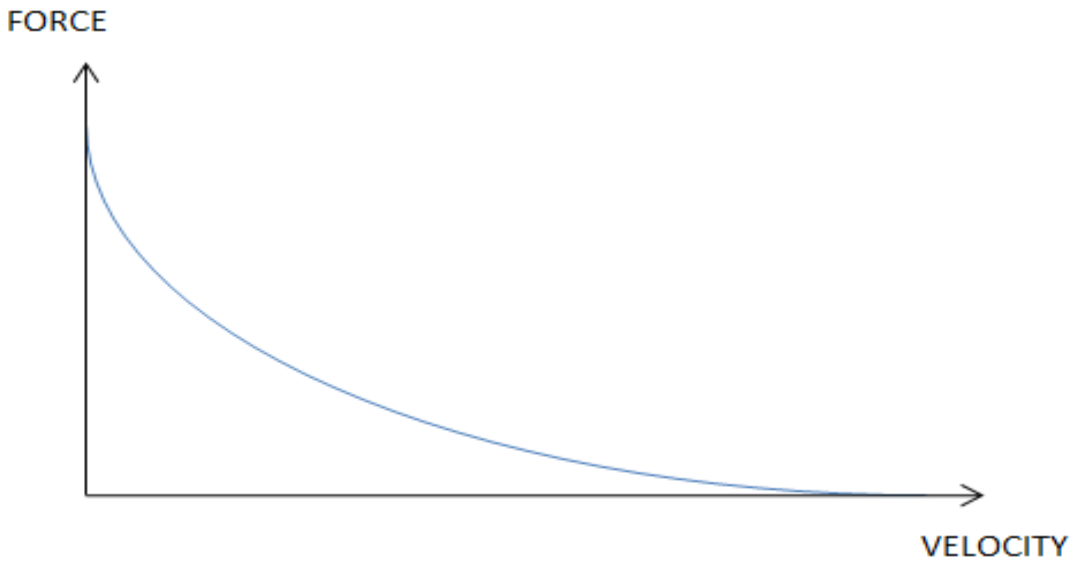


Figure 13. An illustration of the force-velocity relationship of skeletal muscle.

Skeletal muscles are velocity- and length-dependent force producers. Because of this velocity- and length-dependence, movement necessarily alters the force produced by a muscle. Hakkinen et al. (1985) state that only the motor units that are recruited in a specific exercise will produce force and will therefore respond and adapt to a resistance stimulus. However, the force-velocity characteristic of skeletal muscle is actually a fundamental property of the cross bridge cycle rather than motor unit recruitment (Duchateau et al., 2006). Specifically, the force-velocity relationship is underpinned by the cross bridge cycle and the number of myosin heads that are able to contribute to force output during a contraction. Therefore, the profile of myosin heavy chain isoforms has a major influence of force-velocity curve. These aspects of muscle physiology have been studied in animal models to understand the relationship between skeletal muscle design and performance. However, measuring muscle performance in humans *in vivo* is more difficult because of voluntary activation and the effects of synergist and antagonist muscle groups. Nonetheless, it is important to try to measure human muscle outputs because animal models cannot be used to study complex sport-related movements.

Single fibre measurements have been used to investigate the effects of sport-related movements or sports training in humans. Analysis of the force-velocity profile of single muscle fibres in vitro reveals plyometric jump training increases the maximal shortening velocity of myofibres to a greater extent than the increases in cross sectional area and peak isometric force (Malisoux et al., 2006). This may be due to changes in the calcium sensitivity of human single muscle fibres following plyometric training as Malisoux et al., (2006) reported increased calcium sensitivity especially in type I fibres. This is suggests that velocity profile of a particular muscle

fibre type can be changed without altering its MyHC content. These measurements performed on skinned fibres in the absence of neural input provide clear evidence that jump training is able to alter the fundamental properties of skeletal muscle and affect the curvature of the force-velocity relationship. However, traditional biochemical analysis was not able to define the exact protein modification that was responsible for this effect.

We will attempt to explain differences in human muscle performance *in vivo* by proteomic analysis. Proteomics will provide more comprehensive analysis can be conducted in an 'unbiased' non-targeted manner to discover new information. However, relatively large amounts of muscle are required to develop this method so whole samples of muscle needle biopsies (i.e. ~50 mg) of muscle are required, rather than single fibres. Therefore, we were keen to find the most robust or precise measurements of isolated limb movements so that changes in muscle performance could be correlated with the muscle proteome. Moreover, we sought to find a selection of techniques that were capable of analysing the entire force-velocity range of muscle. This is particularly important because changes in maximum force are likely to be explained by myofibre hypertrophy whereas changes in the make-up of the muscle myofibrillar proteome are likely to affect the native of the force-velocity curve and perhaps modulate velocity to a greater extent than force. Lastly, we were interested in relating our findings to sporting movements so we chose to use isolated knee extension as our model of muscle function.

Biopsy samples can be taken from the quadriceps muscles responsible for knee extension and we also hypothesised that the force output of the knee extensors could be correlated with power output during a squat jump, which is a sport-related movement. Analysis of squat jumps has been used to detect significant differences in force-velocity and power-velocity profiles either across different training backgrounds (e.g. Cormie et al., 2009 ; Cormie et al., 2010) or responses to different training interventions (Cormie et al., 2010a). Moreover, squat jump power-output is commonly used as a screening tool or performance indicator in athletic populations. Previous work by Iossifidou et al., (2005) has reported a correlation of power generation between knee extension IKD and the squat vertical jump performance. Iossifidou et al., (2005) measured knee joint power generation during a concentric knee extension isokinetic test and a squat vertical jump and they found a stronger correlation at the highest velocities ($300^{\circ}.s^{-1}$). However, there is a large difference in knee angular velocity and peak power between squat vertical jump which typically > $450^{\circ}.s^{-1}$ and knee extension at $300^{\circ}.s^{-1}$ IKD. For example, significant differences were found between knee joint power in the two tests performed by Iossifidou et al., (2005) to trained basketball player and track and field athletes (squat vertical jump: 2255 ± 434 W; isokinetic knee extension: 771 ± 81 W).

Several studies (Aragon & Gross, 1997 ; Bobbert et al., 1986) investigating joint kinematics during jumping state that knee angular velocity during vertical jump is approximately 15 $rad.s^{-1}$, which is equal to $860^{\circ}.s^{-1}$. However, in interpreting the relationships, the squat vertical jump involves both legs and is also influenced by the action of the bi-articular muscles and there is a combined transfer of energy between joints. So the velocity during isolated knee extension is likely to be less.

Techniques for investigating the force-velocity profile of the knee extensors

There are various techniques that can be implement to test the muscles capability to produce force with difference speed of contraction. Isokinetic testing can be used to investigate power output and also differences in the force-velocity profile of muscle groups. Isokinetic dynamometers control the velocity of joint flexion or extension by applying variable resistance throughout the range of motion. Therefore the velocity of joint rotation is constant, excluding acceleration to and deceleration from the designated velocity and the force is dependent on how hard the individual pushes against the load cell (Huang et al., 2003). IKD can be used at low, moderate and high velocities depending on the different evaluations or programs and provides reliable data. Data acquisition and analysis have been improved by using computer systems interfaced to isokinetic dynamometers. Recently developed computer systems provide correction for gravitational and inertial errors, accurate computation of isokinetic parameters and real-time display of the torque output.

Kanehisa & Fukunaga (1999) reported isokinetic muscle contractions at velocities of 60, 180 and 300 deg/s and investigated difference in force output of weight lifters compared to untrained participants. This study was designed to determine velocity-specific isokinetic forces and cross sectional areas of reciprocal muscle groups measured by B-mode ultrasonic apparatus in weight lifters (n = 34) and untrained male participants (n = 31). Force declined at higher velocity movements (as expected from muscle force-velocity curve) in both sedentary individuals and Olympic weight lifters. However, this effect was less pronounced in Olympic weight lifters and this trend may be due to adaptations induced by their training regimen, which places

emphasis on high velocity movements. These findings suggest isokinetic dynamometry could be used to study the force velocity profile of human muscle groups in vivo but first we wanted to confirm that a commercial isokinetic dynamometer was able to measure the entire range of velocities produced during knee extension.

2.2 Pilot Studies

Pilot study I

The aim of this pilot work was to establish a simple technique for measuring peak angular velocity of knee extension during unloaded kicking and to determine the maximum angular velocity of the knee during unloaded. An appropriately positioned linear encoder (Figure 14) may be used to calculate knee angular velocity during unloaded kicking. Therefore we investigated the correlation between linear encoder and kinematic measurement of angular velocity using a camera system during unloaded kicking. If successful, this technique could provide a simple measurement of maximum velocity.

One healthy male participant volunteered to participate in the study and performed 10 unloaded kicks (knee extension) at different velocities in order to determine maximum angular velocity at knee extension. Angular velocity, acceleration, moment of force, and power development during maximal unloaded knee extension were determined using ProReflex Motion Capture System (Qualisys, Sweden) (Figure 14). Ten maximal unloaded knee extensions were performed at varying

velocities separated by rest periods of 30 s. Trials with a visible countermovement were discarded. A cuff was strapped around the lower leg, approximately 2 cm above the medial malleoli and connected to a linear velocity encoder (MuscleLab Ergotest version 4010, Norway). Average velocity (AV) was recorded during each kick. The lower leg is allowed to move freely. The participant was instructed to extend their right knee "as fast as possible" into a kicking pad (Figure 14). The movement range was from 90° knee flexion, which is the relaxed starting position, to ~5–10° from full extension. During the test sessions, three-dimensional motion analysis was performed with an Oqus Motion Capture System (Qualisys, Sweden). Twelve markers were used in total for dynamic trials (Iliac crest, knee medial epicondyle, knee lateral epicondyle, upper leg proximal anterior and posterior, upper leg distal anterior and posterior, lower leg proximal anterior and posterior, lower leg distal anterior and posterior, maleolus lateral) and attached on the right side. Data were transferred to Windows-based data acquisition software (Qualisys Track Manager). This system includes an advanced optoelectronic camera system that produces clean and accurate 3D data and also recording velocity. All modelling and analysis was undertaken in visual D (v.4.83, C-Motion, Germantown, MD, USA) using geometric volumes to represent segments (Hanavan, 1964) based on cadaver segmental data (Dempster, 1955).

Data from the linear encoder (MuscleLab) were record as meter per second linear velocity, therefore, to compare between the camera systems, which give angular velocity, the MuscleLab data must be converted into angular velocity (Table 15). Angular velocity is equal to theta (degree/one radian=57.2958) multiplied by

distance (length in metres) from participant's knee to the attachment of the linear encoder at their ankle.

A B

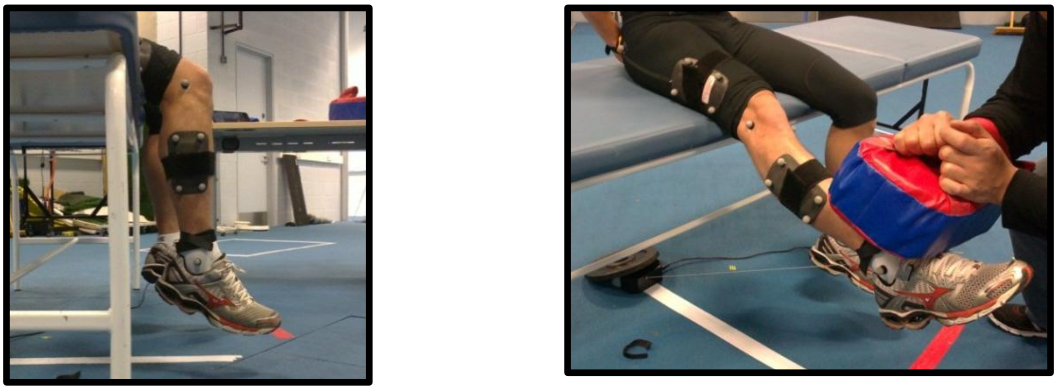


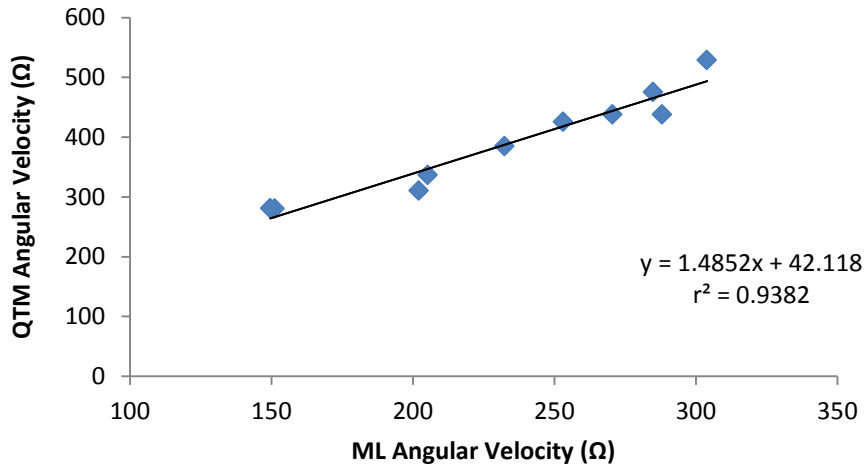
Figure 14. Apparatus for measuring maximum velocity of unloaded knee extension (A) Leg in rest position and (B) Leg extended to kicking pad.

Table 15. Conversion of MuscleLab data to angular velocity.

Velocity (m/s)	(/)ℓ In meter	ω (sec x 57.2958)	Peak angular velocity (degree/sec)
1.46	0.36	4.06	232.4
1.27	0.36	3.53	202.1
0.95	0.36	2.64	151.2
0.94	0.36	2.61	149.6
1.29	0.36	3.58	205.3
1.59	0.36	4.42	253.1
1.79	0.36	4.97	284.9
1.81	0.36	5.03	288.1
1.70	0.36	4.72	270.6
1.91	0.36	5.31	304.1

Equating angular velocity, v=ωxℓ. Equating velocity on linear speed, v=m/s where v=velocity, m=meter, s=second, ω=angular speed, ℓ=length (participant's leg) Each one of the degree represent one kick or attempt.

The correlation between the velocity of unloaded kicks measured by the Qualisys Track Manager and calculated from the linear encoder is shown in Figure 15. Correlation coefficients measure the strength of association between two variables, and we found a strong positive relationship 0.9382 between the two measurement techniques.



ML	232.4	202.1	151.2	149.6	205.3	253.1	284.9	288.1	270.6	304.1
QTM	384.6	310.3	280.2	280.9	336.4	425.4	475.5	438.1	438.1	528.9

Figure 15. Correlation between ML and QTM.
Angular velocity is reported in degrees per second (Ω) measured by the Qualisys Track Manager (QTM) camera system or calculated from the MuscleLab (ML) linear encoder.

We conclude that measurement of linear displacement using MuscleLab can be used to measure angular velocity at knee during maximum unloaded kicking. However, the average maximum angular velocity of knee extension measured using the QTM system was 362°.s^{-1}, and the highest value was 528°.s^{-1}. This contrasts with Andersen

et al., (2005) that reports changes in the human muscle force velocity relationship during training and detraining, and found peak unloaded kicking angular velocity after detraining was ~800˚.s^{-1} measured by flexible goniometer. This maybe due to differences in the methods or participants of the two studies. We recruited a recreationally active person, whereas Andersen et al., (2005) studied trained athletes. Nonetheless, this range of velocities is close to the range (0-500 ˚.s^{-1}) that can be measured by the Biodex 3 isokinetic dynamometer (Multi-Joint System 3 Pro; Biodex Medical Systems, New York, USA). Therefore a further study was conducted to investigate whether IKD was capable of measuring the entire range of velocities in an untrained population.

Pilot study II

Five healthy sedentary male volunteered to participate in the high velocity isokinetic dynamometry study. The mean (± SD) age, height, body mass were 25 ± 2.5 years, 179.4 ± 8.6 cm and 79.4 ± 8.8 kg, respectively. Consistent with Drouin et.al (2004) the biodex was set to 500˚.s^{-1} and knee extension and flexion performance was measured according to standard protocols using an isokinetic dynamometer. Strict adherence to the guidelines of the Biodex isokinetic dynamometer operations manual was followed. Participants were familiarized with use of the dynamometer and testing procedures prior to the measurements. Each participant completed a warm-up protocol prior to muscular performance. The warm-up session included 5 minutes cycling at 150 W on a cycle ergometer (Monark Ergomedic 824E) and a standardised routine of quadriceps and hamstrings stretching exercises. Then 3 to 5 specific warm-up trials (50 % effort) were conducted on the isokinetic dynamometer

prior to completing 5 maximal velocity attempts. We found none of the participants were capable at achieving the maximum $500^{\circ}.s^{-1}$ angular velocity. Because the Biodex software only measures power when the prescribed velocity is reached, force and power data are not reported for these experiments. We conclude that the maximum pre-selected angular velocity for future work should be $450^{\circ}.s^{-1}$ because participants can achieve that speed and power data will be recorded.

In summary, our pilot findings suggest isokinetic dynamometry can be used to investigate the entire range of velocities (i.e. from isometric to velocity maximum) of knee extension in normal human participants. Therefore it is not necessary use a separate analysis technique (unloaded kicking). However, it was still necessary to prove that IKD is a reliable measurement tool across this range of velocities because few other studies report IKD at velocities > $300^{\circ}.s^{-1}$. Also it was still unknown whether there is a correlation between IKD knee extension and Max power (or similar) during vertical jumping, i.e. external validity.

Therefore, the information that we gathered from pilot studies will be applied in the main study of this chapter (i) to investigate the feasibility of using isolated knee performance to measure the force-velocity profile of human muscle in vivo and (ii) to test the reliability by using standard isometric and IKD in analyses of isolated knee exercise.

Rational of this study

Previously, analysis of squat jumps has been used to detect significant differences in force-velocity and power-velocity profiles either across different training backgrounds (e.g. Cormie et al, 2009 ; Cormie et al, 2010) or responses to different training interventions (Cormie et al., 2010a). The squat jump technique is used extensively to assess the performance of athletes but it encompasses simultaneous extension of ankle, knee and hip joints and delineating links between this complex movement and the myofibrillar proteome of a specific muscle would be challenging. To overcome this difficulty we will investigate the feasibility of using isolated knee performance to measure the force-velocity profile of human muscle in vivo.

Aim of this study

We will conduct force-velocity and power-velocity analyses of isolated knee exercise using standard isometric and IKD and test the reliability at these measurements. If these are found to be reliable we will use this protocol as the main physiological assessment in our subsequent studies. A further aim of this study was to investigate the reliability of jump analysis by force platform and investigate whether isolated knee extension data can be correlated with indices of jump performance.

2.3 METHODS

Measurement on force platform and isokinetic dynamometry

2.3.1 Participants

Eleven male participants with different sporting backgrounds (e.g sprinter, rugby player, footballer etc.) participated in this study, which was approved by LJMU ethics committee reference number 12/SPS/013. The mean (± SD) age, height, body mass were 24.9 ± 3.1 years, 176.2 ± 7.1 cm and 80.2 ± 10.1 kg, respectively. Participants were questioned on their training history and the Physical Readiness Questionnaire (PAR-Q; Thomas et al., 1992) was used as a screening tool to assess each volunteer's level of physical fitness and ability to engage in physical activity. Each participant was required to perform 4 sessions (1 introductory session and 3 repeated experiment sessions). Briefly, the sessions consisted of squat jumps on a force platform and then isokinetic dynamometry of knee extension movement at 5 different velocities. All measurements were performed at the same time of day for each participant to limit diurnal variation (Reilly and Waterhouse, 2009).

2.3.2 Force platform

Jump performance was assessed in order to investigate whether a correlation exists between isolated knee performance measured by IKD and whole body jump performance. Performance during jump squat was measured by force platform and linear encoder (Muscle Lab, version 4010, Norway). Objective of this testing was to investigate the correlation of average velocity data collected by linear encoder and force platform. Each participant performed an extensive dynamic warm-up (consisting of squat, lunge and jump squats), which was standardised and repeated

before each session. After completing the warm-up, each participant performed a countermovement jump for three repetitions. Participants held a 0.4-kg rigid plastic pole across their shoulders. The linear encoder was attached at the pole via a fine string. The linear encoder was placed directly under the axis of travel.

During each jump, participants were instructed to step on the force platform and stand still while the force platform offset was zeroed. They were then instructed to jump as high as possible, using a single countermovement jump. The basic description process was STEP ON, JUMP and then STEP OFF. Only trials in which participant reached a relative knee angle (i.e., angle between the midline of the lower leg and the midline of the thigh) <90° of flexion were considered successful. Participants were reminded to squat as low as they can during the jumping phase.

Acknowledge software (FPL-960.acq file) was used to display the force data from analogue signals collected from the floor-mounted force platform (Kistler, UK). MuscleLab software (Ergotest, Norway) of the linear encoder made simultaneous measurements during each jump. To provide a comparison between force platform data and the linear encoder output data were extracted from force platform analysis and used to calculate average power force and velocity. Force and time data were extracted from the point of transition between counter movement and jump.

2.3.3 Isokinetic dynamometry

IKD was performed at different velocities to investigate the reliability of data across f-v curve. Knee extension and flexion performance was measured according to standard protocols using an isokinetic dynamometer (Multi-Joint System 3 Pro; Biodex Medical Systems, New York, USA). Strict adherence to the guidelines of the Biodex isokinetic dynamometer operations manual was followed. Participants were familiarized with use of the dynamometer and testing procedures prior to the baseline measurements to reduce possible influences of test habituation on muscular performance. Each participant completed a warm-up protocol prior to analysis of their muscular performance. The warm-up session include 5 minutes cycling on a cycle ergometer (Monark Ergomedic 824E) set with 150 W resistance and a standardise routine of quadriceps and hamstrings stretching exercises. After a 5 minutes rest on completion of the warm-up, the participants were instructed to continue with the testing procedure.

The participant was seated; leaning against a backrest inclined at 16˚ from vertical and with the seat inclined 6˚ from horizontal. The axis of the knee was aligned with the axis of the Biodex dynamometer exercise arm. The accuracy of alignment was checked by allowing the participant to extend the leg while pushing against the shin pad that was positioned over the lower third of the leg. If the pad did not move up or down the leg over the range of motion to be tested, the knee was considered to be aligned with the axis of the lever arm.

The participant's position was secured using thigh, pelvic and torso straps in order to minimize body movements during the test procedure. Shoulder straps were applied diagonally across the chest with the participant's arms crossed and their palms on opposite shoulders to minimize excessive upper body movement and muscular substitution. The lateral femoral epicondyle was used as a bony landmark for matching the axis rotation of the knee joint and the axis rotation of the dynamometer shaft.

Each participant used the same radius for each test but the angular velocities were tested in a different order for each session using balanced Latin Square design. Test angular velocities were set at $60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, $240^{\circ}.s^{-1}$ and $450^{\circ}.s^{-1}$. Participants were asked to do 3 repetitions of extension and flexion for each angular velocity. One minute rest was given between each angular velocity. Peak torque (PT) and average power (AV) were recorded and exported as an excel file for further analysis through the report after the testing.

After completing the isokinetic test, participants were asked to remain at the same position and rest for 5 minutes. Meanwhile the protocol was changed to isometric mode. The participants performed isometric contractions at a static knee joint angle at 60°. They were instructed to extend their knee "as fast and hard as possible" and sustain the maximal voluntary isometric contraction for two seconds. The participants were asked to do 3 repetitions with 60 seconds rest between each attempt.

2.3.8 Data analysis

The data were analysed using Statistical Package for Social Sciences (SPSS) version 17.0 software (SPAA v.17, SPSS Inc, Chicago). To assess reliability across the three testing, one way ANOVA analysis repeated measures was used to analyse the main effects of time. Bonferroni test for multiple comparisons was used to determine the differences when one way ANOVA repeated measure showed a significant effect of time. Significance level was set at <0.05, and all data were presented as mean + SD. Furthermore we look at the reproducibility. Typically reproducibility is expressed as a coefficient variation (CV) relative to measurement value. There are various methods of calculating CV, the simplest method is with data from repeated measurements on a single case, where the standard deviation (SD) of the error is divided by the total mean and multiplied by 100 (Sale, 1991).

2.4 RESULTS

Force platform and isokinetic dynamometry

Eleven participants completed each of the 3 repeated trials according to the protocol design and were included the analysis. The average jump height across the 3 trials was 0.334 ± 0.07 m and the peak ground reaction force and power output were 1739.82 ± 212.86N and 4.54 ± 0.90W/kg respectively. Average velocity measured by MuscleLab was 1.73 ± 0.14 m/s, data were also extracted from force platform and average velocity was calculated during the jump phase D – F in figure 16.

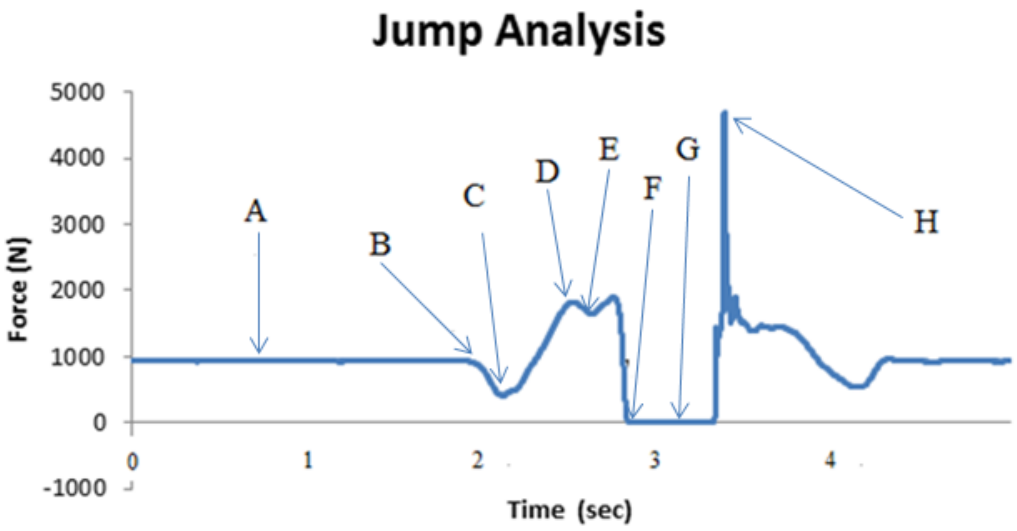


Figure 16. Typical output from force platform jump analysis.
Force (N) was recorded during CMJ using AcqKnowledge 3.9 software. A. Standing phase. B. Initial momentum phase. C. Start of amortization. D. Peak plyometric force. E. Final momentum phase. F. Take off. G. Flight phase. H. Peak plyometric force upon landing.

This phase relates to the dynamic concentric phase of the jumping movement. When calculated from force plot data the average take off velocity was more (2.442 ± 0.312 m/s) than that recorded by MuscleLab system. Moreover there was no correlation (R^2 = 0.3677) between the data recorded using the force platform and linear encoder (MuscleLab system). This demonstrates that ground reaction force is a poor indicator of vertical acceleration measured at the shoulder (i.e. bar across shoulder).

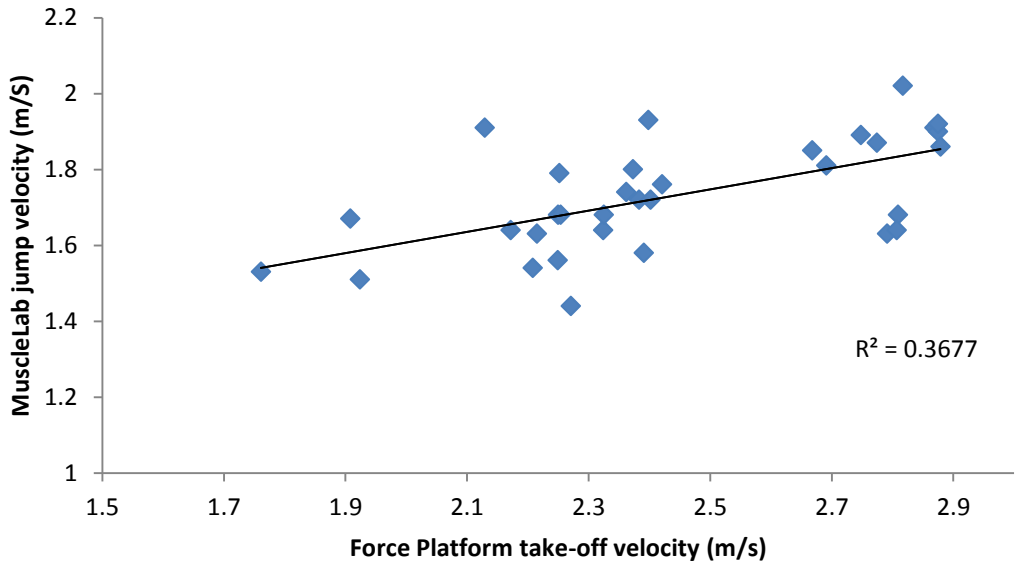


Figure 17. Correlation of jump velocity measured by MuscleLab and take-off velocity measured by Force platform.
Correlation of jump velocity and take-off jump velocity (m/s) measured by two different methods. The correlation coefficient at r^2 = 0.3677.

Isokinetic dynamometry devices control the velocity of limb movement by applying variable resistance throughout the range of motion. Thus, excluding acceleration and deceleration phase outside of when the velocity of joint rotation is constant and the force (N.m) measured is dependent on the maximum strength of the individual at that velocity of movement. As such IKD applied over a series of velocities is able to provide information relating to the f-v profile of the major muscles contributing to the movement. Moreover, because force is recorded at a known velocity of movement muscle power output can also be derived from IKD data. Figure 18 shows an example trace of IKD data during knee extension-flexion movements.

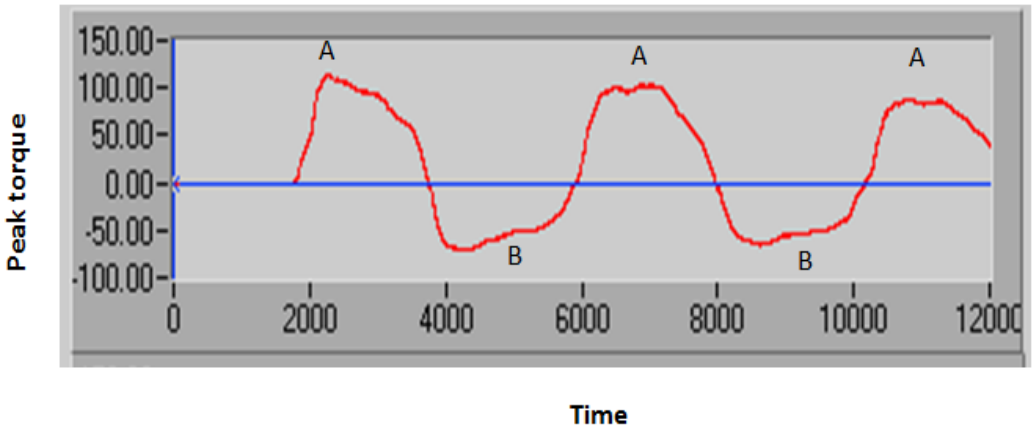


Figure 18. Example torque output during knee extension-flexion movements. Torque (N.m) was recorded during 3 isokinetic knee extension (A) and flexion (B) movement at angular velocity of 60˚.s^{-1}.

Peak torque during knee extension at velocities of 0˚.s^{-1}, 60˚.s^{-1}, 120˚.s^{-1}, 240˚.s^{-1} and 450˚.s^{-1} is shown in Figure 19. Consistent with force-velocity characteristics of skeletal muscle, peak torque was greatest during isometric contraction and declined as the velocity of contraction increased.

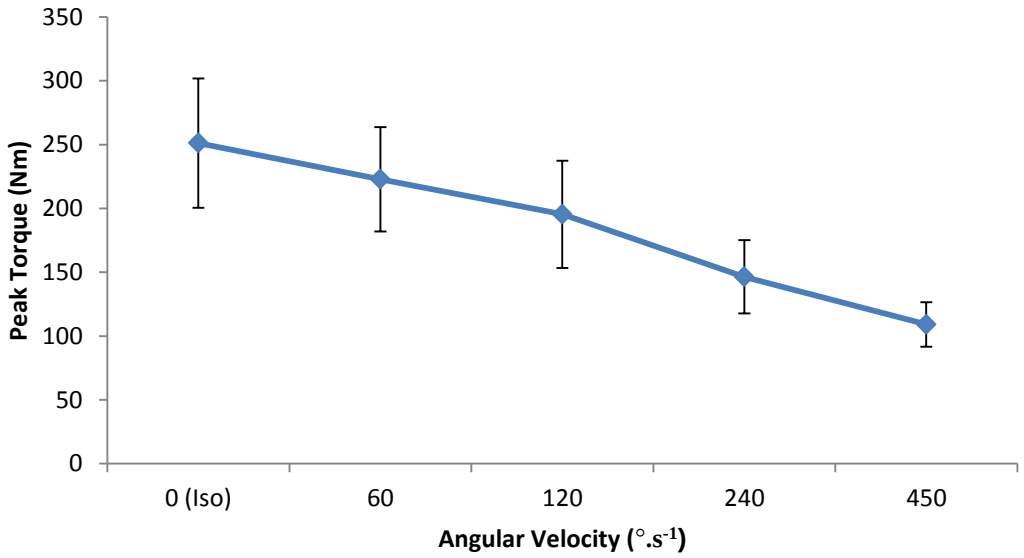


Figure 19. Knee extension torque-velocity relationship measured using isokinetic dynamometry.
Peak torque data are presented as mean ± SD across 3 repeated trials (*n* = 11, in each trial). Peak torque (Nm) was recorded during isokinetic knee extension at angular velocities of 0 (isometric), 60˚.s^{-1}, 120˚.s^{-1}, 240˚.s^{-1} and 450˚.s^{-1}.

Figure 20 reports average power output measured during 3 repeated trials of the 11 participants. Average power at 60˚.s^{-1} was 149.87 ± 32.44 W whereas the greatest power output (357.03 ± 83.72W) was recorded during knee extension at 450˚.s^{-1}. Therefore peak power output occurs with low load and high movement velocity.

Power output at 60˚.s^{-1} was 42 % of power output, developed at 450˚.s^{-1} and was significantly (p<0.05) different from all other velocities investigated. Power output 120˚.s^{-1} was also significant difference from 240˚.s^{-1} and 450˚.s^{-1}. However there was no significant difference between 240˚.s^{-1} and 450˚.s^{-1}(p=0.11).

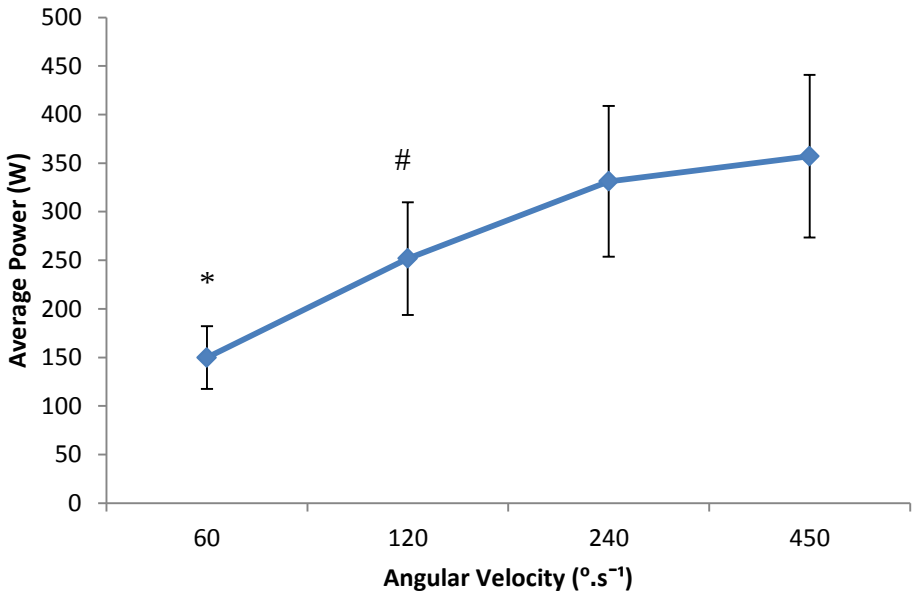


Figure 20. Knee extension power-velocity relationship measured using isokinetic dynamometry.
Average power data are presented as mean ± SD across 3 repeated trials (n = 11, in each trial). Average power (W) was recorded during isokinetic knee extension at angular velocities of 60˚.s^{-1}, 120˚.s^{-1}, 240˚.s^{-1} and 450˚.s^{-1}.
* p<0.05 significant different from 120, 240 and 450˚.s^{-1}.
# p<0.05 significant different from 240 and 450˚.s^{-1}.

To investigate this further angular acceleration during knee extension at 450˚.s^{-1} was interrogated. Participants were segregated into two groups based on whether or not they achieved peak torque within 0.1 sec of starting the movement. Figure 21 shows the mean angular acceleration for the 2 groups. Group 1 includes individuals that did not achieve peak torque within the first 0.1 sec of the movement and were characterized by a gradual increase in acceleration from 28.12 ± 10.45 rad/s² (Group 1). In contrast, individuals in group 2 were able to achieve peak torque within the first 0.1s and exhibited an average acceleration of 76.31 ± 33.56 rad/s².

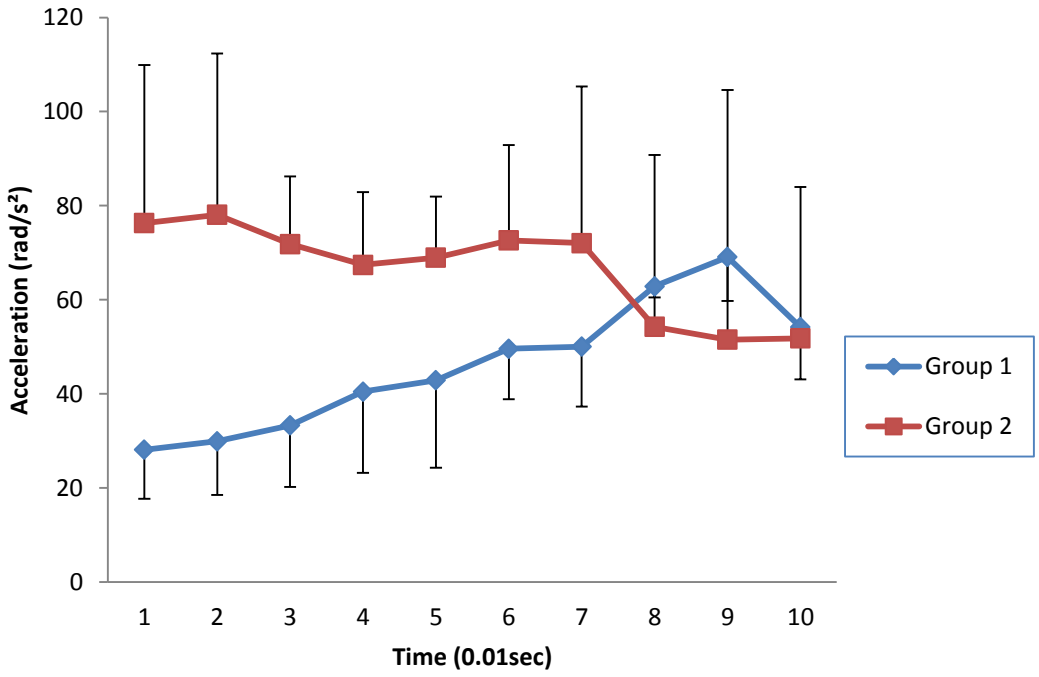


Figure 21. Knee extension angular acceleration measured using isokinetic dynamometry.
Angular acceleration data are presented as mean ± SD across 3 repeated trials with group 1 (*n* = 5, in each trial) and group 2 (n = 6, in each trial). Angular acceleration (rad/s²) was recorded during intervals 0.1 sec isokinetic knee extension at angular velocity of 450˚.s^{-1} ranging from 0-1 sec.

To predict variable that correlate most to sport movement like jumping, we used backwards stepwise regression. Backwards stepwise regression procedures work to deduct from a group the one variable at each stage which makes the smallest contribution to R^2. The dependent variable is regressed on all independent variables. If any variables are statistically insignificant, the one making the smallest contribution is dropped. Then the remaining variables are regressed on Y, and again the one making the smallest contribution is dropped. The procedure continues until all remaining variables are statistically significant.

Table 16. Regression analysis for maximum power (jump analysis).

IKD velocities	1	2	3	4
60	0.473	0.439	0.406	
120	0.927			
240	0.858	0.823		
450	0.004*	0.002*	0.000*	0.000*
Isometric	0.026*	0.011*	0.004*	0.001*
Adjusted R^2	0.763	0.763	0.762	0.756

Isometric and highest velocities of movement correlated significant with jumping power. *P<0.01

Test re-test reliability of jump analysis and IKD measurements was assessed using repeated measures ANOVA and the coefficient of variation (CV) was calculated for major parameters. Jump and IKD data (Table 17) were not significantly different across the 3 repeated trials, demonstrating there was no evidence of systematic bias. Moreover, post-hoc analysis comparing trials 1-2, 1-3 and 2-3 did not reveal significant random effects. The average CV of jump parameters was 3.87 ± 1.9% ranging from 1.5 to 5.1%. The CV of IKD measures was relatively greater. The average CV was 9.38 ± 3.0 % ranging from 7.0 to 15.7 %.

Table 17. Reliability of muscle performance test.

	Mean	ANOVA (p) value	% Coefficient of Variation (CV)
Force Platform			
Height Flight (m)	0.334 ± 0.07	0.300	0.0
Average Force (N)	1001 ± 163.34	0.487	5.1
Max Force (N)	1739.82 ± 212.86	0.633	4.6
Normalised Force (N/kg)	2.18 ± 0.22	0.302	4.8
Max Power (W)	3649.27 ± 863.44	0.526	5.0
Normalised Power (W/kg)	4.54 ± 0.90	0.319	5.7
MuscleLab			
Average Power(W)	1755.55 ± 201.95	0.241	4.4
Average Force(N)	1014.11 ± 85.9	0.187	1.5
Average Velocity(m/s)	1.73 ± 0.14	0.231	3.7
IKD Peak Torque(Nm)			
Isometric	251.22 ± 50.59	0.273	7.9
60	222.95 ± 40.91	0.181	9.2
120	195.43 ± 41.95	0.322	7.0
240	146.39 ± 28.68	0.087	8.4
450	109.17 ± 17.46	0.213	6.4
IKD Average Power(W)			
60	149.87 ± 32.44	0.337	12.7
120	251.74 ± 58.02	0.308	7.2
240	331.22 ± 77.55	0.240	15.7
450	357.03 ± 83.72	0.269	9.9

Data are presented as means ± SD; *n* = 11 participants averaged from 3 separate test sessions. Jump squat was performed on force platform and recorded using linear encoder (MuscleLab). Force and power were normalised by dividing participant body weight (kg). Isokinetic dynamometry (IKD) was performed at 5 different velocities: 0 (isometric), 60, 120, 240 and 450˚.s^{-1}. Data presented are peak torque (Nm) and average power (W) over 3 kicks during knee extension.

2.5 DISCUSSION

The overall aim of this study was to investigate the reliability of data produced by IKD of knee extension and flexion movement and correlation with jump analysis.

To assess the reliability of force platform and IKD data, ANOVA with repeated measures including a correction for 'sphericity' (Vincent, 1994) was used for assessing systematic bias across repeated tests (Vincent, 1994; Thomas & Nelson, 1990). Using appropriate post hoc multiple comparisons, random bias between individual tests was also assessed. To investigate the correlation of jump and IKD methods/protocol, participants performed squat jumps on force platform and then the isokinetic dynamometry testing at 5 different velocities at $0^{\circ}.s^{-1}$, $60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, $240^{\circ}.s^{-1}$ and $450^{\circ}.s^{-1}$. In the present study, after 3 repeated sessions of the squat jump and isokinetic dynamometry testing, one way ANOVA for repeated measures revealed that there was no significant difference across the 3 sessions for any of the performance variables that were recorded.

Typically reproducibility is expressed as a coefficient variation (CV) relative to measurement value. There are various methods of calculating CV, the simplest method is with data from repeated measurements on a single case, where the standard deviation (SD) of the error is divided by the total mean and multiplied by 100 (Sale, 1991). The use of CV assumes that the largest test-retest variation occurs in the individuals scoring the highest values on the test (Bland & Altman, 1995). Some scientists have arbitrarily chosen an analytical goal of the CV being 10% or

less (Stoke, 1985). This does not mean that all variability between tests is always less than 10% of the mean. In the current work, percentage of CV was below 10% except on $60^{\circ}.s^{-1}$ (12.7%) and $240^{\circ}.s^{-1}$ (15.7%) isokinetic knee extension average power (W). Cormack et al (2008) reported in their study on reliability of measures obtained during countermovement jumps that all CV were below 10%. In most parameters in this study showed no significant different in CV% with current study, for instance peak power (W), relative peak power (W/kg), peak force (N), relative peak force (N/kg), mean force (N) and height (cm). In height parameter the CV% was 5% compared to current study was 0.002%.

Force-velocity relationship

The force generated by a muscle is a function of its velocity of shortening. The force-velocity relationship has been used to define the dynamic properties of the cross-bridges which cycle during muscle contraction. Muscles are strengthened based on the force placed across the muscle. Higher forces during training produce greater adaptation in strength. Therefore, exercise performed with muscle activated in a way that allows them to contract at high velocities, necessarily imply that they are also contracting with relatively low force. Metaxas et al., (2009) reports the physiological profile of soccer and basketball players over a range of peak torques based on different velocities using IKD. In soccer division IV players for $60^{\circ}.s^{-1}$ testing, the peak torque was 256.1±36.1 Nm and basketball division IV players was 284.9±51.6 Nm. In $180^{\circ}.s^{-1}$ testing for soccer the peak torque was 175.5±25.3 Nm and basketball was 194.5±33.2 Nm. Meanwhile for velocity $300^{\circ}.s^{-1}$, soccer player peak torque was 123.1±17.5 Nm and basketball 151.5±25.6 Nm. The quite similar

peak torque range also detected in professional soccer player in Fousekis et al., (2010) study.

Another study made by Alangari & Al-Hazzaa (2004) reports the reference data for peak isokinetic muscle strength of hamstring and quadriceps muscles in sedentary young adult Saudi males. The peak torque at extension $60^{\circ}.s^{-1}$ was 172.2±32.9 Nm. In $180^{\circ}.s^{-1}$ testing, the peak torque was 118.2±20.7 Nm and for $300^{\circ}.s^{-1}$ testing the peak torque was 84.5±16.8 Nm. Based on those data, the peak torque data in this study is within the expected range for recreational athletes. The result of present study indicated that mean knee extension peak torque at $0^{\circ}.s^{-1}$, $60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, $240^{\circ}.s^{-1}$ and $450^{\circ}.s^{-1}$ of the participans during three different test sessions was similar with the force-velocity relationship concept. The peak torque at isometric contraction was 251.2±49.9 Nm and at extension $60^{\circ}.s^{-1}$ was 222.9±40.6 Nm. In $120^{\circ}.s^{-1}$ testing, the peak torque was 195.4±40.9 Nm, for $240^{\circ}.s^{-1}$ the peak torque was 146.3±28.3 Nm and for $450^{\circ}.s^{-1}$ testing the peak torque was 109.2±16.9 Nm. The mean peak torque at isometric test was high and the peak torque began to decline when the test velocity was increased. A previous study by Kanehisa and Fukunaga (1999) reported for isokinetic muscle contractions at velocities of $60^{\circ}.s^{-1}$, $180^{\circ}.s^{-1}$ and $300^{\circ}.s^{-1}$ isokinetic force declined at higher velocities (as expected from muscle f-v curve) in both sedentary individuals and Olympic weight lifters.

Angle peak torque and muscle acceleration

Angle at peak torque is the joint angle at which peak torque occurs. The characteristic torque-joint angle curve describes relative torque increasing until an optimal joint angle is achieved (Margison & Eston, 2001). We observed that the angle at which peak torque occurred was affected by movement velocity for some participants. This may be due to individual differences in peak angular velocity, which only become evident during high movement velocities (i.e. $450^{\circ}.s^{-1}$). Previous study by Kawakami et al., (2002) revealed that angle at peak torque negatively correlates with angular velocity, that is as angular velocity increases the angle at peak torque decreases.

Kawakami et al., (2002) tested velocities covering a range from 30˚.s-1 to $200^{\circ}.s^{-1}$ and reported angles at peak torque occurs ranging from 50-70˚. In the present study we also found a distribution of angles at which peak torque occurs of approximately 60˚ to 80˚ at velocities of $60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, and $240^{\circ}.s^{-1}$. However at velocities of $450^{\circ}.s^{-1}$ the distribution was larger, suggesting that the velocity affects the angle at which peak torque is developed more so at high movement velocities. The individual capability of muscle contraction could be one of the reasons underpinning this observation. Therefore, analysis of limb acceleration during $450^{\circ}.s^{-1}$ knee extension may provide important additional information that can be used to correlate with molecular difference in muscle composition.

2.6 CONCLUSION

The feasibility of isolated knee performance to measure the f-v profile of human muscle in vivo using the isokinetic dynamometry (IKD) was investigated. This is an appropriate exercise for vastus lateralis, which is a suitable muscle that can be used for muscle phenotype/biochemical analysis and muscle function/performance testing. Our work began with a pilot study to find the peak angular velocity of human knee extension during unloaded kicking. Contrary to previous estimations, we found the average peak angular velocity of human knee extension was within the range (0-500 ^{o}/s) of a commercial isokinetic dynamometer. Therefore, we created an IKD protocol that encompassed the entire force-velocity range of human knee extension. Next we investigated the reliability of isolated knee performance measures across the f-v profile of human muscle encompassing a range of velocities using isokinetic dynamometry (IKD).

To attempt to relate our work to wider applications in sports performance testing, we also assessed the reliability of jump analysis using either a force platform or linear encoder. This enabled us to investigate the strength of correlation between indices of isolated knee performance and a more complex sport-related movement such as jumping. Our knee extension data was consistent with the f-v concept of striated muscle and the reliability of each of the measures of knee extensor performance was acceptable (<15 % CV). The reliability of jump performance measures was also good and we found modest correlations amongst each of the knee measures. Reverse regression analysis found isometric and highest velocity knee kicking on IKD was the strongest predictor of jump performance.

In the next chapter, the IKD protocol will be combined with measurement of peak isometric force and rate of force development in order to create a continuum tests that span the entire f-v range. Currently it is not known which measure of muscle output exhibits the greatest diurnal variation.

Chapter 3

Diurnal Variation in Human Skeletal Muscle Performance

3.1 INTRODUCTION

Chapter background

Diurnal variation in sports performances/muscle function

The power output of human muscle and human sports performance exhibit diurnal variation (Drust et al., 2005; Reilly and Waterhouse, 2009). World records in sporting events, such as athletics, are usually broken by athletes competing in the early evening, which is the time of day at which muscle function and body temperature are at their greatest (Reilly et.,al 1997). Table 1 summaries research studies that have investigated the effects of diurnal variation on muscle performance (see Table 1). Regardless of the dependant variable measured (e.g. maximum isometric force, peak torque etc.) muscle performance is consistently better during the evening (~17:00 h) as opposed to the morning (~07:00 h). The magnitude of the change in muscle output from morning to evening is reported to range between 5-20%. The underlying cause of this variation has been attributed to variations in metabolism, circulating levels of hormones, and differences in core or local muscle temperature, all of which have shown patterns of circadian rhythms and may therefore affect performance (Araujo et al., 2011; Racinais & Oksa, 2010).

Life on earth evolved in an environment that changed cyclically and as a result, environmental fluctuations such as the light-dark cycle and recurring seasons take place (Reilly & Brooks, 1990). As the human is essentially a diurnal creature, oscillations occur with a frequency similar to that of a solar day in human processes and effect physiological, biochemical and psychological events. Chronobiology is

the biology of time and internal biological clocks, the terms is derived from the Greek phrases, chronos meaning time, bios meaning life and logos meaning study (Dunlap et al., 2004). Minor & Waterhouse (1981) defined chronobiology as the science concerned with investigating and objective quantification of the mechanism of timing manifestations of life.

Beside the light-dark cycle and melatonin, there are other non-photic signals help synchronise the body clock to solar day such as social interaction and the timing of food (see review by Mistleberger & Skene, 2005). The body's internal time keeper is localised to a small pair of nuclei in the hypothalamus called the suprachiasmatic nuclei (SCN) located at either side of the midline brain, and just above the location where the optic nerves cross prior to their entry to the brain (Ralph et al, 1990). The SCN expresses 'clock genes' that are regulated by a series of feedback loops and are considered to be the molecular mechanism of the circadian clock that generates a rhythmic response with a singles cycle period of 24-25 h (Piggins, 2002). The SCN may function as a master clock, synchronising other peripheral molecular and protein clocks, found in muscle and other tissues, in a rhythmical fashion. Because of its anatomical location the SCN influence body temperature, and core temperature is one of the most easily observed outputs of the SCN circadian clock.

Diurnal variation in core and muscle temperature

The circadian rhythm of core temperature has been used as a surrogate marker for the body clock and has often been described as the fundamental variable (Reilly et

al., 2000). Core body temperature is tightly controlled at approximately 37ºC but exhibits a daily range of oscillation of about 0.6-1.0ºC. In participants living a normal lifestyle, circadian changes in core body temperature are phased such that temperature minimum at about 05:00 h and the temperature maximum occurs between 14:00 h to 20:00 h (Waterhouse et al., 2002). Local muscle temperature in vastus lateralis also varies with time of day and evening (17:00 h) values are higher than the morning (07.30 h) values, by ~0.35ºC (Robinson et al., 2013; Edwards et al., 2013).

Sargent et al. (2010) reports muscle handgrip strength was greater in the evening than in the morning and this difference correlated with the circadian rhythm in core temperature. The superior muscle force output in the evening has been suggested to be evidence of a causal link between core body and muscle temperatures and muscle performance. However recent work (Edwards et al., 2013) that used active or passive warm-up strategies to manipulate body temperature in the morning did not report an improvement in muscle force production despite elevations in core and muscle temperature to evening values. Furthermore, Atkinson et al. (2005) used an active warm-up (25 min of cycle ergometer at 60% VO2max) in the morning which they predicted would improve performance in a 20 km cycle time-trial to afternoon values, thus removing any influence of diurnal variation. Even though pre-performance intra-aural temperature increased by ~0.5°C to values statistical similar to afternoon values, finishing times did not decrease to levels achieved in the evening. Therefore diurnal variation in muscle force output and cycling performance cannot be completely dependent on core body temperature variation and other mechanisms may exist.

Hormonal rhythms

Many hormonal secretions are episodic, evidencing significant ultradian oscillations. Rhythm with period less than 20 hours is called 'ultradian'. Cortisol and growth hormone concentrations exhibit marked peaks at several times during sleep (Veldhuis et al., 1992). Indeed, growth hormone and cortisol rhythms are strongly influenced by sleep characteristics, which are, in turn, affected by habitual levels of physical activity. The time structure of the endocrine system is highly complex and interactive among its different components, other signalling mechanisms, and the environment. It is rhythmic in multiple frequencies. At many sites, the rhythmic variations of a hormone like melatonin, prolactin, thyroid stimulating hormone (TSH), corticotrophin releasing hormone (CRH) or related messenger are deterministic of its effect and efficacy upon a target tissue (Haus, 2007). Testosterone levels are higher in the morning compare than evening (Gray et al., 2004) and chronic testosterone administration can significantly increase muscle strength (e.g Bhasin et al., 1996). However this pattern does not correlate with studies reporting diurnal variation in muscle performance (see Edwards et al., 2013). As yet, it is not clear how relatively acute (i.e. diurnal) changes in testosterone or cortisol could influence muscle performance.

Activation – nerve transduction

Circadian rhythms in motor neuron activity might be responsible for these time of day effects. However, many studies showed the higher muscle performance in the evening was observed without modification of electrical activity (e.g Guette et al., 2005). Guette et al., (2005), conducted the test at 06:00, 10:00, 14:00, 18:00, and

22:00 h to distinguish the neural and muscular mechanisms that influence muscle strength. The electromyographic and mechanical muscle responses associated with electrically evoked and/or voluntary contractions of the human quadriceps and semi-tendinosus muscles for each leg were recorded and compared. The central activation of the quadriceps muscle remained unchanged during the day. Furthermore, in an investigation into time of day effect on electromyography (EMG) parameters, Souissi et al., (2012) found that the root-mean-square (RMS) values produced by electromyograph was unaffected by the time of day of testing.

Clock gene and molecular mechanism

The presence of molecular clock within a cell or organism provides the necessary timekeeping for anticipation of daily changes in environmental (Albrecht, 2002; Gekakis et al., 1998). The timing of these circadian rhythms is synchronized to the environment by external cues (Green, et al., 2008). At the molecular level, the circadian clock represents as well defined gene regulatory network composed of transcriptional-translational feedback loops such as CLOCK, BMAL1 and E-box on target genes like Per1 for instance (Lowrey & Takahashi, 2004). Zhang et al., (2009) suggests there may also be a skeletal muscle molecular clock modulating skeletal muscle physiology. Recent research has focused on the components of molecular clock in the SCN but there is still little known about the regulation and function of molecular diurnal mechanisms in skeletal muscle.

Rational of this study

Diurnal variation in muscle force and sports performance in young healthy individuals is widely reported but the mechanism underpinning this phenomenon are not yet understood. Muscle force production is least between the hours of 06.00 – 08.00 in the morning and greatest from 16.00 – 18.00 in the afternoon.

Fluctuations in force production co-occur alongside biological rhythms in core temperature but we have found diurnal variation in muscle performance is not entirely explained by differences in muscle temperature. For example, passive heating in the morning to stimulate the warmer afternoon muscle temperature did not bring about similar elevation in muscle performance. Similarly, passive cooling in the afternoon to replicate the cooler muscle temperature of the morning was not associated with the decrease in performance. Thus intrinsic differences occur in the ability of skeletal muscle to produce force during the course of the day. The parameters of muscle performance (i.e. maximum force and maximum speed) are determined by collections of contractile proteins. Differences in genetic background, habitual activity or training status affect the relative amounts of each muscle protein and are one of the main reasons for the broadly different physique and physical performance of sprinters compared to marathon runners. The same contractile proteins can also be rapidly modified to cope with short-term changes in demand. This latter mechanism contributes to the positive effect of warming up prior to strenuous exercise, and similar mechanisms could able responsible for the greater output of skeletal muscle in the afternoon compared to the morning. Therefore

further investigation involving proteomic analysis of muscle biopsies needs to be done.

Prior to invasive analysis of muscle biopsy samples we wanted know which aspect of muscle performance exhibits the greatest level of diurnal variation. Previous studies related to this area are summarised in Table 1. The most common techniques that have been used include isokinetic dynamometer and the measurement of peak isometric force produced during maximal voluntary contraction (MVC). Because these data were collected during individual studies that used different populations of participants it is not clear which muscle output (i.e. peak isometric force, power, velocity maximum, etc.) or which measurement technique exhibits the greatest diurnal variations and is therefore optimal for subsequent studies that aim to correlate differences in muscle output with individual proteins.

Therefore we choose a selection of isolated knee extension exercise that cover the entire range of velocities from isometric to velocity maximum. In the current work we used the IKD protocol from Chapter 2 that encompasses 4 different angular velocities (60, 120, 240 and 450 degrees per second). In addition, we measured MVC peak isometric force using the twitch interpolation technique and also the maximum rate of force development (RFD) during the initial 150 ms of the isometric contraction. During this technique, participants perform voluntary rapid sub-maximal isometric contraction (1s duration to approximately 80% of MVC) in order to calculate the maximum rate of force development (RFD; $Nm.s^{-1}$). Previously, Aagaard et al., (2002) reported increasing in the rate of force development and

neural drive of in response to resistance training. Currently, it is not known whether this measure of muscle output exhibits diurnal variation.

The aim of this study

To find which method of measurement or which feature of muscle function shows the greatest in diurnal variation e.g four different velocities on IKD, MVC and RFD.

3.2 METHODS

3.2.1 Participants

Twenty male participants were recruited. Procedures were explained in full to participants with questions answered prior to participation. The study was approved by the local Ethics Committee of University. Inclusion criteria required previous strength training experience (≥ 2 years) with exclusion criteria stating recent shift-work or travel across multiple time zones.

3.2.2 Protocol

Participants performed two separate experimental sessions in this experiment (Figure 22). These were an isokinetic dynamometry (IKD) protocol of knee extension and flexion, and an isometric protocol of maximal voluntary contractions (MVC) and rate force development (RFD) of the quadriceps group.

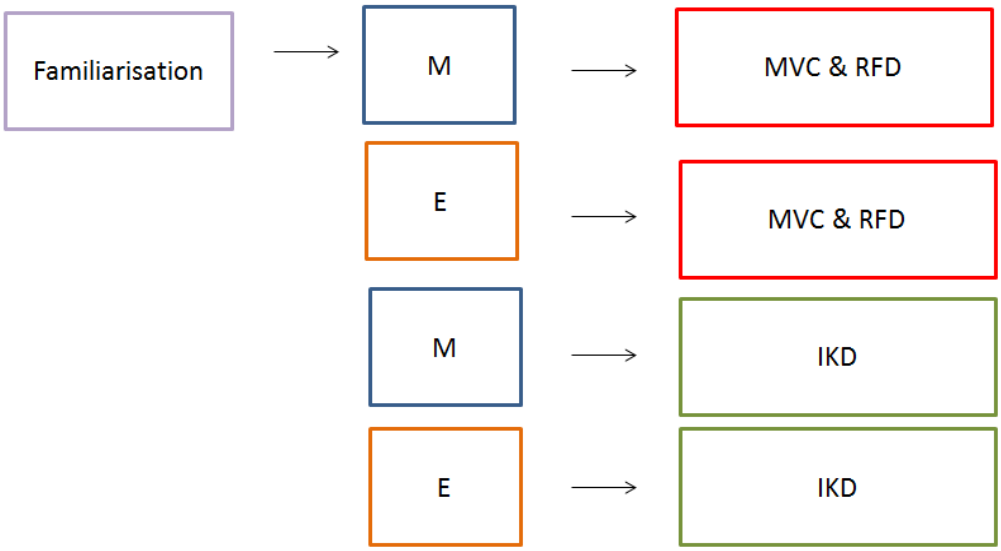


Figure 22. Two separate experimental sessions; MVC & RFD and IKD. Each protocol was completed by the participants in the morning (M) and evening (E) in a counter balanced order.

Each participant completed two experimental conditions a minimum of 48 h apart in either the morning (07:30 h) or evening (17:30 h) for both the IKD and ISO protocols. A limitation to our experiment is that participants were free to live normally between sessions, however they were asked to refrain from caffeinated or alcoholic beverages and from other training or intense exertion 48 h before experiments. Participants reported in a fasted state for ≥ 7 h prior the morning sessions and were instructed not to consume food for ≥ 4 h prior to the evening session so we cannot rule out difference due to 'fed/fasted state. Prior to familiarization participants completed Composites Morningness, Sleep Flexibility/Rigidity [F/R] and Languid/Vigour [L/V] questionnaires (Smith et al., 1989) in order to profile their chronotype. In addition, during each laboratory visit, participants completed a Profile of Mood States (POMS) questionnaire (McNair, 1971). Thermal comfort (TC) (Bakkevig, & Nielsen, 1994) was measured at rest and during warm-up, and perceived exertion (RPE) (Birk & Birk, 1987) and effort (0-10 liekert scale; "0" representing no effort and "10" maximal) were measured during warm-up following each MVC.

Rectal, Skin and Muscle Temperatures

Participants arrived 30 min prior to experimental procedures and lay down in the laboratory after inserting a flexible temperature probe ~10 cm beyond the external anal sphincter (Mini Thermistor; Grant Instruments, Shepreth, UK). Skin temperature (T_{sk}) was measured as the average value of four sites using skin thermistors (Mini Thermistor, Grant Instruments, Shepreth, UK). The thermisters were affixed via tape (Micropore, 3 M) to the left calf, left thigh, left forearm and

lower sternum according to (Ramathan, 1964). Participans remained awake and semisupine while rectal (T_{rec}) and T_{sk} continuously recorded for 30 min using a data logger (Squirrel 1000, Grant Instruments, Shepreth, UK). This standardised body position was used because core temperature is influenced by other factors than that of the endogenous 24 h oscillator, such as feeding, activity and sleep. After participants had rested for 30 min, a 5 min active warm-up was performed at 150 W on a cycle ergometer (Monark Ergomedic 839E, Vansbro, Sweden). Measurement of T_{rec} was the average value of the final 5 min recorded at rest and both T_{rec} and T_{sk} were recorded at rest and following active warm-up, MVC and RFD. Muscle temperature (T_m) was assessed in the vastus lateralis of the right leg using the method described by Gregson et al. (2003) at rest, after active warm-up and between MVC and RFD by needle thermistor (13050; ELAB electronic measurement system (CTF 9004; ELAB, Rodovre, Denmark).

3.2.3 Maximum voluntary contractions

Participants completed five familiarization sessions separated by at least 3 d to ensure full acquaintance with the experimental protocol. During familiarisation sessions, participants alternated between performing MVCs with and without twitch interpolation, so that approximately three trials of each were performed within each session. This approach was suggested by Morton et al. (2005), whom reported naïve participants perform weaker contractions when they anticipate stimulation (attributed to the potential apprehension due to the prospect of receiving noxious stimuli) compared to when they were not expecting stimulation. Familiarisation sessions were conducted until the participant's MVC force and voluntary activation

demonstrated a plateau effect and the average percentage of voluntary muscle activation was greater than 80 % (consistent with Morton et al. 2005). This level of initial consistency was achieved within 3 sessions, therefore data from sessions 4 and 5 were used to calculate the reproducibility of the peak isometric force data.

3.2.4 Maximal isometric quadriceps Force and Percutaneous Stimulation

For each session, the position of the participant in the isometric chair (Lido Active, Loredan, Davis, CA, USA) was standardized in accordance with the guidelines set by the manufacturers and also taking into account any adjustment required by the individual (established during familiarisation sessions). In brief, these guidelines are as follows: The backrest was positioned to ensure 80° of hip flexion and the knee joint was aligned with the axis of the actuator. A belt and straps were tightly secured across the shoulders, hips and chest in order to stabilize the upper body. Quadriceps muscle force was measured from the ankle where the attachment was connected to a strain gauge (Tadea, Tension/Compression cell, Huntleigh, Sweden) by a metal force transducer (previously calibrated with known weights). Participants performed isometric maximum voluntary contractions (MVCs) of the quadriceps muscles (4-s duration), both with and without twitch interpolation. During the initial session, participants practiced performing MVCs without twitch interpolation, to become accustomed to achieving and maintaining voluntary force for the time required. This session was also used to obtain maximal current tolerance (mA) and establish the supra-maximal current amplitude for superimposition during an MVC (Digimeter, DS7, Hertfordshire, UK). Whilst remaining at rest, the amperage of a 240 V square-wave pulse (100 μs, 1 Hz) was progressively increased until the point that further

increases in intensity caused no further increase in twitch force (Newman et al. 2003; Morton et al. 2005).

The quadriceps were electrically stimulated using two moistened surface electrodes (Dura-stick II, Chattanooga Group, Hixson, TN, USA, 7 x 12.7 cm) positioned on the anterolateral side of the thigh, on the belly of the rectus femoris and vastus lateralis muscles. The skin was prepared prior to the placement of each electrode by shaving and light abrasion of the skin followed by cleansing with an isopropyl alcohol swab. A permanent marker pen was used to identify the position of each electrode in order to minimise electrode placement variability between sessions (Giacomoni et al. 2005; Keogh et al. 1999). Eight single square-wave electrical impulses (100 μs) were delivered during the 8-s sampling period. Each impulse was computer driven and was delivered at 240 V AC (RMS) at 50 Hz. Two impulses were delivered before and after the contractions; the other four impulses were administered during the contraction period and tested the peak value of the MVC. The peak forces of the pre- and post-contraction twitches were then averaged, allowing comparison of resting twitch amplitudes in both an un-potentiated and potentiated condition (Oskouei et al. 2003). The amplitude of supra-maximal superimposed current was to 10% above the level required to evoke a resting muscle twitch of maximal amplitude (Todd et al. 2004), determined in familiarization sessions.

Standardised verbal encouragement was provided throughout with real-time bio-feedback of force development projected on a large display screen in front of

participants. Data were acquired for 8 s and analysed with commercially software (AcqKnowledge III software for Windows, Biopac Systems Inc, Aero Camino Goleta, CA, USA). The calculation of voluntary activation was conducted according to an interpolated twitch ratio (as recommended by Merton, 1954), whereby the size of the interpolated twitch is expressed as a ratio of the amplitude elicited by the same stimulus delivered to a relaxed un potentiated muscle. The average force recorded during the 100-ms period before the application of each stimulus during the contraction and, subsequently, the maximal force recorded during the 100-ms period after each stimulus was used. The highest pre-stimulus force (taken as MVC force) and the resulting maximal post-stimulus force were then used to calculate the size of the interpolated twitch. Interpolated twitch size was calculated by subtraction of the mean pre-stimulus force from the maximal post-stimulus force. To calculate voluntary activation of the quadriceps using the following equation:

$$\text{Voluntary Activation} = \left[1 - \left\{\frac{Size\ of\ interpolated\ twitch}{Size\ of\ resting\ twicth}\right\}\right] X\ 100$$

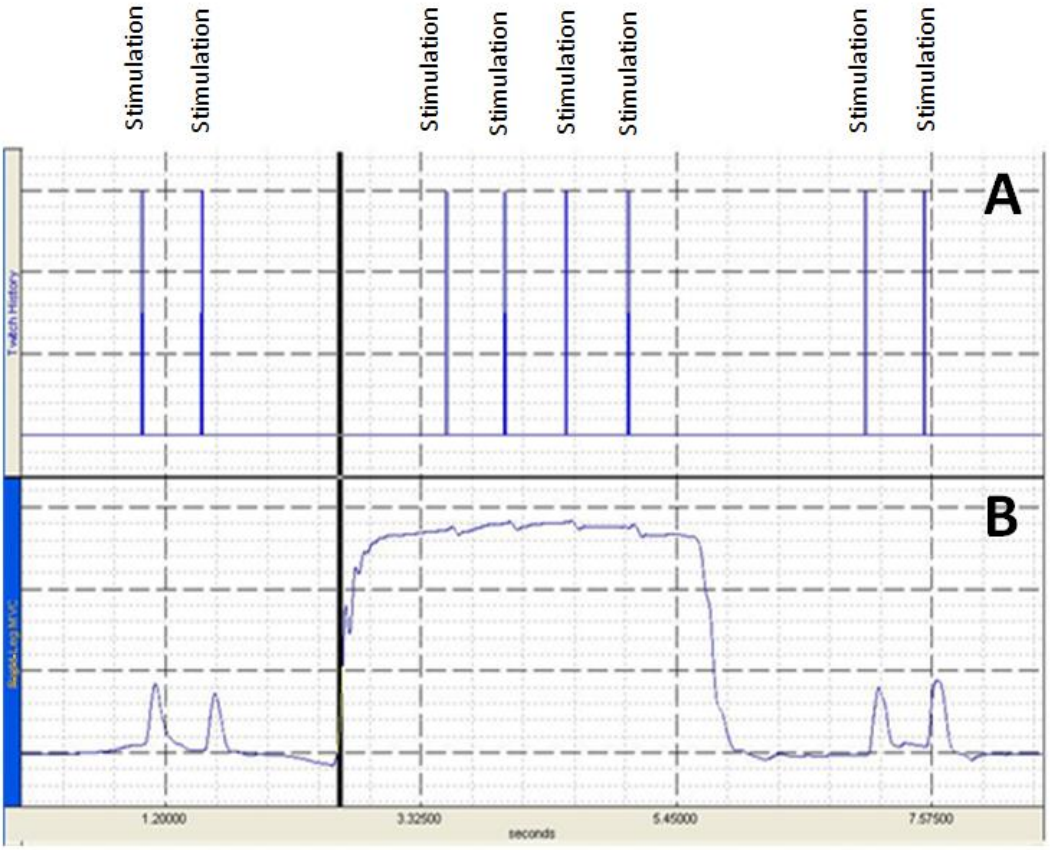


Figure 23. A typical MVC with twitches superimposed. Panel A illustrates the timing of the twitches whilst panel B illustrates the participants achieved force rate.

3.2.5 Rate of Force Development

For each session, the position of the participant in the isometric chair (Lido Active, Loredan, Davis, CA, USA) was standardized in accordance with the guidelines set by the manufacturers and also taking into account any adjustment required by the individual (established during familiarisation sessions). In brief, the process is identical to that described for MVC. Peak rate of force development (RFD) was analysed using AcqKnowledge III software. Participants kicked as quickly and forcefully as possible without producing any countermovement and were encouraged to achieve at least 80 % of maximum isometric force. Visual data representation was utilised to provide feedback and ensure no counter-movement preceded each kick.

Ten kicks were performed interspersed by 30 s rest, with the three efforts of greatest force and no discernable counter-movement selected for analysis. Measurement of RFD represented the highest positive value from the first derivative of the force signal (the greatest slope of the force-time curve).

3.2.6 Isokinetic dynamometer protocol

Consistent with Chapter 2, IKD was performed at different velocities to investigate the data across force-velocity curve in morning and evening. Test angular velocities were set at $60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, $240^{\circ}.s^{-1}$ and $450^{\circ}.s^{-1}$. Participants were asked to do 3 repetitions of extension and flexion for each angular velocity. Thirty seconds rest was given between each repetitions and three minutes between each angular velocity delivered in randomised order. Three warm-up trials were be performed prior each velocity during which the participant were instructed to complete the protocol using up to 50 % of maximal effort. Trails were interspersed by 3 min recovery periods and standard performance parameters, including torque and power output was recorded.

The schematic of IKD protocol is shown in Figure 24. Meanwhile for maximal isometric quadriceps force, percutaneous stimulation and RFD were measured as described in 3.2.4 and 3.2.5 and the schematic protocol for MVC and RFD showed as figure 25.

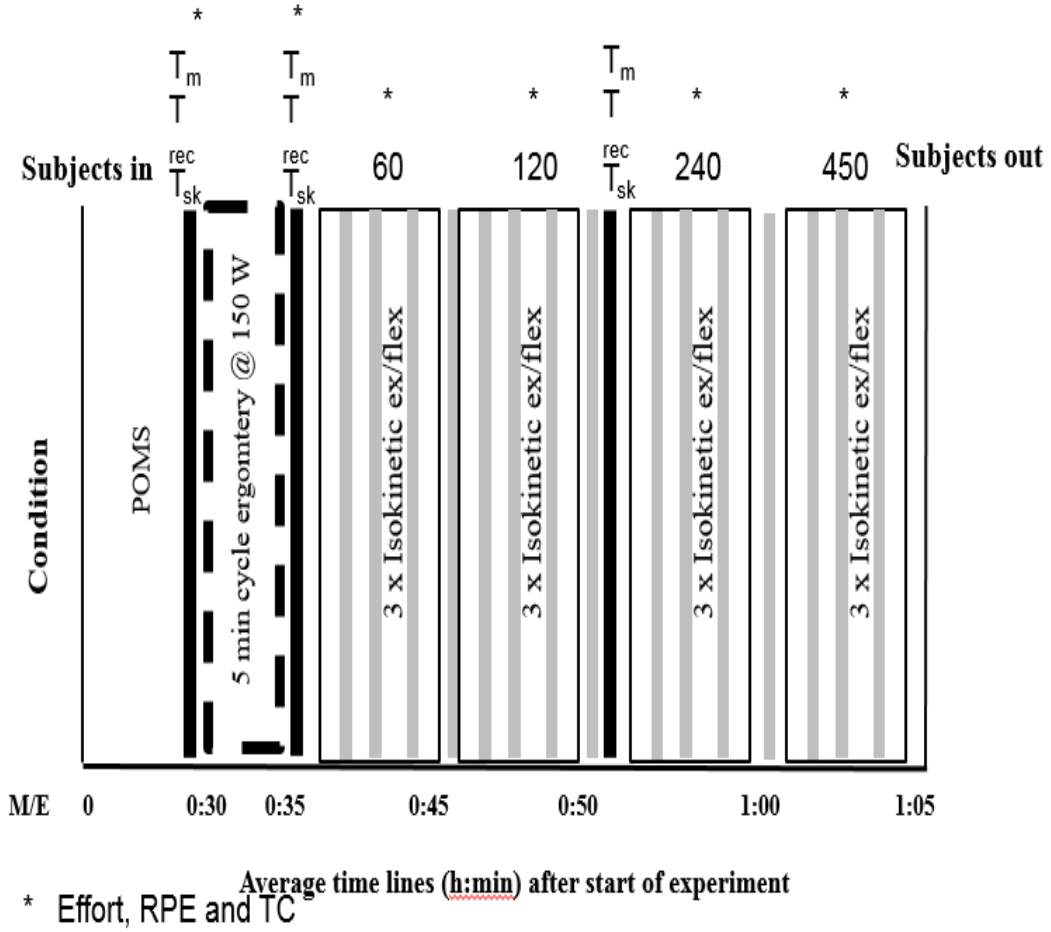


Figure 24. Schematic of the protocol for IKD in the morning (M, 07.30 h) and evening (E, 18:00 h) conditions. Rectal (T_{rec}), skin (T_{sk}) and muscle (T_m) temperatures, thermal comfort (TC) and perceived onset of mood (POMS) were measured after the participants had reclined for 30 min at the start of the protocol, after the warm-up and after 120˚.s^{-1}. Rating of perceived exertion (RPE) was taken during warm-up and after each velocity kicking. Black bars indicate muscle temperature taken at these points; grey bars indicate recovery between tests.

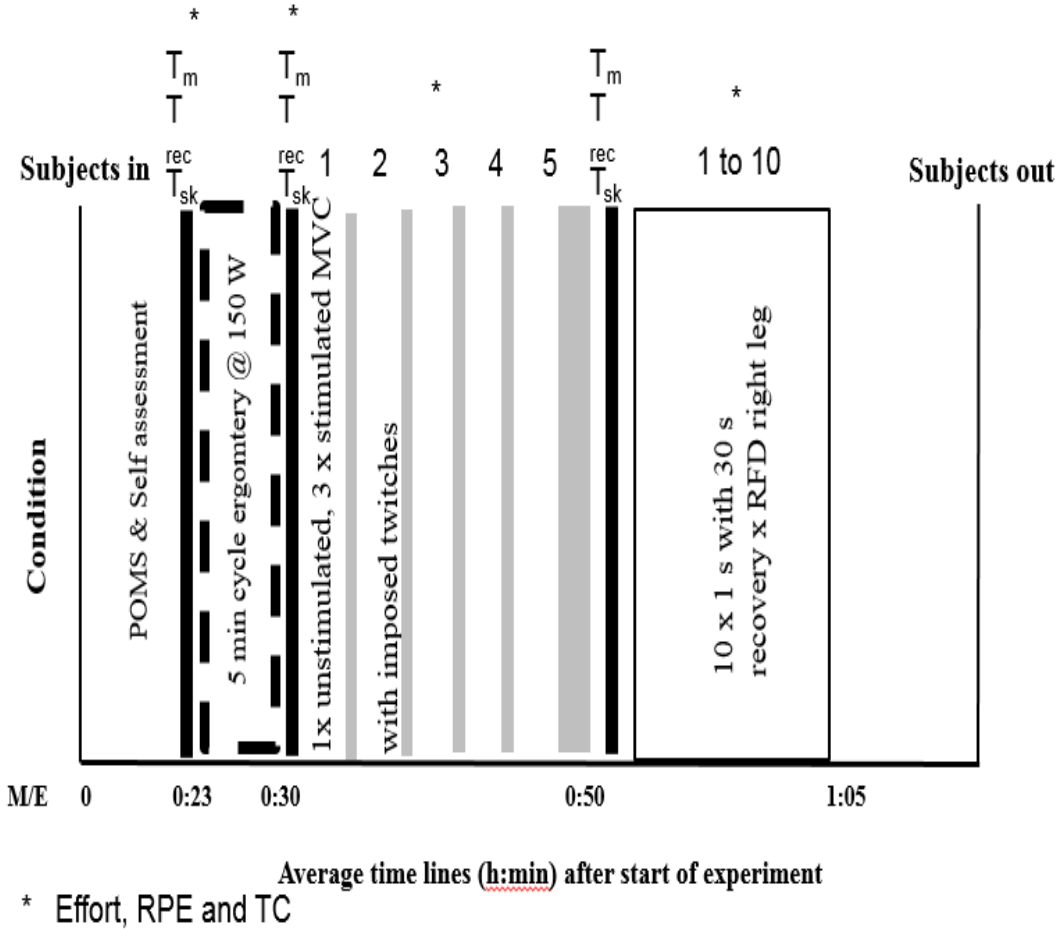


Figure 25. Schematic of testing protocol for MVC and RFD in the morning (M, 07.30 h) and evening (E, 18:00 h) conditions. Rectal (T_{rec}), skin (T_{sk}) and muscle (T_m) temperatures and thermal comfort (TC) were measured after the participants had reclined for 30 min at the start of the protocol and after the warm-up. Rating of perceived exertion (RPE) was taken during warm-up and after each MVC, with rating of effort taken after each MVC. Black bars indicate muscle temperature taken at these points; grey bars indicate recovery between tests.

3.2.7 Statistical Analysis

The data were analysed using the Statistical Package for Social Sciences (SPSS) for Windows (SPSS, Chicago, IL, USA) version 20. Comparisons by paired sample t-test were used to test the primary hypothesis that muscle output is greater in the evening. The results are presented as the mean ± the standard deviation (SD) throughout the text unless otherwise stated. Ninety-five percent confidence interval (CIs) are presented where appropriate. The alpha level of significance was set at 5% ($p < 0.05$).

3.3 RESULTS

3.3.1 Participants

Twenty male participants were recruited of age (mean±SD) 25.9 ± 4.4 years, height 177.3 ± 6.8 cm, body mass 75.1 ± 8.2 kg with habitual retiring and waking times 11:36 ± 0:36 p.m. and 06:24 ± 0:18 a.m., respectively. Mean chronotype score was 35.2 ± 4.5 (all intermediate type); F/R score 43.0 ± 5.6; and L/V score, 43.0 ± 9.3. POMS data for both IKD and MVC sessions identified significantly greater ($p < .05$) "happy" and "vigour" in the evening. As anticipated, both T_{rec} and T_m were significantly greater in the evening than the morning, whereas diurnal variation in Tsk was not robust. Effort levels of 10 (i.e. maximum effort) were consistently reported contributing no difference between conditions. Thermal confort was not significantly different between morning and evening, but mean RPE was significantly greater during the MVC protocols performed in the morning compared to the evening.

3.3.2 Isokinetic measures

Peak torque during knee extension at velocities of 60˚.s^{-1}, 120˚.s^{-1}, 240˚.s^{-1} and 450˚.s^{-1} is shown in Figure 26. Consistent with force-velocity characteristics of skeletal muscle, peak torque was greatest during slow contraction and declined as the velocity of contraction increased. At each of the velocities tested, peak torque during extension was significantly ($p<0.05$) greater in the evening than the morning. The magnitude of diurnal variation ranged from 9.8 % at 60˚.s^{-1} (245.4 ± 42.8 Nm vs. 223.4 ± 35.7 Nm) to 8.1 % at 240˚.s^{-1} (143.5 ± 24.1Nm vs. 132.8 ± 26.6 Nm) .

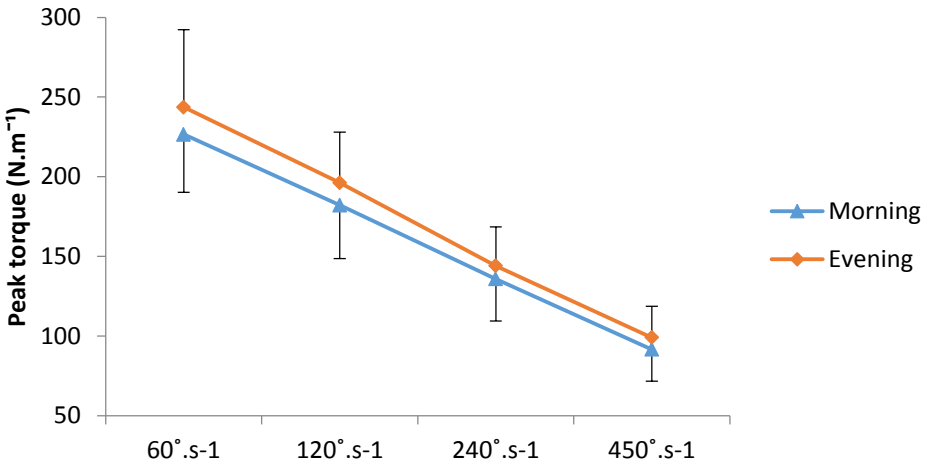


Figure 26. Knee extension torque-velocity relationship measured using isokinetic dynamometry.
Peak torque data are presented as mean ± SD (*n* = 20, in each trial). Peak torque (Nm) was recorded during isokinetic knee extension at angular velocities of 60˚.s^{-1}, 120˚.s^{-1}, 240˚.s^{-1} and 450˚.s^{-1}.

Table 18. Analysis IKD measurements were assessed using paired t-test and the percentage of difference was calculated for major parameters.

	Morning	Evening	(p) value	Diff. (%)
Rectal Temperature (˚C)				
Rest	36.8 ± 0.3	37.3 ± 0.3*	0.000	
Post Warm-Up	36.8 ± 0.3	37.3 ± 0.3*	0.000	
T_{rec} (post 60˚.s^{-1})	37.0 ± 0.4	37.4 ± 0.3*	0.000	
T_{rec} (post 120˚.s^{-1})	37.0 ± 0.4	37.4 ± 0.4*	0.000	
T_{rec} (post 240˚.s^{-1})	37.0 ± 0.4	37.3 ± 0.4*	0.004	
T_{rec} (post 450˚.s^{-1})	37.0 ± 0.4	37.3 ± 0.4*	0.006	
Muscle Temperature (˚C)				
Rest	35.9 ± 0.4	36.3 ± 0.5*	0.003	
Post Warm-Up	37.3 ± 0.5	37.8 ± 0.4*	0.002	
T_m (post 120˚.s^{-1})	36.9 ± 0.4	37.4 ± 0.4*	0.000	
Skin Temperature (˚C)				
Rest	32.3 ± 0.9	32.5 ± 0.9	0.664	
Post Warm-Up	31.6 ± 0.6	31.9 ± 0.8	0.078	
T_{sk} (post 60˚.s^{-1})	32.3 ± 0.5	32.6 ± 0.6*	0.041	
T_{sk} (post 120˚.s^{-1})	32.2 ± 0.7	32.5 ± 0.6	0.127	
T_{sk} (post 240˚.s^{-1})	32.3 ± 0.5	32.4 ± 0.6	0.467	
T_{sk} (post 450˚.s^{-1})	32.3 ± 0.6	32.5 ± 0.6	0.320	
IKD Peak Torque(Nm)				
Extension (60˚.s^{-1})	223.4 ± 35.7	245.4 ± 42.8*	0.000	9.8
Flexion (60˚.s-1)	124.4 ± 26.7	130.9 ± 28.5*	0.018	5.2
Extension (120˚.s^{-1})	180.2 ± 32.1	195.4 ± 29.1*	0.001	8.4
Flexion (120˚.s^{-1})	118.5 ± 24.2	128.2 ± 21.5*	0.003	8.2
Extension (240˚.s^{-1})	132.8 ± 26.6	143.5 ± 24.1*	0.003	8.1
Flexion (240˚.s^{-1})	84.0 ± 19.7	93.7 ± 19.8*	0.010	11.5
Extension (450˚.s^{-1})	90.3 ± 19.3	98.1 ± 18.8*	0.033	8.6
Flexion (450˚.s^{-1})	62.8 ± 12.1	70.3 ± 12.9*	0.048	11.9
IKD Average Power(W)				
Extension (60˚.s^{-1})	151.1 ± 28.1	167.2 ± 27.7*	0.001	10.6
Flexion (60˚.s^{-1})	81.8 ± 21.3	89.9 ± 20.4*	0.003	9.9
Extension (120˚.s^{-1})	217.1 ± 37.8	244.4 ± 43.1*	0.000	12.5
Flexion (120˚.s^{-1})	152.2 ± 27.7	167.3 ± 32.3*	0.000	9.9
Extension (240˚.s^{-1})	272.4 ± 52.8	299.8 ± 60.6*	0.002	10.1
Flexion (240˚.s^{-1})	163.8 ± 36.2	203.2 ± 49.2*	0.000	24.1
Extension (450˚.s^{-1})	283.2 ± 72.8	306.4 ± 68.8	0.087	8.2
Flexion (450˚.s^{-1})	180.9 ± 48.6	190.2 ± 51.3	0.535	5.1

Data are presented as means ± SD; *n* = 20 participants. Isokinetic dynamometry (IKD) was performed at 4 different velocities: 60, 120, 240 and 450˚.s^{-1}. Data presented are peak torque (Nm) and average power (W) over 3 kicks during knee extension and flexion.

3.3.3 MVC and RFD

MVC was significantly greater (10.2 %) in the evening compared to morning. There was no significant difference in voluntary activation level ($p > .05$) between morning and evening conditions. RFD Peak was greater in E vs. M (+953.2 $N.s^{-1}$, see Table 19). There was no difference in the time to RFD peak between M and E ($p > .05$).

Table 19. Analysis MVC & RFD measurements were assessed using paired t-test and the percentage of difference was calculated for major parameters.

	Morning	Evening	(p) value	(%)
Rectal Temperature (˚C)				
Rest	36.8 ± 0.3	37.2 ± 0.4*	0.000	
Post Warm-Up	36.9 ± 0.3	37.2 ± 0.4*	0.000	
Post MVC	37.1 ± 0.3	37.3 ± 0.2	0.056	
Post RFD	37.1 ± 0.3	37.2 ± 0.2	0.090	
Muscle Temperature (˚C)				
Rest	36.1 ± 0.5	36.4 ± 0.4*	0.008	
Post Warm-Up	37.5 ± 0.4	37.8 ± 0.5*	0.042	
Post MVC	37.0 ± 0.6	37.4 ± 0.4*	0.009	
Skin Temperature (˚C)				
Rest	32.1 ± 0.7	32.2 ± 0.9	0.449	
Post Warm-Up	31.5 ± 0.7	31.6 ± 0.8	0.682	
Post MVC	32.1 ± 0.5	32.3 ± 0.6	0.378	
Post RFD	32.0 ± 0.5	32.1 ± 0.5	0.281	
Performance Measures				
MVC force ($N.m^{-1}$)	654.0 ± 78.9	720.8 ± 79.7*	0.000	10.2
MVC activation (%)	82.8 ± 7.7	86.5 ± 6.4	0.061	4.5
Peak RFD ($N.s^{-1}$)	5972.3 ± 1500.7	7051.6 ± 1261.1*	0.002	18.0
Time to Peak RFD (ms)	72.8 ± 17.2	67.2 ± 11.7	0.141	7.8
RPE				
During Warm-Up	10.6 ± 2.0	10.9 ± 2.0	0.863	
During MVC efforts	13.9 ± 2.8	12.7 ± 2.9	0.002	
Thermal Comfort				
Resting	5.0 ± 0.9	5.1 ± 0.2	0.825	
During Warm-Up	5.4 ± 0.6	5.6 ± 0.8	0.287	

Data are presented as means ± SD; *n* = 20 participants. MVC and RFD were performed on isometric chair (Lido Active, Loredan, Davis, CA, USA).

Test re-test reliability of MVC and RFD measurements was assessed using paired t-test and the coefficient of variation (CV) was calculated for major parameters. MVC and RFD data (Table 20) were not significantly different across the 2 repeated trials, demonstrating there was no evidence of systematic bias. The CV of the parameters was ranging from 1.1 to 6.7%.

Table 20. Reliability of muscle performance test on MVC and RFD.

	Mean	Paired t-test (p) value	% Coefficient of Variation (CV)
MVC			
MVC force ($N.m^{-1}$)	679.6 ± 120.2	0.188	1.1
MVC activation (%)	84.6 ± 7.3	0.138	3.1
RFD			
Peak RFD ($N.s^{-1}$)	6447.3 ± 1847.5	0.949	6.7
Time to Peak RFD (ms)	72.9 ± 15.8	0.480	6.2

Data are presented as means ± SD; *n* = 20 participants averaged from 2 separate test sessions.

3.4 DISCUSSION

The investigations of daily variation in muscle force output have been establish using methods of measurement including Biodex Isokinetic Dynamometry and maximal voluntary isometric contractions. Many studies show the diurnal variation in performance by using IKD (Reilly, 2007; Edwards et al., 2013) and MVC (Martin et al., 1999; Racinais et al., 2005; Reilly, 2007; Edwards et al., 2013). The main idea of this study was to optimize the technique in muscle performance and determine which measure of muscle function exhibits the greatest diurnal variation. The measurement techniques chosen covered the entire range from isometric to velocity maximum using IKD at 4 velocities ($60^{\circ}.s^{-1}$, $120^{\circ}.s^{-1}$, $240^{\circ}.s^{-1}$ and $450^{\circ}.s^{-1}$), MVC and RFD.

Our work investigated IKD knee extension and flexion at movement speeds of 60, 120, 240 and $450°s^{-1}$, paying particular attention to the 450 speed as it has rarely been studied in the literature. There was a diurnal variation in temperature and IKD peak torque extension at all velocities. This data is in agreement with the current literature suggesting that performance and core temperature follower similar patterns of diurnal variation, with lows in the morning and peaks around the evening (Atkinson et al., 2005; Edwards, et al., 2013; Pullinger, et al., 2014; Robinson et al., 2013).

Measures of MVC, RFD and IKD all displayed significant time-of-day variation with higher values in the evening when compared to the morning ($P<0.05$) in current study. The range of morning to evening diurnal variation displayed by this cohort of participants varied from 8.1 % to 18 % for all measurements. It was established that MVC and RFD showed the greatest diurnal variation when compared to IKD (10.2 % and 18 %, respectively). The present study used a counterbalanced design and considered all factors such as light and room temperature (within the test laboratories), prior training history and status; and calorific intake. The only parameter which was not directly controlled was sleep, and this was due to the live-normal conditions of protocol. The principal finding was that in highly motivated individuals muscle output applied to range of measurements that have been shown in this study and in the literature (see Giacomoni et al., 2005, Sedliak et al., 2007 and Edwards et al, 2013) to exhibit diurnal variation when measured under a rigorous research design still peak in the late afternoon/early evening (~18:00 h).

During this live-normal protocol there was a significant diurnal variation effect for the perception of mood state (POMS) variables vigour and happy which revealing higher in evening. These observations coincided with measured factors of muscle force output, with vigour and happy corresponding with later time-points and highest values of muscle force output. These results lend support towards the statements made by Reilly and Bambaeichi (2003), who proposed that there might be times of day when an individuals are less inclined to produce a maximal effort. Other than the physiological mechanisms discussed previously it has been suggested that participant motivation can mask diurnal variation in performance (Edwards et al., 2013; Robinson et al., 2013). This limitation can be assessed through subjective measures

of motivation, effort and arousal. However, MVC performance was implemented with the superimposed technique which has been shown to override the central mechanisms related to motivation (Morton et al. 2005). Indeed, this is evident by the fact that no significant difference was found between voluntary muscle activation levels for all conditions which is further supported by all participants giving a maximum of 10 for effort rate. Based on this data it can be assumed that the MVC data is a true reflection of diurnal variation of performance and not heavily influenced by other central mechanisms.

Possible factors to be considered other than temperature could be that previously observed of hormonal behaviours. Hayes et al., (2010) have proposed that, whilst basal levels of testosterone are greater in the morning, responsiveness of testosterone to exercise is increased later in the day, which might therefore be related to this observed increase in muscle temperature. It is widely recognised that morning resting levels of cortisol are higher than those observed during the afternoon or evening (Sedliak et al., 2007). As cortisol is generally regarded to be a catabolic hormone which counteracts the effects of testosterone, this is an important factor when considering muscle force output. Sedliak et al., (2007) concluded that, whilst training at a specific time of day reduced morning resting cortisol concentration, but did not affect the diurnal pattern of maximum strength. Therefore, further work is needed to determine the mechanisms underpinning the diurnal variation in muscle performance.

In current work test retest in quadriceps MVC was showed with no significant different after three familiarisation of performance in activation, as previously observed that this level of initial consistency was typically achieved within three to five sessions, after which participants were then considered eligible to participate (Edwards et al., 2013; Robinson et al., 2013). We present comparable force and activation data to previous work in this muscle group (Edwards et al., 2013; Robinson et al., 2013) and suggest participant familiarization and parallels in protocol contribute to similarities. Nevertheless, 679.58 ± 120.20 (Nm) mean in maximum isometric force is consistent with literature where force is apparent in isometric contractions (Racinais et al., 2005). Contraction velocity is key to time-restricted explosive actions such as kicks, sprints and jumps (Andersen & Aagard, 2006) and with peaks occurring inside 100 ms of force onset and durations > 300 ms required to achieve maximum force (Thorstensson et al., 1976). We report there was not significantly difference in test retest for RFD and showed the RFD_{Force} in this study is within the expected range for athletes (Tillin et al., 2010). With greatest velocity occurring between 50 and 100 ms as observed in previous knee extensors studies (Andersen & Aagard, 2006) and those in other musculature (Erskine et al., 2014).

In current study of the RFD diurnal difference (18 %) was almost double that of the diurnal difference (10.2 %) in peak isometric force. Pereira et al., (2011) reported explosive muscle force was greater in the evening than morning. The difference in magnitude of diurnal variation reported here between MVC and RFD could represent a physiological phenomenon or could be due to a difference in precision of the analytical techniques. Taylor et al., (2011) reported if single trial protocol was used,

the error associated with mean force was lower compare to RFD. When a six-trial protocol was used, the error was reduced however RFD still remained high at approximately 13-16 %. Herein we performed 5 familiarisation sessions and found that participants produced consistent results after the third session. Therefore, data from sessions 4 and 5 were used to assess the reproducibility of the MVC and RFD techniques (Table 20). Consistent with Taylor et al., 2011, we report the reproducibility of MVC data is greater than RFD. Nonetheless, RFD data was not associated with significant error and the coefficient of variation was approximately 6 %.

3.5 CONCLUSION

Twenty young healthy males completed measures spanning maximum force production through to maximum speed of contraction. Diuranl variation ranged from 8.1 % to 18 % across six different measures. Rate of force development (RFD) and isometric force during maximal voluntary contraction (MVC) exhibited the greatest diurnal variation (5972.3 ± 1500.7 - 7051.6 ± 1261.1 N/s, p= 0.002 and 654.0 ± 78.9 - 720.8 ± 79.7 Nm, p= 0.000 respectively). The proposed work were used these robust measures of performance and attempt to discover changes within muscle that correlate with the differences in muscle output.

In summary, irrespective of the type of testing modality, the evidence provided here supports the previously reported circadian variability of muscle force output and strength performance. However, it was established that RFD and MVC showed the greatest diurnal variation when compared to IKD (10.2 % and 18%, respectively). Therefore these methods will be used as diurnal variation performance before the biopsies taken to investigate whether modifications occur to the contractile proteins of the skeletal muscle that effect its ability to produce force at different time of day.

Chapter 4

Proteomic Analysis of Diurnal Variation in Human Myofibrillar Proteome

4.1 INTRODUCTION

Chapter background

The proteome dictates cellular function, therefore differences in function that occur between morning and evening must be underpinned by differences in the proteome. Candidate mechanisms such as temperature, arousal and hormones etc. are associated with diurnal differences in muscle output but none of these mechanisms have been causally linked to daily changes in muscle force. Irrespective of the upstream stimulus, the aim of this chapter was to investigate whether modifications occur to the contractile proteins of the skeletal muscle that underpin its ability to produce force at different times of day. By using non-targeted proteomic analysis to measure differences in the abundance of dozens of important myofibrillar proteins new information might be discovered that can help to understand the mechanisms of diurnal variation. The main proteins of interest (in addition to myosin heavy chain isoforms) were the isoform, splice variant and post-translational sub-species of regulatory and essential myosin light chains, tropomyosins and troponins, which may modulate muscle contractile characteristics.

Proteomics is the large-scale comprehensive study of proteins using various separation techniques and non-targeted mass spectrometry-based analysis. To date, 33 peer-review journal articles report proteomic analysis of human skeletal muscle, i.e. from 2003 to 2015 (Table 21). The first proteomic analysis of human skeletal muscle was by Gelfi et al., (2003) reporting a 2DGE map of human vastus lateralis

that provides a valuable resource for the definition of the functional properties of muscle. Since this initial publication, proteomic analysis of human samples has been used to address experimental questions including diabetes (Hojlund et al., 2003; Al-Khalili et al., 2014), aging (Gelfi et al., 2006; Lanza et al., 2008; Staunton et al., 2012), muscular dystrophies (Moriggi et al., 2010; Brocca et al., 2012), obesity (Hittel et al, 2005; Hojlund et al., 2010; Hwang et al., 2010; Giebelstein et al., 2012) and exercise training (Holloway et al., 2009; Hody et al., 2011; Egan et al, 201; Son et al., 2012; Malik et al., 2013; Schild et al., 2015).

Typically biopsy samples from human vastus lateralis have been analysed (Gelfi et al., 2003; Lefort et al., 2009; Wijers et al., 2010; Zhao et al., 2011; Son et al., 2012), but some researchers have also used deltoideus (Capitanio et al., 2005), rectus femoris (Hody et al., 2011) and soleus (Moriggi et al., 2010). In addition, primary myoblasts have been cultured from human muscle (e.g. Norheim et al., 2011; Hartwig et al., 2014). Many of the studies included proteome mining and provide important catalogues of accessible muscle proteins (Gelfi et al., 2003; Yi et al., 2008; Lefort et al., 2009; Baraibar et al., 2011; Zhao et al., 2011). By considering the number of proteins identified it is clear the combination of SDS-PAGE with LC-ESI-MS/MS, known as GeLC-MS/MS is of particular utility in skeletal muscle protein mining. Alternatively, protein profiling has been conducted using 2DGE or DIGE which offers robust comparative analysis of protein species, proteome profiling and differential expression (Hojlund et al., 2003; Gelfi et al., 2006; Holloway et al., 2009; Giebelstein et al., 2012;; Hartwig et al., 2014; Schild et al., 2015). More recently, some studies (Hwang et al., 2010; Hussey et al., 2013; Malik et al., 2013) have also applied LC-MS profiling in human skeletal muscle.

Table 21. Summary of studies on proteomics of human skeletal muscle

Reference	Sample	Area of research	Technique	Number of proteins
Hojlund et al., (2003)	VL	Diabetes	2DGE, MS	489 spots, 11 sig diff
Gelfi et al., (2003)	VL	Mining	2DGE, MALDI-MS, LC-ESI-MS/MS	>500 spots, 124 identified
Hittel et al., (2005)	RA	Obesity	2DGE, MS	7 sig diff
Capitanio et al., (2005)	VL, D	Mining	2DGE, MS	37 sig diff
Gelfi et al., (2006)	VL	Aging	DIGE, SDS-PAGE, LC-ESI-MS/MS.	~2700 spots, 39 sig diff
Viganò et al., (2008)	VL	Altitude	2DGE, LC-ESI-MS/MS	122 spots, 56 sig diff
Yi et al., (2008)	VL	Mining	GeLC-MS/MS	507 identified
Lanza et al., (2008)	VL	Aging	LC-ESI-MS/MS, iTRAQ	50 sig diff
Hojlund et al., (2008)	VL	Mining	GeLC-MS/MS	741 identified
Lefort et al., (2009)	VL	Mining	GeLC-MS/MS	823 identified
Højlund et al., (2009)	VL	Mining	GeLC-MS/MS	127 identified
Parker et al., (2009)	VL	Mining	GeLC-MS/MS	>2000 identified
Holloway et al., (2009)	VL	Exercise	2DGE, iTRAQ, SDS-PAGE, LC-MALDI MS/MS.	256 spots, 168 identified, 38 sig diff
Højlund et al., (2010)	VL	Obesity	SDS-PAGE, LC-ESI-MS/MS	7 sig diff
Hwang et al., (2010)	VL	Obesity	LC-ESI-MS/MS	334 identified, 15 sig diff
Wijers et al., (2010)	VL	Thermo-genesis	2DGE MALDI-MS/MS	224 spots, 52 sig diff
Moriggi et al., (2010)	VL, S	Muscular dystrophies	DIGE, MALDI- MS/MS, LC-ESI MS/MS	33 sig diff
Hody et al., (2011)	RF	Exercise	2DGE, MALDI- MS/MS	191 spots, 61 identified, 52 sig diff
Baraibar et al., (2011)	PM	Primary cell	2DGE, SDS-PAGE, MALDI-MS/MS	~4000 spots
Zhao et al., (2011)	VL	Mining	LC-ESI-MS/MS	878 identified
Egan et al., (2011)	VL	Exercise	DIGE, LC-ESI-MS/MS	800 spots, 31 sig dif
Norheim et al., (2011)	VL, T, PM	Exercise & Primary	SDS-PAGE, LC-ESI-MS/MS	236 identified, 25 sig diff

		cell		
Brocca et al., (2012)	VL	Muscular dystrophies	2DGE, MALDI MS/MS, LC-ESI-MS/MS	>800 spots, 33 sig diff
Giebelstein et al., (2012)	VL	Obesity	2DGE, LC/ESI-MS/MS	2852 spots, 107 identified, 44 sig diff
Staunton et al., (2012).	VL	Aging	DIGE, LC-ESI-MS/MS	19 sig diff
Son, et al., (2012)	VL	Exercise	2DGE, MALDI-MS/MS	~600 spots, 16 sig diff
Malik et al., (2013)	VL	Exercise	LC-ESI-MS/MS	207 identified, 16 sig diff
Hussey et al., (2013)	VL	Exercise & Diabetes	GeLC-MS/MS	438 identified, 15 sig diff
Conti et al., (2014)	VL, G	Muscle	2DGE, LC-ESI-MS/MS	11 sig diff
Hartwig et al., (2014)	VL, PM	Primary cell	2DGE, SDS-PAGE, MALDI-MS/MS	1200 spots, 171 identified, 12 sig diff
Al-Khalili, et al. (2014a)	VL, PM	Diabetes	DIGE, LC-ESI-MS/MS	166 identified, 11 sig diff
Al-Khalili, et al. (2014b)	VL, PM	Diabetes	DIGE, LC-ESI-MS/MS	1804 spots, 47 sig diff
Schild, et al. (2015)	VL	Exercise	LC-ESI-MS/MS	3481 identified, 115 sig diff

Summary of proteomic studies of human muscle, including vastus lateralis (VL), rectus abdominus (RA), deltoid (D), gracilis (G), primary myoblast (PM), soleus (S), rectus femoris (RF), trapezius (T). Proteomic techniques that have been used on human muscle include two-dimensional gel electrophoresis (2DGE), one-dimensional denaturing gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption tandem mass spectrometry (MALDI-MS/MS), liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS), isobaric tags for relative and absolute quantitation (iTRAQ), difference in-gel electrophoresis (DIGE) and one-dimensional denaturing gel electrophoresis coupled with liquid chromatography-electrospray ionisation-tandem mass spectrometry (GeLC-MS/MS).

Proteome mining work (Hojlund et al., 2008) has identified 954 different proteins in human vastus lateralis muscle obtained from three healthy, non-obese subjects using GeLC-MS/MS. Meanwhile Parker et al., (2009) investigated various combinations of SDS-PAGE and HPLC separations coupled with ESI-MS/MS to identify almost 2000 proteins, and determined how 370 very abundant proteins behave upon differential solubilisation. These results provide the comprehensive characterization of the human skeletal muscle proteome. The current thesis also used the GeLC-MS/MS techniques to catalogue the proteins present in the myofibrillar fraction.

Work from our laboratory (Holloway et al., 2009) was the first to report proteomic analysis of the response of human skeletal muscle to exercise training. Since our initial publication, exercise physiology has become a major theme in muscle proteomics and to date 7 further studies have investigated the effects of exercise. Son et al., (2012) investigated the effect of resistance and endurance training on muscle proteome expression, samples of vastus lateralis from 10 physically active young men were analysed by 2DGE and MALDI-MS/MS. They concluded that endurance and resistance exercise training differently alter the expression of individual muscle proteins, and that the response of muscle protein expression may be associated with specific myofibre adaptations to exercise training. Our current study focuses on muscle performance and diurnal variation and, to date, no other human muscle chronobiology study has attempted to use proteomic analysis.

Indeed it was difficult to find chronobiology studies reporting effects in human muscle. Rather the majority of studies (e.g. Bae et al. 2006; McCarthy et al., 2007;

Andrews et al., 2010; Wolff et al., 2013) have used laboratory animal models involving genetic deletion of molecular clock genes. Bae et al., (2006) reported the differential effects of the two period genes (Per1 and Per2) on the physiology and proteomic profiles of mouse anterior tibialis muscles using 2DGE. Loss of the Per2 clock gene affected forced locomotor performance in mice but did not alter muscle contractility. At the proteome level, Per2 knockout altered the abundance of glycolytic enzymes (e.g. triosephosphate isomerase and beta-enolase) and the chaperone protein, Hsp90. However, relatively few biological replicates were analysed in Bae et al., 2006, which makes it difficult to know if the reported changes are statistically relevant. Moreover, this work failed to recognise that changes in the abundance of individual spots on a 2D gel often reflect changes in the post-translational state of a protein rather than a change in its total abundance (see Burniston 2008 and Burniston & Hoffman 2011 for discussion).

Muscle phenotype can be determined biochemically by analysing the relative abundances of each MyHC isoform. However, diurnal variations in muscle performance are unlikely to be due to changes in protein abundance because for most muscle proteins the time required to degrade and synthesise new proteins is much greater than 12 h. Using the proteomic technique of 2DGE it is possible to measure the relative abundance of each myofibrillar protein species encompassing different protein isoforms, splice-variants and post-translational states. Of particular interest are changes in reversible post-translational modifications (PTM) because these can occur within minutes to hours and could represent a feasible mechanism by which the muscle proteome differs during the course of the day.

Post-translational modifications are chemical modifications that are crucially important in regulating the basic biological functions and dynamics of proteins. There are many different types of PTM, among them, the most common and important PTM are phosphorylation, acetylation, glycosylation, methylation, sulfation, nitration, deamidation, and disulfide bond formation (Mann & Jansen, 2003). PTM function in various ways, for example, phosphorylation can reversibly activate/inactivate enzyme activity, regulate cellular processes including cell cycle, growth, apoptosis and signal transduction pathways; acetylation can regulate protein–deoxyribonucleic acid (DNA) interactions, and stabilize the structures of proteins; glycosylation can regulate cell-cell recognition and signalling, and affect the folding, conformation, stability, and activity of proteins; disulfide bonds commonly stabilize the secondary structure of proteins (Mann & Jansen, 2003). The study of PTMs can therefore provide invaluable insight into the cellular function processes.

Aim of this study

The current study will use state of art proteomic techniques described in Chapter 1 to simultaneously investigate modifications to numerous contractile proteins in human skeletal muscle. Information regarding changes at the protein level will be compared with measures of muscle performance to determine the level of association between muscle output and a particular protein modification. If successful, this work will provide candidate biomarkers that could be used to develop and optimise interventions aimed at forestalling morning decrements in muscle performance.

4.2 METHODS

To correlate functional and molecular responses it is necessary to obtain muscle samples by percutaneous needle biopsy. While invasive, this technique is established and routinely used in the Department for studies of this nature (e.g. Holloway 2009). A trained practitioner obtained biopsies from the vastus lateralis at 2 different time points (morning and evening). Samples of vastus lateralis were fractionated and sub-fractions enriched and myofibrillar proteins were 'mined' using GeLC-MS/MS, whereas differential profiling by 2DGE was used to investigate diurnal variations in the myofibrillar proteome.

4.2.1 Participants

Participants were selected from the cohort reported in Chapter 3, and their muscle function was assessed again using MVC and RFD, also described in the Chapter 3. Procedures were explained in full to the participants and questions were answered prior to participation. The study was approved by the National Research Ethics Committee (14/WM/0065) and was registered with the Liverpool John Moores University Research Ethics Committee (14/SPS/015 "Mechanisms underpinning diurnal variation in human muscle"). Inclusion criteria required previous strength training experience (≥ 2 years) with exclusion criteria stating recent shift work or travel across multiple time zones.

Muscle Performance testing

4.2.2 Protocol and Measures

The protocol and measurement were identical to the MVC, RFD and temperature measures reported in Chapter 3 and, for convenience, are illustrated in Figure 27. In the current work samples were provided from ten healthy young males randomly assigned to 2 counterbalanced groups: Group 1 (morning-evening) and Group 2 (evening-morning).

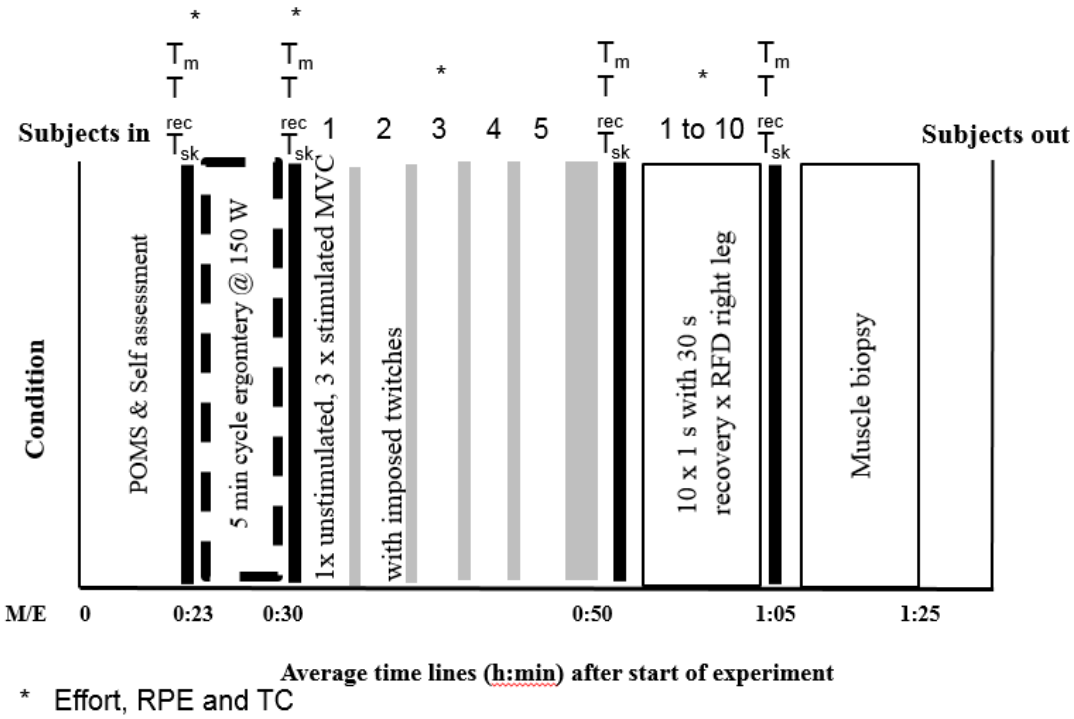


Figure 27. Schematic diagram of the testing protocol following biopsy for all conditions, rectal (T_{rec}), skin (T_{sk}) and muscle (T_m) temperatures and thermal comfort (TC) were measured after the participants had reclined for 30 min at the start of the protocol and after the warm-up. Rating of perceived exertion (RPE) was taken during warm-up and after each MVC, with rating of effort taken after each MVC. Black bars indicate muscle temperature taken at these points; grey bars indicate recovery between tests.

4.2.3 Quadriceps Muscle biopsies

Each participant underwent a quadriceps (vastus lateralis) muscle biopsy of their right leg in the morning (0700h) and evening (1700h). The biopsies were taken within 5 min of completing MVC and RFD measurements. Participants were placed comfortably on an examination couch in the supine position. Dorsiflexion of the ankle was used to help define the rectus femoris muscle, and the vastus lateralis was identified as lying along its lateral border. The biopsy site was marked as the junction between the upper third and lower two-thirds of the thigh (Figure 28). Muscle biopsies were obtained using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge x 10 cm length (Bard Biopsy System, Tempe, AZ). Samples (~15 mg) were obtained under local anesthesia (0.5% Marcaine). A small 1cm longitudinal stab incision was made and extended deeper down until the muscle fascia was pierced. The core biopsy needle was attached to the biopsy gun and inserted through the incision. A muscle biopsy was obtained by pressing the trigger button on the biopsy gun, which unloads the spring and activates the needle to collect muscle tissue. The sample was immediately frozen in liquid nitrogen and stored at -80C for further analysis. Using the same skin incision, 2 to 3 further biopsies were taken to ensure adequate quantities (~70 mg) of muscle tissue were available for analysis. At the end of the procedure, firm pressure was applied over the biopsy site for 5 minutes to prevent excessive bleeding. Sterile strips were then placed over the incision site and covered by a water-proof dressing. Finally a crepe bandage was firmly applied around the thigh in order minimize bleeding and bruising when the participant starts to mobilize. Instructions were provided to remove the crepe bandage after 8 hours, and to keep the biopsy site dry for about 2 to 3 days. No adverse effects were reported by participants of this study.

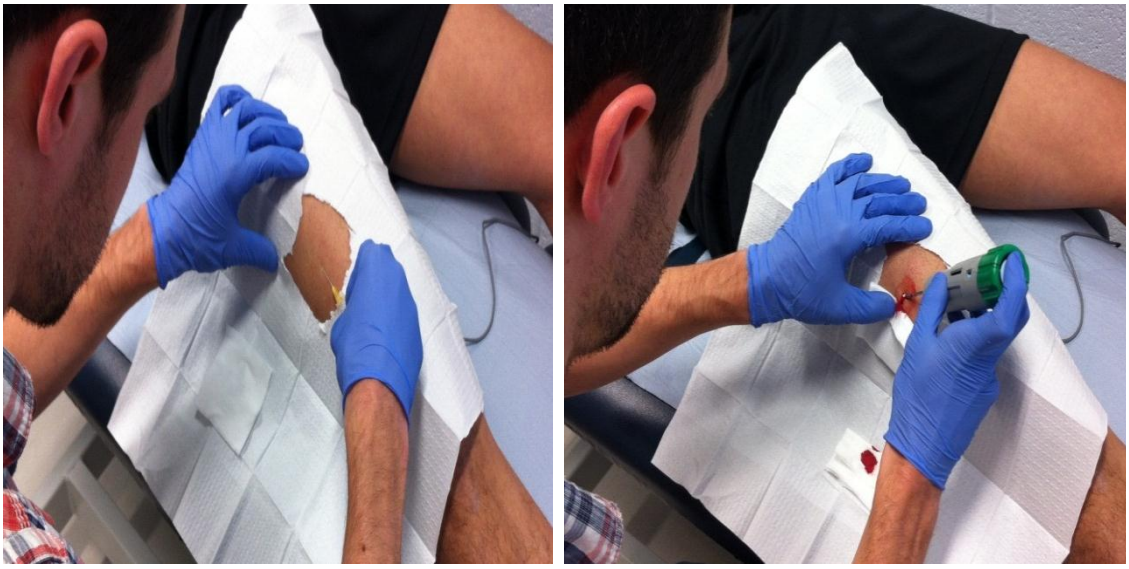


Figure 28. Quadriceps muscle biopsy procedure

4.2.4 Muscle processing

Muscle samples were ground using a pestle and mortar, under liquid nitrogen. The powder was accurately weighed (70 mg) and homogenised on ice in 10 volumes of lysis buffer, using a glass-teflon homogeniser. The lysis and solubilising buffer consisted of 1 % (v/v) Triton X-100, 40 mM Tris pH 7.4, phosphatase inhibitor and complete protease inhibitor cocktail (Roche, Indianapolis, USA). Homogenates were centrifuged at 1,000 x g, 4 °C for 10 min and the supernatant (containing soluble proteins) was decanted and stored in 100 µl aliquots at -80 °C. The current thesis reports analysis of the pellet that contains myofibrillar proteins. The pellet was re-suspended in 2DGE Lysis buffer containing 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 30 mM Tris including phosphatase and protease inhibitor cocktails (Roche). A Bradford Assay (Bradford, 1976) was performed as described previously in 1.2.7 and the samples were analysed using a variety of different protein/proteomic analysis (Figure 29).

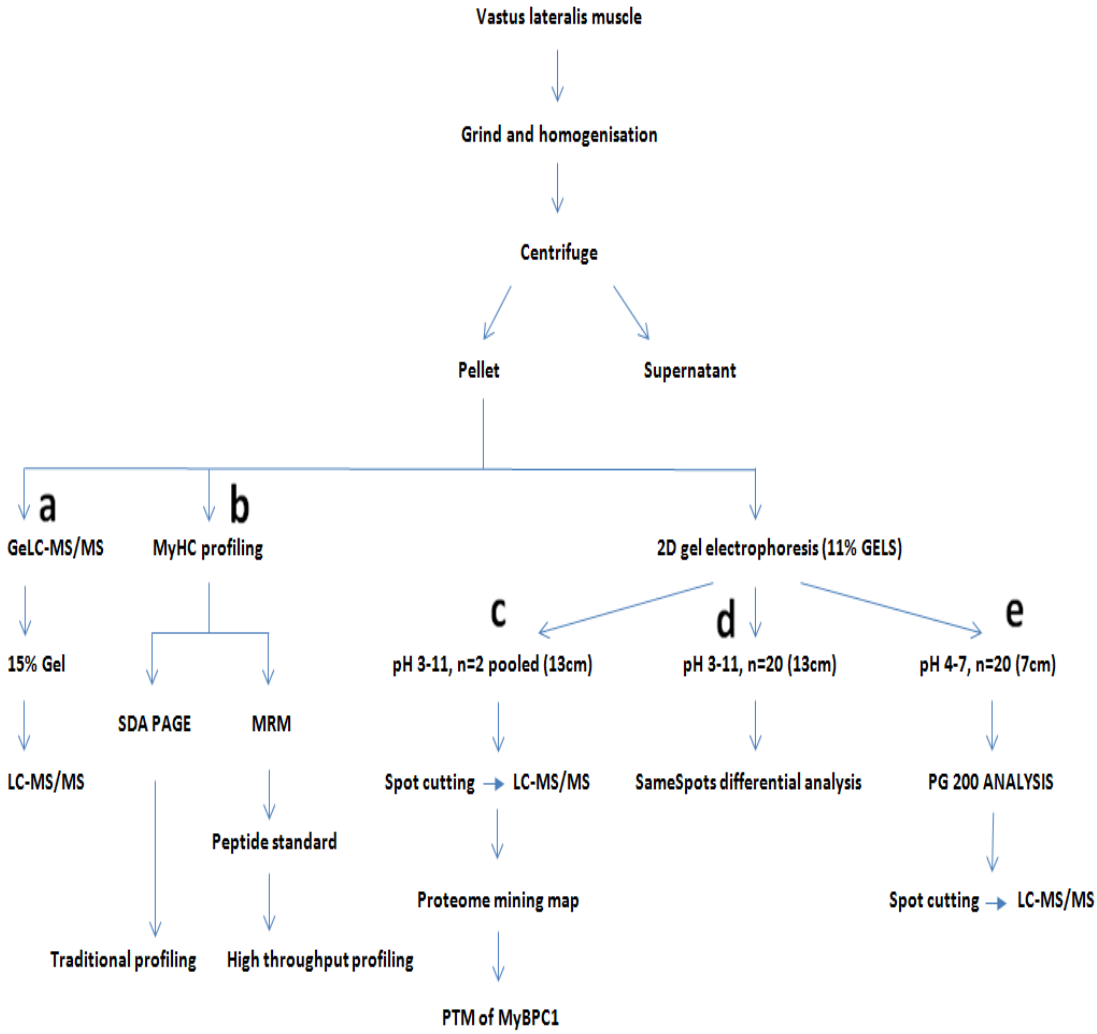


Figure 29. Several methods were used in this study including GeLC-MS/MS (**a**). MyHC profiling used traditional method by 1D SDS-PAGE and latest method with high throughput profiling (**b**). 2D gel electrophoresis was used to discover which myofibrillar proteins were resolved in to multiple species by several pH separation and size of gel like preparative pH 3-11 in 13 cm gel (**c**), pH 3-11 in 13 cm gel (**d**) and pH 4-7 in 7 cm gel (**e**).

4.2.5 Mining of the myofibrillar proteome by GeLC-MS/MS

A pooled sample was created from aliquots of the individual myofibrillar fractions and 50 μg was precipitated in 5 volumes of acetone and then resuspended in Laemmli buffer. The pooled protein sample was boiled (5 min at 95 ºC) separated by 15 % SDS-PAGE as described in Chapter 1 (Section 1.2.9). The entire gel lane, encompassing was cut in to 10 equal segments. Each segment was diced into 1 mm cubes and the proteins were digested with trypsin, as described in section 1.2.13. LC-ESI-MS/MS analysis of each of the 10 molecular weight fractions was performed according to section 1.2.15 and the protein identifications were combined in to a single non-redundant list that describes the entire catalogue of MS-accessible proteins in the myofibrillar fraction.

4.2.6 Analysis of Myosin heavy chain isoforms.

MyHC isoforms were analysed according to molecular weight by 8 % SDS-PAGE as described in Chapter 1. In addition, multiple reaction monitoring (MRM) of isoform specific peptides was performed. An aliquot of the myofibrillar fraction of human muscle was prepared for in-solution digest, by precipitating supernatants in 5 volumes of acetone and resuspended in Lysis Buffer: 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 30 mM Tris, containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK). After clearing by centrifugation (12,000 xg, 4 ºC for 45 min) protein concentrations were measured using the Bradford assay (Sigma, Poole, Dorset, UK) and each sample adjusted to 5 μg μl^{-1}. Aliquots containing 100 μg protein were precipitated in 5 volumes of acetone for 1 h at -20 ºC. Pellets were resuspended in 0.1% (w/v) Rapigest SF (Waters; Milford, MA) in 50 mM

ammonium bicarbonate and incubated at 80 ºC for 15 min. DTT was added (final concentration 1 mM) and incubated at 60 ºC for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide at 4 ºC. Sequencing grade trypsin (Promega; Madison, WI) was added at a protein ratio of 1:50 and digestion allowed to proceed at 37 ºC overnight. Digestion was terminated by the addition of 2 µL concentrated TFA and peptide solutions were cleared by centrifugation at 13 000 xg for 5 min.

MRM transitions (parent ion – fragment ion) for isoform specific peptides were 767.33-987.52 *m/z* for residues 387-400 (AAYLQNLNSADLLK) of MYH1 (MyHC IIx), 577.2-912.32 *m/z* for residues 1286-1295 (LQTESGEFSR) of MYH2 (MyHC IIa) and 773.35-917.38 *m/z* for residues 745-757 (LLSSLDIDHNQYK) of MYH7 (MyHC I). In addition, a pan-MyHC isoform peptide (LASADIETYLLEK) was monitored using the transition 733.3-895.41 *m/z*.

4.2.7 Differential profiling of Myofibrillar proteins by 2DGE

Samples were precipitated in acetone and resuspended in rehydration containing 7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 20 mM dithiothreitol, and 0.5% (v/v) ampholytes. Broad-range analysis of myofibrillar proteins was conducted using large-format (13 cm) 2D gels over the range pH 3-11. In addition, smaller format (7 cm) narrow-range (pH 4-7) gels were used to specifically investigate differences in myosin light chain protein species.

Large-format 2D gels were loaded with 250 µg of protein and isoelectric focusing was conducted as follows: 300 V for 3 h, 600 V for 3 h, linear gradient to 8,000 V over 3 h and 8,000 for 4 h. The IPG strips were then equilibrated in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) sodium dodecylsulfate, and a trace of bromophenol blue. Proteins were electrophoresed through a linear 11 % polyacrylamide gel at 20 °C; initially at a constant current of 15 mA per gel for 30 min and then at 30 mA per gel. Following the process, the gel was removed from the glass plates and washed 3 times in ddH_2O, with each wash taking ~5 minutes. The ddH_2O was poured off and then the gels were stained in 100 ml of colloidal Coomassie blue (Bio-safe, Bio-rad laboratories, Hercules, USA) for 1 hour before being de-stained overnight in ddH_2O. The gel image was analysed using Progenesis SameSpots software to detect within participant differences between morning and evening samples.

Small-format 2D gels, similar to those used in section 1.2.11, were loaded with 40 µg protein. Isoelectric focusing was conducted as follows: 50 V for 15 min, 250 V for 15 min, linear gradient to 4,000 V over 2 h and 4,000 for 2 h. The IPG strips were then equilibrated in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) sodium dodecylsulfate, and a trace of bromophenol blue. Proteins were electrophoresed through a linear 15 % polyacrylamide gel at 20 °C; initially at a constant voltage of 100 V per gel for 30 min and then at 200 V per gel. After electrophoresis, the gel was removed from the glass plates and washed 3 times in ddH_2O, with each wash taking ~5 minutes. The ddH_2O was poured off and then the gels were stained in 100ml of colloidal Coomassie blue (Bio-safe, Bio-rad

laboratories, Hercules, USA) for 1 hour before being de-stained overnight in ddH_2O. Progenesis PG 2000 software was used to analyse within participant differences.

4.2.8 In-gel digestion and LC-MS

Standard in-gel digestion was performed as described in section 1.2.13. In addition for identification of phosphorylated proteins, HyperSep^{TM} SpinTip Porous Titanium Dioxide was used to enrich phosphopetpides according to the manufacturer instructions. Briefly, each SpinTip was washed by 50 µl of 50 mM formic acid and repeated 3 times. Fifty microliters of sample digest was added in the SpinTip and air pressured was applied to force the solution through the porous TiO_2 material. Then the packed bed was washed 3 times with 50 µl of ddH_2O and the solution labelled as Non-Phosphorylation. The bound molecule from the packed bed was eluted with 50 µl of 50 mM Ambic and the solution labelled as Phosphorylated peptides.

4.2.9 Protein identification

Mass spectrometry analysis was performed using a quadrupole-high capacity ion-trap (HCT Ultra ETD II; Bruker Daltonics, Bremen, Germany) coupled online via an electrospray ionisation source to a nano-flow HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA). MS/MS spectra were exported in Mascot generic format and searched against the Swiss-Prot database (2011.6) restricted to 'Human' using a locally implemented Mascot (www.matrixscience.com) server (version 2.2.03). The enzyme specificity was trypsin allowing 1 missed cleavage, carbamidomethyl modification of cysteine (fixed), deamidation of asparagine and glutamine (variable),

oxidation of methionine (variable) and an m/z error of ± 0.5 Da. Post-translational modifications were analysed using error tolerant searches followed by modification specific searches that included either phosphorylation (S,T,Y) or acetylation (K) as variable modifications.

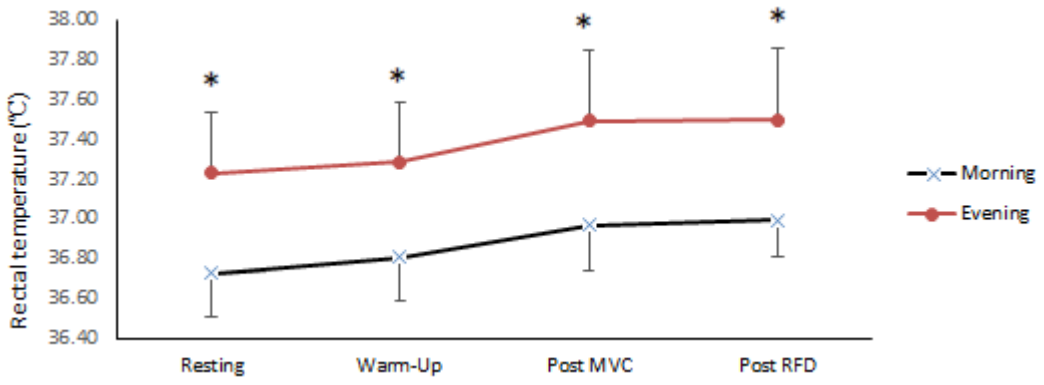
4.2.10 Statistical Analysis

The data were analysed using the Statistical Package for Social Sciences (SPSS) for Windows (SPSS, Chicago, IL, USA) version 20. Comparison by paired sample t-test was used to test the primary hypothesis and identify presence of a diurnal variation. The results are presented as the mean $\pm$ the standard deviation (SD) throughout the text unless otherwise stated. Ninety-five percent confidence interval (CIs) are presented where appropriate. The alpha level of significance was set at 5% ($p < .05$).

4.3 RESULTS

4.3.1 Characteristics of human muscles samples

Ten male participants were recruited of age (mean±SD) = 26.7 ± 3.7 years; height 177.8 ± 8.4 cm, body mass 74.9 ± 9.8 kg with habitual retiring and waking times of 11:24 ± 0:36 p.m. and 06:12 ± 0:12 h a.m., respectively. Mean chronotype score was 35.5 ± 3.1 (all intermediate types), F/R score 44.9 ± 5.9; and L/V score, 44.8 ± 8.0. Procedures were explained in full to participants with questions answered prior to participation. POMS data identified significantly greater "happy" and "vigour" score in the evening than in the morning. Throughout the testing protocol, both core temperature (T_{rec}) and muscle temperature were significantly greater in the evening than in the morning (Figure 30).



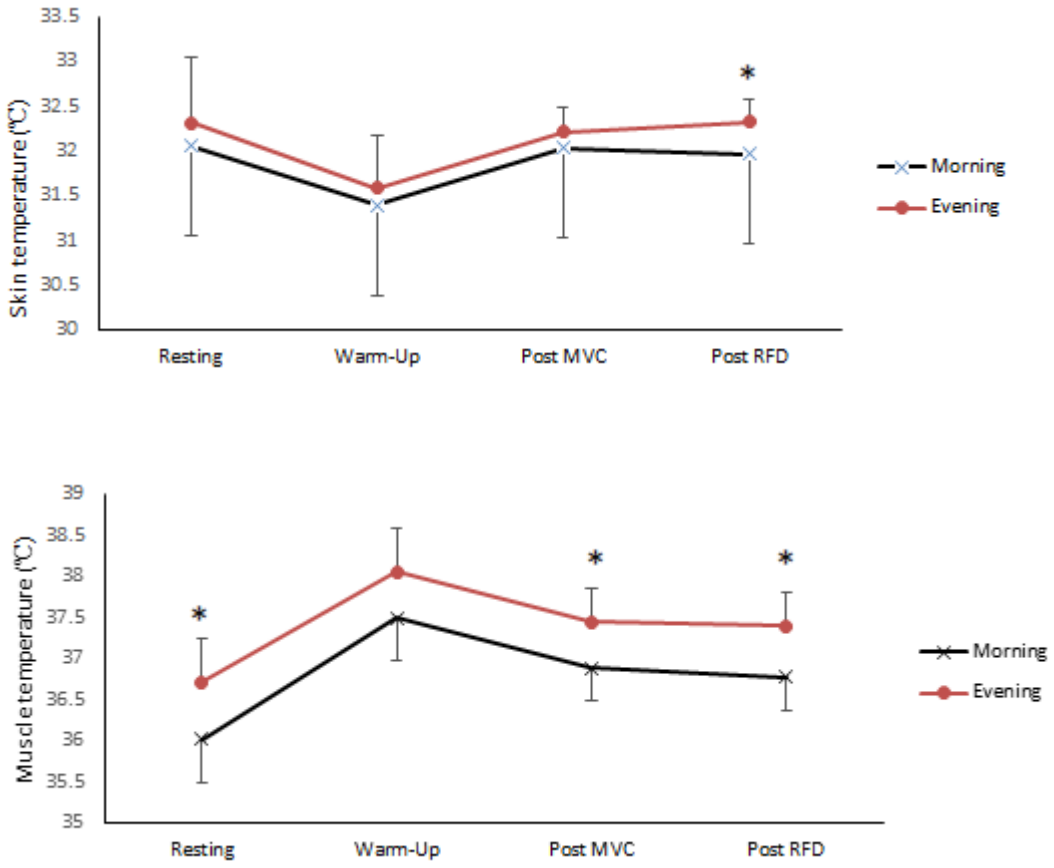


Figure 30. Means and standard deviations for rectal, skin and muscle temperatures measured at rest, post warm-up, post MVC and post RFD. * Significantly higher than morning ($p<0.05$).

Muscle Performance testing

4.3.2 MVC and RFD

Consistent with our findings in Chapter 3, MVC was significantly greater (11%) in the evening compared to morning (Table 22). There was no statistically significant difference in the level of voluntary activation between morning and evening conditions. Peak RFD ($N \cdot s^{-1}$) was significantly greater (15.7 %) in the evening than in the morning. There were no significant differences in the rate of perceived exertion (RPE) or thermal comfort (TC).

Table 22. Diurnal differences in muscle performance.

	Morning	Evening	P value	(%)
MVC force (Nm)	679.9 ± 104.9	754.4 ± 112.9*	0.004	11.0
MVC activation (%)	85.5 ± 8.7	87.2 ± 7.9	0.532	2.0
Peak RFD ($N.s^{-1}$)	6077.3 ± 1371.7	7030.5 ± 1100.7*	0.008	15.7
Time to Peak force (ms)	76.6 ± 15.2	70.8 ± 12.5	0.214	8.2
RPE				
During Warm-Up	10.9 ± 2.2	10.9 ± 2.0	1.000	
During MVC efforts	14.0 ± 3.1	13.2 ± 3.3	0.104	
Thermal Comfort				
Resting	4.8 ± 0.3	4.9 ± 0.3	1.000	
During Warm-Up	5.9 ± 0.6	6.0 ± 0.8	0.591	

Data are presented as means ± SD; n = 10 repeated measures taken in the morning and evening. * Significantly different from morning condition.

Analysis of human muscle proteins

4.3.3 GeLC-MS/MS mining of the human myofibrillar fraction

GeLC-MS/MS analysis was used to catalogue proteins in the myofibrillar fraction. In total 193 proteins were detected (Table 23) and 33 of these proteins were specifically associated with the myofibrillar apparatus and identified with high degrees of confidence and the protein list (Table 24) encompassed the majority of expected thin and thick filament proteins of the myofibrillar apparatus (Figure 31).

Table 23. Entire list of proteins detected in the myofibrillar fraction.

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
1433E	14-3-3 protein epsilon	217	29326	4.63	26.7	10
1433G	14-3-3 protein gamma	118	28456	4.8	16.6	6
GRP78	78 kDa glucose-regulated protein	103	72402	5.07	4.1	3
THIL	Acetyl-CoA acetyltransferase, mitochondrial	74	45456	8.98	10.1	4
ASM3A	Acid sphingomyelinase-like phosphodiesterase 3a	41	51741	5.88	1.8	5
ACTC	Actin, alpha cardiac muscle 1	1554	42334	5.23	67.1	133
ACTS	Actin, alpha skeletal muscle	3750	42366	5.23	73.2	288
ACTA	Actin, aortic smooth muscle	1515	42381	5.23	64.2	126
ACTB	Actin, cytoplasmic 1	1597	42052	5.29	64.3	157
ACTG	Actin, cytoplasmic 2	99	42108	5.31	9.9	8
ACTH	Actin, gamma-enteric smooth muscle	1445	42249	5.31	60.9	116
ACYP2	Acylphosphatase-2	64	11190	9.52	30.3	3
ADT1	ADP/ATP translocase 1	217	33271	9.78	24.5	12
ADT	ADP/ATP translocase 2	101	33059	9.71	12.8	7
ADT3	ADP/ATP translocase 3	113	33073	9.76	16.4	8
A1AT	Alpha-1-antitrypsin	96	46878	5.37	10.5	6
ACTN1	Alpha-actinin-1	122	103563	5.25	2.6	3
ACTN2	Alpha-actinin-2	1387	104358	5.31	38	103
ACTN3	Alpha-actinin-3	407	103917	5.37	23.5	34
ENOA	Alpha-enolase	410	47481	7.01	32.3	23
ALMS1	Alstrom syndrome protein 1	37	463078	5.87	0.4	4
AATC	Aspartate aminotransferase, cytoplasmic	132	46447	6.52	18.9	7
AATM_	Aspartate aminotransferase, mitochondrial	77	47886	9.14	6.5	3

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
ATPA	ATP synthase subunit alpha, mitochondrial	421	59828	9.16	29.3	23
ATPB	ATP synthase subunit beta, mitochondrial	457	56525	5.26	35.9	22
ATPG	ATP synthase subunit gamma, mitochondrial	57	33032	9.23	10.1	4
ACTBL	Beta-actin-like protein 2	592	42318	5.39	23.1	41
ENOB	Beta-enolase	767	47244	7.59	48.2	45
CASQ1	Calsequestrin-1	319	44471	4.02	23.8	16
CAH1	Carbonic anhydrase 1	446	28909	6.59	57.1	22
CAH2	Carbonic anhydrase 2	142	29285	6.87	27.7	8
CAH3	Carbonic anhydrase 3	477	29824	6.86	55	24
CATA	Catalase	58	59947	6.9	6.5	3
CHCH3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial	137	26421	8.48	15	4
KCRM	Creatine kinase M-type	982	43302	6.77	54.1	61
KCRS	Creatine kinase S-type, mitochondrial	97	47988	8.46	15	7
CSRP3	Cysteine and glycine-rich protein 3	159	21867	8.89	23.2	5
QCR1	Cytochrome b-c1 complex subunit 1, mitochondrial	88	53297	5.94	8.5	5
DESM	Desmin	1955	53560	5.21	57.4	90
EF1A1	Elongation factor 1-alpha 1	92	50451	9.1	11.5	7
EF1A2	Elongation factor 1-alpha 2	92	50780	9.11	8.9	6
CAZA2	F-actin-capping protein subunit alpha-2	68	33157	5.57	12.6	3
FABPH	Fatty acid-binding protein, heart	90	14906	6.29	12.8	4
FHL1	Four and a half LIM domains protein 1	129	38006	9.25	7.1	3
ALDOA	Fructose-bisphosphate aldolase A	342	39851	8.3	41.2	28
ALDOB	Fructose-bisphosphate aldolase B	47	39961	8	1.9	4
ALDOC	Fructose-bisphosphate aldolase C	70	39830	6.41	8.2	7
LEG1	Galectin-1	79	15048	5.34	23.7	3

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
ENOG	Gamma-enolase	173	47581	4.91	12	10
GELS	Gelsolin	47	86043	5.9	3.1	3
G3P	Glyceraldehyde-3-phosphate dehydrogenase	688	36201	8.57	47.5	38
PYGB	Glycogen phosphorylase, brain form	85	97319	6.4	4.6	7
PYGL	Glycogen phosphorylase, liver form	74	97486	6.71	3.7	7
PYGM	Glycogen phosphorylase, muscle form	207	97487	6.57	18.8	32
HSP71	Heat shock 70 kDa protein 1A/1B	165	70294	5.48	16.8	11
HSP7C	Heat shock cognate 71 kDa protein	290	71082	5.37	17	13
HSPB1	Heat shock protein beta-1	253	22826	5.98	47.8	17
HSP72	Heat shock-related 70 kDa protein 2	202	70263	5.56	11.4	9
HB	Hemoglobin subunit alpha	620	15305	8.72	62	51
HB	Hemoglobin subunit beta	1925	16102	6.75	84.4	133
HBD	Hemoglobin subunit delta	895	16159	7.85	83	71
HBE	Hemoglobin subunit epsilon	113	16249	8.67	6.8	6
HBG1	Hemoglobin subunit gamma-1	113	16187	6.64	6.8	6
HBG2	Hemoglobin subunit gamma-2	113	16173	6.64	6.8	6
H4	Histone H4	70	11360	11.36	17.5	3
IGHG1	Ig gamma-1 chain C region	163	36596	8.46	18.2	11
IGHG2	Ig gamma-2 chain C region	44	36505	7.66	8.6	7
IGHG3	Ig gamma-3 chain C region	161	42287	8.23	11.4	9
IGHG4	Ig gamma-4 chain C region	96	36431	7.18	13.1	7
IGKC	Ig kappa chain C region	241	11773	5.58	65.1	13
LAC2	Ig lambda-2 chain C regions	80	11458	6.92	46.2	5
LAC7	Ig lambda-7 chain C region	77	11467	8.49	32.1	4
IGLL5	Immunoglobulin lambda-like polypeptide 5	64	23391	9.08	17.8	3
IDHP	Isocitrate dehydrogenase [NADP], mitochondrial	386	51333	8.88	28.3	19

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
KBTBA	Kelch repeat and BTB domain-containing protein 10	328	68792	5.14	29.9	22
KT81L	Keratin-81-like protein	107	54972	6.05	7.8	8
K1H1	Keratin, type I cuticular Ha1	43	48633	4.84	7	5
K1H	Keratin, type I cuticular Ha2	41	51793	4.78	4.5	4
KT33A	Keratin, type I cuticular Ha3-I	32	47166	4.78	17.1	8
KT33B	Keratin, type I cuticular Ha3-II	43	47325	4.81	6.7	4
KRT36	Keratin, type I cuticular Ha6	36	53354	4.9	3.4	3
KRT38	Keratin, type I cuticular Ha8	36	52044	4.79	3.5	3
K1C10	Keratin, type I cytoskeletal 10	1038	59020	5.13	39.2	52
K1C13	Keratin, type I cytoskeletal 13	64	49900	4.91	4.1	3
K1C14	Keratin, type I cytoskeletal 14	255	51872	5.09	15.3	15
K1C15	Keratin, type I cytoskeletal 15	135	49409	4.71	6.1	6
K1C16	Keratin, type I cytoskeletal 16	238	51578	4.99	7.8	10
K1C17	Keratin, type I cytoskeletal 17	67	48361	4.97	8.8	8
K1C19	Keratin, type I cytoskeletal 19	36	44079	5.04	6	5
K1C24	Keratin, type I cytoskeletal 24	47	55567	4.89	3.4	4
K1C25	Keratin, type I cytoskeletal 25	229	49858	5	4	8
K1C26	Keratin, type I cytoskeletal 26	137	52620	4.86	1.9	4
K1C27	Keratin, type I cytoskeletal 27	111	50419	4.98	3.9	5
K1C28	Keratin, type I cytoskeletal 28	236	51163	5.33	5.6	12
K1C9	Keratin, type I cytoskeletal 9	362	62255	5.14	29.4	19
KRT81	Keratin, type II cuticular Hb1	110	56832	5.4	12.1	13
KRT83	Keratin, type II cuticular Hb3	114	55928	5.54	13.8	14
KRT85	Keratin, type II cuticular Hb5	77	57306	6.28	11.6	12
K2C1	Keratin, type II cytoskeletal 1	1154	66170	8.15	34.9	49
K2C1B	Keratin, type II cytoskeletal 1b	183	62149	5.73	8.3	11

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
K22E	Keratin, type II cytoskeletal 2 epidermal	867	65678	8.07	40.7	37
K22O	Keratin, type II cytoskeletal 2 oral	75	66370	8.38	4.7	5
K2C3	Keratin, type II cytoskeletal 3	69	64549	6.12	7.2	8
K2C5	Keratin, type II cytoskeletal 5	179	62568	7.59	11.7	10
K2C6A	Keratin, type II cytoskeletal 6A	130	60293	8.09	13.3	11
K2C6B	Keratin, type II cytoskeletal 6B	167	60315	8.09	12.1	13
K2C7	Keratin, type II cytoskeletal 7	42	51411	5.4	2.6	3
K2C73	Keratin, type II cytoskeletal 73	43	59457	6.93	11.7	8
K2C75	Keratin, type II cytoskeletal 75	148	59809	7.6	8.5	7
K2C79	Keratin, type II cytoskeletal 79	90	58085	6.75	4.5	4
K2C8	Keratin, type II cytoskeletal 8	42	53671	5.52	2.5	3
LDHA	L-lactate dehydrogenase A chain	199	36950	8.44	22.6	11
LDHB	L-lactate dehydrogenase B chain	70	36900	5.71	8.4	3
LDB3	LIM domain-binding protein 3	548	78226	8.47	19.5	25
MDHC	Malate dehydrogenase, cytoplasmic	117	36631	6.91	14.4	7
IMMT	Mitochondrial inner membrane protein	61	84026	6.08	8.6	6
MYG	Myoglobin	304	17230	7.14	42.9	15
MYL1	Myosin light chain 1/3, skeletal muscle isoform	767	21189	4.97	69.1	27
MYL3	Myosin light chain 3	418	22089	5.03	54.4	16
MYL10	Myosin regulatory light chain 10	65	25520	5.56	11.5	4
MLRS	Myosin regulatory light chain 2, skeletal muscle isoform	698	19116	4.91	69.8	26
MLRV	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	334	18777	4.92	61.4	15
MYH1	Myosin-1	1273	223976	5.59	24.4	101
MYH13	Myosin-13	162	224605	5.54	4.2	15
MYH2	Myosin-2	1765	223932	5.64	31	133

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
MYH3	Myosin-3	137	224850	5.62	3.6	6
MYH4	Myosin-4	163	223902	5.65	3.6	6
MYH6	Myosin-6	645	224394	5.58	12.4	32
MYH7	Myosin-7	1726	223757	5.63	30.2	125
MYH	Myosin-8	732	223594	5.59	11.9	40
MYPC1	Myosin-binding protein C, slow-type	207	129240	5.78	14.1	23
MYOTI	Myotilin	456	55760	9.18	29.7	27
MYOZ1	Myozenin-1	398	31725	8.86	35.8	14
NDUAD	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	53	16688	8.04	20.8	4
NDUA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	99	9421	9.42	37	6
NDUV1	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	54	51469	8.51	5.8	4
NEB	Nebulin	103	775406	9.11	1.7	15
SYNE1	Nesprin-1	41	1017069	5.38	0.4	5
NFL	Neurofilament light polypeptide	62	61536	4.64	1.7	3
PDLI5	PDZ and LIM domain protein 5	274	65102	8.55	17.3	17
PRDX6	Peroxiredoxin-6	53	25133	6	21	4
PGM1	Phosphoglucomutase-1	268	61696	6.3	22.2	13
PGK1	Phosphoglycerate kinase 1	43	44985	8.3	12	3
PGAM1	Phosphoglycerate mutase 1	69	28900	6.67	13.8	6
PGAM2	Phosphoglycerate mutase 2	94	28919	8.99	30.4	10
POTEE	POTE ankyrin domain family member E	603	122882	5.83	8.3	39
POTEF	POTE ankyrin domain family member F	603	123020	5.83	6.1	37
POTEI	POTE ankyrin domain family member I	88	122858	5.83	3.4	11

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
POTEJ	POTE ankyrin domain family member J	182	118740	5.66	3.8	20
LMNA	Prelamin-A/C	43	74380	6.57	10.1	7
ABEC2	Probable C->U-editing enzyme APOBEC-2	93	25915	4.81	19.6	6
PGAM4	Probable phosphoglycerate mutase 4	51	28930	6.19	9.4	3
PHB_H	Prohibitin	44	29843	5.57	8.5	3
AHNK2	Protein AHNAK2	39	617383	5.2	0.1	3
NIPS2	Protein NipSnap homolog 2	75	33949	9.42	23.1	8
ACTBM	Putative beta-actin-like protein 3	276	42331	5.91	7.2	14
YI016	Putative tubulin beta chain-like protein ENSP00000290377	64	42204	4.77	11.3	5
TBB4Q	Putative tubulin beta-4q chain	59	48917	5.11	6.5	4
ODPB	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	88	39550	6.2	7.5	3
KPYM	Pyruvate kinase isozymes M1/M2	492	58470	7.96	36	28
ALBU_	Serum albumin	1323	71317	5.92	63.9	110
TBC19	TBC1 domain family member 19	40	60455	5.67	1.5	3
TITIN	Titin	62	3843119	6.01	0.2	8
ECHB_	Trifunctional enzyme subunit beta, mitochondrial	158	51547	9.45	18.4	11
TPIS_	Triosephosphate isomerase	706	26938	6.45	55	29
TRI72	Tripartite motif-containing protein 72	155	53609	6.05	8.6	5
TPM1_	Tropomyosin alpha-1 chain	1767	32746	4.69	51.1	131
TPM3_	Tropomyosin alpha-3 chain	1650	32856	4.68	52.8	113
TPM4_	Tropomyosin alpha-4 chain	1022	28619	4.67	21.8	61
TPM2_	Tropomyosin beta chain	1390	32945	4.66	50.4	103
TNNC1	Troponin C, slow skeletal and cardiac muscles	347	18505	4.04	24.2	24
TNNI2	Troponin I, fast skeletal muscle	120	21496	8.87	20.3	6

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	Number of peptides
TNNI1	Troponin I, slow skeletal muscle	204	21850	9.61	23	7
TNNT2	Troponin T, cardiac muscle	40	35902	4.94	6.7	7
TNNT3	Troponin T, fast skeletal muscle	526	31805	5.71	36.8	34
TNNT1	Troponin T, slow skeletal muscle	381	32985	5.86	30.9	31
TBA1A	Tubulin alpha-1A chain	66	50788	4.94	9.5	3
TBA4A	Tubulin alpha-4A chain	45	50634	4.95	11.6	6
TBB5_	Tubulin beta chain	226	50095	4.78	29.5	13
TBB1_	Tubulin beta-1 chain	59	50865	5.05	6.2	4
TBB2A	Tubulin beta-2A chain	160	50274	4.78	23.4	10
TBB2B	Tubulin beta-2B chain	160	50377	4.78	23.4	10
TBB2C	Tubulin beta-2C chain	160	50255	4.79	23.4	10
TBB4_	Tubulin beta-4 chain	160	50010	4.78	18.9	8
TBB6_	Tubulin beta-6 chain	88	50281	4.77	10.8	6
TBB8_	Tubulin beta-8 chain	64	50257	4.79	9.5	5
TBB8B	Tubulin beta-8 chain B	64	50168	4.75	9.5	5
CH074	Uncharacterized protein C8orf74	46	33942	7.74	2.7	3
ACADV	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	194	70745	8.92	9.9	5
VIME_	Vimentin	375	53676	5.06	35.6	27
VDAC1	Voltage-dependent anion-selective channel protein 1	199	30868	8.62	25.8	11
VDAC2	Voltage-dependent anion-selective channel protein 2	128	32060	7.49	21.4	6
VDAC3	Voltage-dependent anion-selective channel protein 3	214	30981	8.85	19.1	6

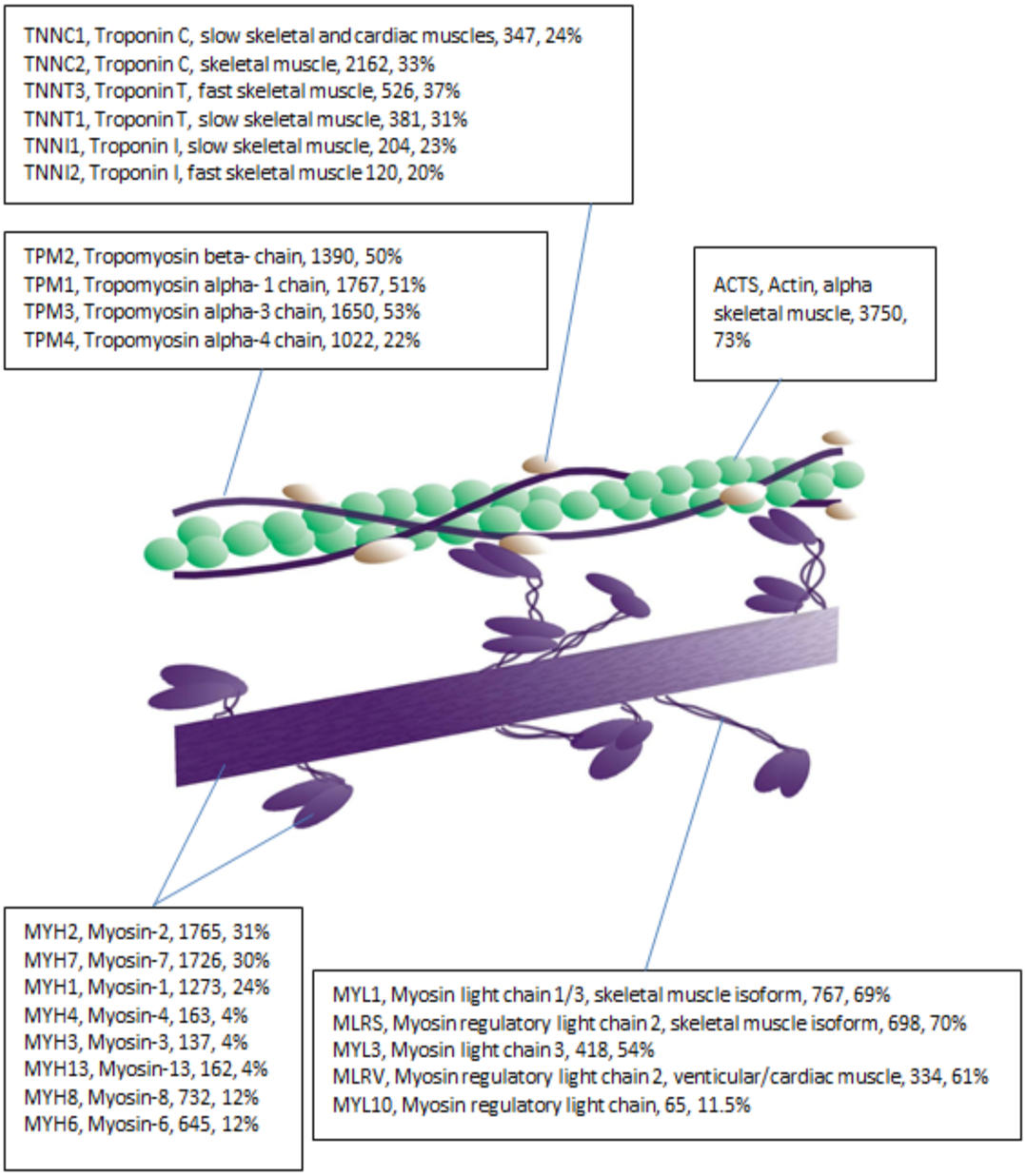


Figure 31. Scheme of structure of thin filament and thick filament; and its main protein components that are known to exist as multiple isoforms from 15% gels.

Table 24. Proteins detected by GeLC-MS/MS of the myofibrillar fraction of human vastus lateralis.

UniProt Accession	Protein Description	MOWSE score	Mass (Da)	Isoelectric point (pH)	Sequence coverage (%)	No of peptides
ACTS	Actin, alpha skeletal muscle	3750	42366	5.23	73.2	288
DESM	Desmin	1955	53560	5.21	57.4	90
TPM1	Tropomyosin alpha-1 chain	1767	32746	4.69	51.1	131
MYH2	Myosin-2	1765	223932	5.64	31	133
MYH7	Myosin-7	1726	223757	5.63	30.2	125
TPM3	Tropomyosin alpha-3 chain	1650	32856	4.68	52.8	113
ACTC	Actin, alpha cardiac muscle 1	1554	42334	5.23	67.1	133
TPM2	Tropomyosin beta chain	1390	32945	4.66	50.4	103
MYH1	Myosin-1	1273	223976	5.59	24.4	101
TPM4	Tropomyosin alpha-4 chain	1022	28619	4.67	21.8	61
MYL1	Myosin light chain 1/3, skeletal muscle isoform	767	21189	4.97	69.1	27
MYH8	Myosin-8	732	223594	5.59	11.9	40
MLRS	Myosin regulatory light chain 2, skeletal muscle isoform	698	19116	4.91	69.8	26
MYH6	Myosin-6	645	224394	5.58	12.4	32
TNNT3	Troponin T, fast skeletal muscle	526	31805	5.71	36.8	34
MYOTI	Myotilin	456	55760	9.18	29.7	27
MYL3	Myosin light chain 3	418	22089	5.03	54.4	16
MYOZ1	Myozenin-1	398	31725	8.86	35.8	14
TNNT1	Troponin T, slow skeletal muscle	381	32985	5.86	30.9	31
TNNC1	Troponin C, slow skeletal and cardiac muscles	347	18505	4.04	24.2	24
MLRV	Myosin regulatory light chain 2, ventricular/ muscle isoform	334	18777	4.92	61.4	15
MYPC1	Myosin-binding protein C, slow-type	207	129240	5.78	14.1	23
TNNI1	Troponin I, slow skeletal muscle	204	21850	9.61	23	7

MYH4	Myosin-4	163	223902	5.65	3.6	6
MYH13	Myosin-13	162	224605	5.54	4.2	15
MYH3	Myosin-3	137	224850	5.62	3.6	6
TNNI2	Troponin I, fast skeletal muscle	120	21496	8.87	20.3	6
NEBU	Nebulin	103	775406	9.11	1.7	15
CAZA2	F-actin-capping protein subunit alpha-2	68	33157	5.57	12.6	3
MYL10	Myosin regulatory light chain 10	65	25520	5.56	11.5	4
TITIN	Titin	62	3843119	6.01	0.2	8
GELS	Gelsolin	47	86043	5.9	3.1	3
TNNT2	Troponin T, cardiac muscle	40	35902	4.94	6.7	7

Data represent myofibrillar proteins were identified by mass spectrometry and database searches, proteins are ranked by database score, highest to lowest.

4.3.4 Traditional muscle phenotyping: Analysis of Myosin heavy chain isoforms by SDS-PAGE.

Figure 32 shows representative images and analysis of MyHC band volumes. Using this method we were able to separate MyHC isoforms into 3 distinct bands representing type IIx, type IIa and type I isoforms. The average percentages of each isoform were 42.5 % MyHC I, 48.3 % MyHC IIa and 9.2 % MyHC IIx (Table 25).

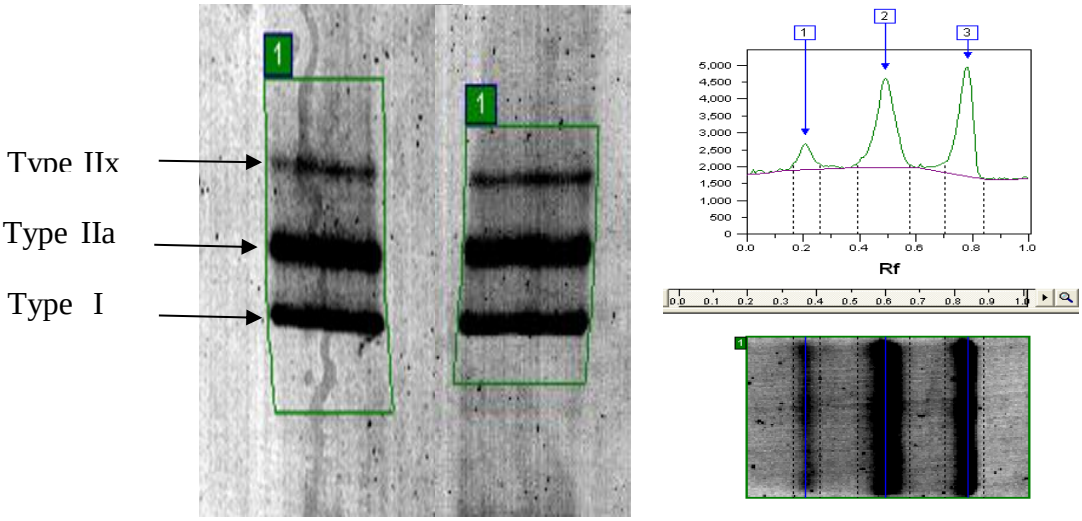


Figure 32. Separation of MyHC isoforms from Diurnal pooled sample by SDS-PAGE. (A) Representative image of gel lanes showing separation of MyHC isoforms. (B) Density plot showing area of interest, in to distinct bands (IIx, IIa, I) based in differences in migration (Pooled standard sample from all participants). (C) Images of lanes aligned with the area of interest.

Table 25. Technical variation of duplicate analysis of 'pooled standard'.

Band	Lane 1 (Volume)	%	Lane 2 (Volume)	%	Volume Mean ± SD	% Mean ± SD
IIx	824	9.3	864	9.0	844 ± 28	9.2 ± 0.2
IIa	4123	46.7	4772	49.9	4448 ± 459	48.3 ± 2.3
I	3881	44.0	3935	41.1	3908 ± 38	42.5 ± 2.1

Data represented 'pooled standard' proteins were identified by T120 software which enables the concentration of each band to be measured by densitometry and can be used to calculate the relative percentage of each MyHC isoform. Data represent band volume (arbitrary units) and relative percentage for each band from pooled standard sample in two different lanes.

Morning and evening samples from each participant were separated using this optimised method (examples from 2 participants are shown in Figure 33). As might be expected, there was no difference in the relative abundance of IIx, IIa and I MyHC isoforms between the morning and evening, but this analysis afforded the calculation of the reproducibility of this technique (Table 26). The coefficient of variation for MyHC isoform analysis ranged from 6.4 % to 3.2 %.

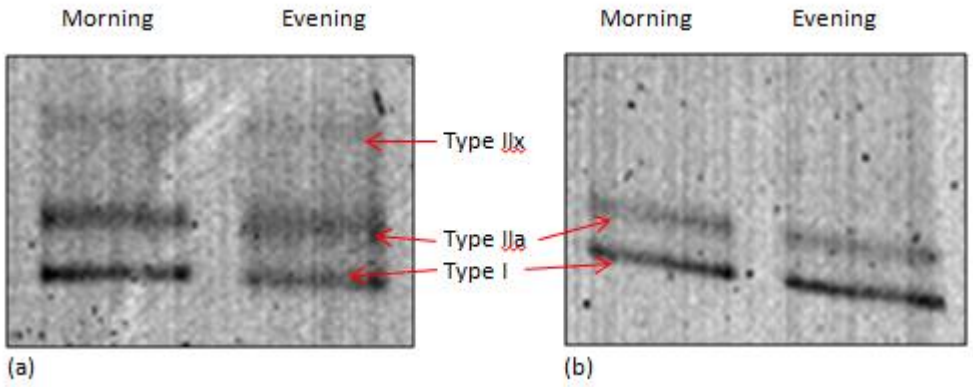


Figure 33. (a) Separation of myofibrillar isoforms by SDS-PAGE into distinct bands (IIx, IIa, I) based in differences in migration. (b) Example of non-existence of type IIx in one of the participant.

Table 26. Coefficient of variation of MyHC isoform analysis.

Band	Average %	Range %	CV %	p value
IIx	6.8 ± 7.8	(0 - 21.5)	6.4	0.194
IIa	52.8 ± 14.5	(33.7 - 93.6)	3.2	0.181
I	40.4 ± 11.9	(6.4 - 57.4)	3.9	0.305

Data are from n = 10 participants (biological replicates)

4.3.5 MRM myofibrillar proteins profiling.

SDS-PAGE analysis of MyHC isoforms exhibits good levels of reproducibility (Table 26) but this technique is relative time consuming so we also investigated the use of multiple reaction monitoring (MRM) to perform similar MyHC isoform profiling. The intensity of a peptide ion is dependent upon both the abundance of the peptide and also its readiness to undergo ionisation, therefore we first analysed mixtures of synthetic peptides produce by a commercial supplier. Table 27 reports the abundance measurements of MRM analysis of an equimolar mixture of isoform-specific and pan-isoform MyHC peptides. The peptide mixture was analysed in duplicate and the coefficient of variation for each peptide was less than 3 %. A multiplication factor was derived from these data that could be used to calculate the abundance of each isoform in 'unknown' muscle samples relative to the pan-isoform internal control (Table 28).

Table 27. MRM analysis of synthetic peptide of MyHC isoforms.

	Pan	Type I	Type IIa	Type IIx
	66758	25350	149791	64820
	69157	26320	143867	66610
Mean	67957	25835	146829	65715
SD	1696	685	4189	1266
CV	0.0249636	0.026531257	0.028527074	0.019260351
%CV	2.5	2.7	2.9	1.9
	Multiplication factor	0.38016714	2.160613026	0.967012822

Table 28. MRM analysis of MyHC isoforms in human muscle.

A

1st measure			2nd measure			Average		
Type I	Type IIa	Type IIx	Type I	Type IIa	Type IIx	Type I	Type IIa	Type IIx
7.6	73.3	19.1	9.6	65.2	25.2	8.6	69.2	22.2
7.4	87.9	4.6	7.5	87.9	4.6	7.5	87.9	4.6
5.5	90.0	4.4	5.0	90.3	4.7	5.3	90.2	4.6
1.7	95.1	3.2	1.7	95.4	2.9	1.7	95.2	3.1
10.0	87.0	3.0	9.8	87.5	2.8	9.9	87.2	2.9
4.6	92.4	2.9	4.7	92.2	3.1	4.7	92.3	3.0
8.0	84.2	7.9	8.1	83.9	8.0	8.0	84.0	7.9
9.0	75.6	15.4	9.5	74.9	15.6	9.2	75.2	15.5
7.4	86.5	6.2	7.2	86.5	6.3	7.3	86.5	6.3
10.2	70.2	19.6	10.0	71.8	18.2	10.1	71.0	18.9
2.9	93.1	4.1	2.7	93.3	4.0	2.8	93.2	4.0
13.4	79.4	7.2	14.2	78.7	7.2	13.8	79.0	7.2
4.5	92.4	3.0	4.7	92.3	3.1	4.6	92.4	3.1
9.2	83.2	7.5	7.6	85.5	6.9	8.4	84.4	7.2
7.3	85.0	7.7	7.3	84.7	8.0	7.3	84.8	7.9

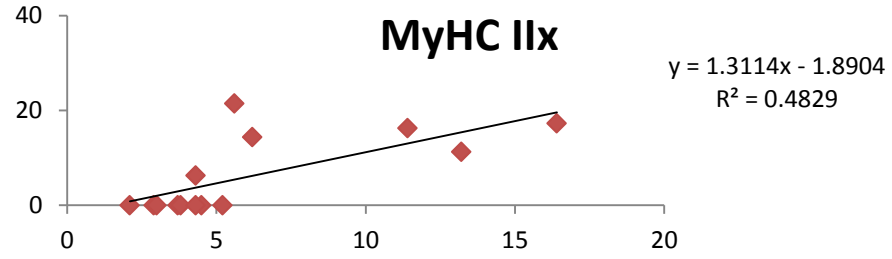
B

SDS PAGE	Type I	Type IIa	Type IIx
(%)	40.4	52.8	6.8

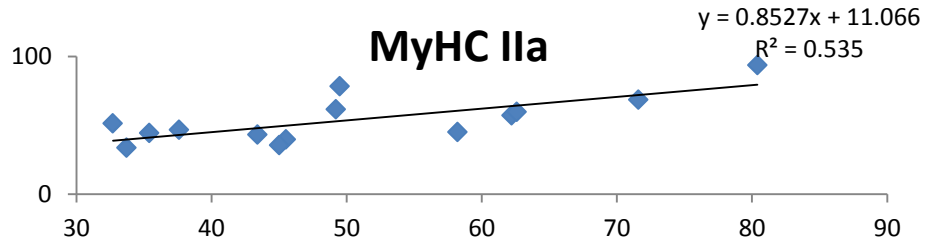
(A) Data represent the calculated relative percentage of each myosin heavy chain isoform by MRM analysis of human muscle and applying the multiplication factor derived from similar analysis of synthetic peptides (reported in Table 27). (B) Represent data from SDS-PAGE analysis for comparison.

There was little to no connection between the MyHC isoform profile measured by traditional SDS-PAGE analysis (Table 28B) and the data collected by MRM analysis (Table 28A). Therefore, we attempted to back-calculate a new correction factor from the average SDS-PAGE results, and we applied this to the MRM data then tested the correlation between SDS-PAGE and MRM on individual biological replicates (Figure 34). In all, the correlation was weak (R^2 less than 0.6) between MyHC isoform relative abundance measured by MRM and SDS-PAGE.

A



B



C

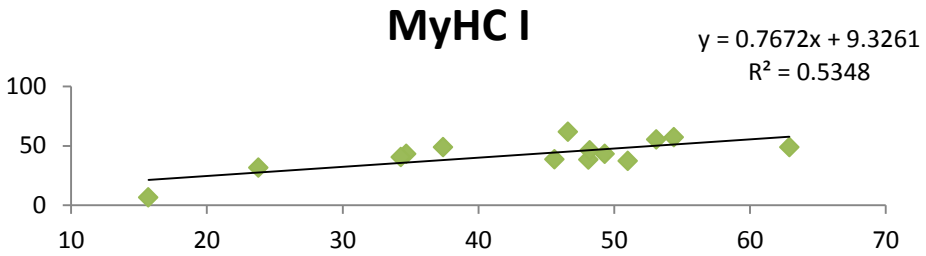


Figure 34. Correlation of percentage MyHC type IIx (**A**) type IIa (**B**) and type I (**C**) from MRM and 1D gel result. Correlation of MyHC percentage measured by two different methods. The correlation coefficient at 0.4829 for type IIx, 0.535 for type IIa and 0.5348 for type I.

4.3.6 Two-dimensional gel electrophoresis.

Individual samples from both morning and evening sessions were analysed and the proteins were separated according to their isoelectric point using isoelectric focusing (IEF) and molecular weight (SDS-PAGE). All gel spots were cut and from the gel and processed by manual in-gel tryplic digestion in preparation for LC-MS/MS analysis. Figure 35 illustrates separation of human myofibrillar proteins by large-format 2DGE. Using this technique we were able to resolve 122 protein spots. Each of the protein spots was identified by LC-ESI-MS/MS analysis and (Table 29) reports the spot number, protein name, database score, number of peptides, sequence percentage, MW and pI. From the 122 spots there were 40 unique proteins, i.e. some proteins were discovered in multiple spots. Meanwhile small format (7 cm) 2DGE (Figure 36) was used to specifically investigate differences in the abundance of essential and regulatory myosin light chains, which were also identified by mass spectrometry (Table 30).

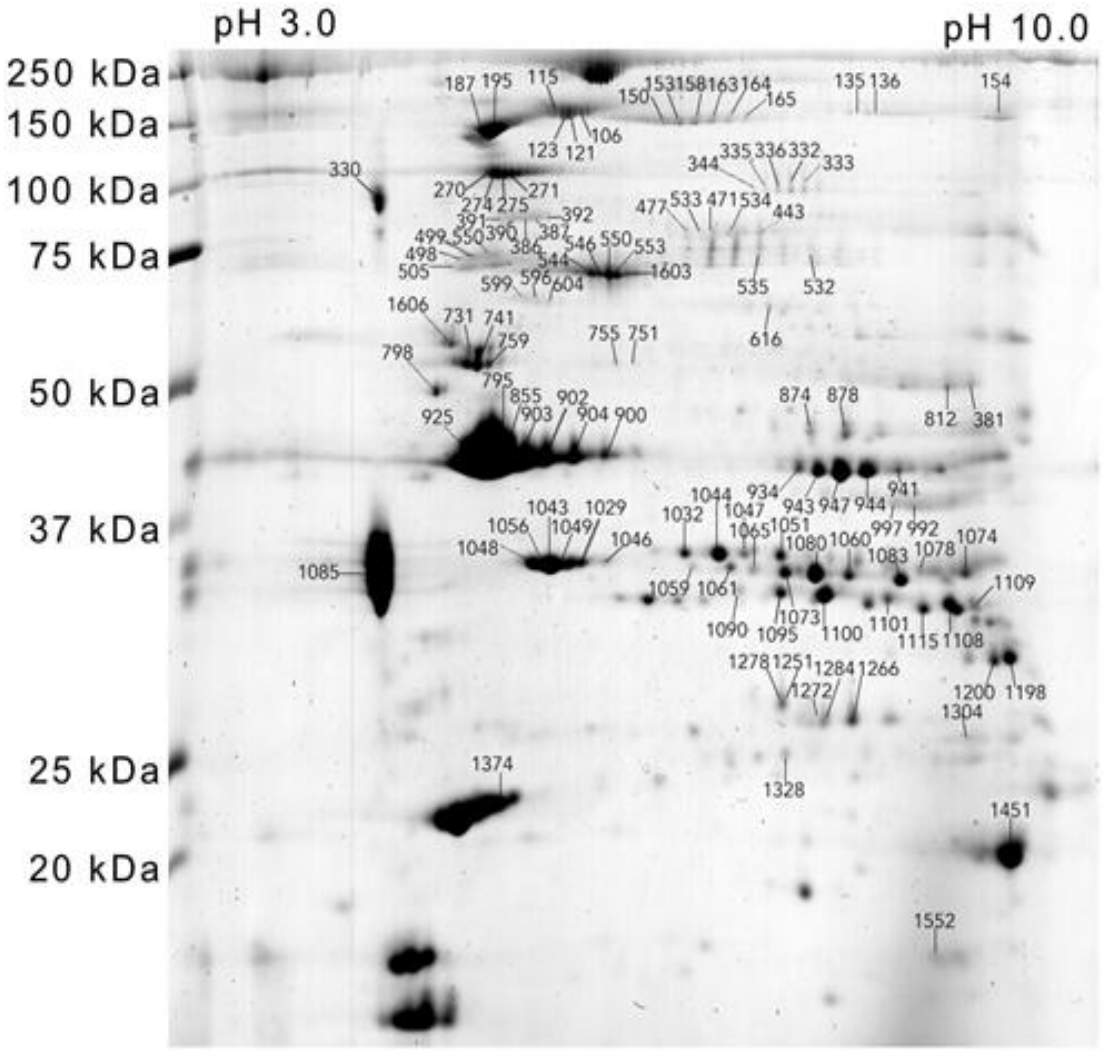


Figure 35. Separation of myofibrillar protein species by 2DGE in human vastus lateralis muscle. Representative colloidal Coomassie stained gel used for protein-expression profiling. Annotations correspond to reference numbers in Table 29.

Table 29. Proteins detected in 2D gel spots.

Spot #	Accession	Name	MOWSE	% coverage	MW	pI
270	ACTN2	Alpha-actinin-2	3558	69	104358	5.2
271	ACTN2	Alpha-actinin-2	3354	71	104358	5.2
274	ACTN2	Alpha-actinin-2	3445	69	104358	5.2
275	ACTN2	Alpha-actinin-2	3512	71	104358	5.2
795	ACTS	Actin, alpha skeletal muscle	1011	54	42366	5.1
855	ACTS	Actin, alpha skeletal muscle	1319	65	42366	5.1
900	ACTS	Actin, alpha skeletal muscle	415	39	42366	5.1
902	ACTS	Actin, alpha skeletal muscle	962	63	42366	5.1
903	ACTS	Actin, alpha skeletal muscle	1009	62	42366	5.1
904	ACTS	Actin, alpha skeletal muscle	877	55	42366	5.1
925	ACTS	Actin, alpha skeletal muscle	1277	63	42366	5.1
544	ALBU	Serum albumin	1539	56	71317	5.9
546	ALBU	Serum albumin	1622	59	71317	5.9
550	ALBU	Serum albumin	1852	65	71317	5.9
553	ALBU	Serum albumin	1838	60	71317	5.9
1603	ALBU	Serum albumin	1625	58	71317	5.9
599	ALBU	Serum albumin	59	8	71317	5.9
992	ALDOA	Fructose-bisphosphate aldolase A	733	58	39851	9.2
997	ALDOA	Fructose-bisphosphate aldolase A	812	60	39851	9.2
812	ATPA	ATP synthase subunit alpha, mitochondrial	1275	45	59828	9.6
813	ATPA	ATP synthase subunit alpha, mitochondrial	963	42	59828	9.6
798	ATPB	ATP synthase subunit beta, mitochondrial	1882	68	56525	5.1
1278	CAH1	Carbonic anhydrase 1	578	54	28909	6.7
1251	CAH3	Carbonic anhydrase 3	419	44	29824	7.1

1266	CAH3	Carbonic anhydrase 3	690	67	29824	7.1
1284	CAH3	Carbonic anhydrase 3	533	54	29824	7.1
616	CATA	Catalase	710	38	59947	7.0
731	DESM	Desmin	2161	72	53560	5.1
739	DESM	Desmin	1884	63	53560	5.1
741	DESM	Desmin	1927	67	53560	5.1
874	ENOB	Beta-enolase	1040	55	47244	8.6
878	ENOB	Beta-enolase	1196	53	47244	8.6
1074	G3P	Glyceraldehyde-3-phosphate dehydrogenase	1068	54	36201	9.3
1552	HERC1	Probable E3 ubiquitin-protein ligase HERC1	52	2	538790	5.6
499	HSP71	Heat shock 70 kDa protein 1A/1B	1070	39	70294	5.4
500	HSP71	Heat shock 70 kDa protein 1A/1B	1470	46	70294	5.4
498	HSP76	Heat shock 70 kDa protein 6	54	5	71440	5.8
505	HSP7C	Heat shock cognate 71 kDa protein	1246	39	71082	5.2
934	KCRM	Creatine kinase M-type	1134	54	43302	6.9
941	KCRM	Creatine kinase M-type	890	54	43302	6.9
943	KCRM	Creatine kinase M-type	1126	59	43302	6.9
944	KCRM	Creatine kinase M-type	1100	53	43302	6.9
947	KCRM	Creatine kinase M-type	1295	57	43302	6.9
455	KPYM	Pyruvate kinase isozymes M1/M2	815	36	58470	9.0
703	MYBPH	Myosin-binding protein H	425	23	52531	6.3
106	MYH2	Myosin-2	3726	33	223932	5.5
121	MYH2	Myosin-2	4044	37	223932	5.5
123	MYH2	Myosin-2	3325	34	223932	5.5
386	MYH2	Myosin-2	1266	13	223932	5.5
387	MYH2	Myosin-2	915	12	223932	5.5
390	MYH2	Myosin-2	1367	14	223932	5.5
391	MYH2	Myosin-2	1510	18	223932	5.5
392	MYH2	Myosin-2	709	12	223932	5.5

443	MYH2	Myosin-2	978	13	223932	5.5
471	MYH2	Myosin-2	1204	15	223932	5.5
477	MYH2	Myosin-2	2036	26	223932	5.5
479	MYH2	Myosin-2	1359	18	223932	5.5
533	MYH2	Myosin-2	1084	13	223932	5.5
534	MYH2	Myosin-2	1360	16	223932	5.5
535	MYH2	Myosin-2	1195	17	223932	5.5
604	MYH2	Myosin-2	1104	13	223932	5.5
1132	MYH2	Myosin-2	616	8	223932	5.5
115	MYH7	Myosin-2	4288	35	223757	5.5
187	MYH7	Myosin-2	3534	31	223757	5.5
195	MYH7	Myosin-2	4307	32	223757	5.5
596	MYH7	Myosin-2	1229	17	223757	5.5
1374	MYL3	Myosin light chain 3	856	77	22089	4.9
1109	MYOZ1	Myozenin-1	596	54	31725	9.3
1078	MYOZ1	Myozenin-1	848	85	31725	9.3
1108	MYOZ1	Myozenin-1	708	50	31725	9.3
1115	MYOZ1	Myozenin-1	378	30	31725	9.3
1272	MYOZ1	Myozenin-1	154	29	31725	9.3
1200	MYOZ3	Myozenin-3	278	36	27254	10.0
1198	MYOZ3	Myozenin-3	538	37	27254	10.0
150	MYPC1	Myosin-binding protein C, slow-type	2849	50	129240	5.7
153	MYPC1	Myosin-binding protein C, slow-type	2648	51	129240	5.7
158	MYPC1	Myosin-binding protein C, slow-type	2460	49	129240	5.7
163	MYPC1	Myosin-binding protein C, slow-type	2135	42	129240	5.7
164	MYPC1	Myosin-binding protein C, slow-type	2581	51	129240	5.7
165	MYPC1	Myosin-binding protein C, slow-type	2366	51	129240	5.7
135	MYPC2	Myosin-binding protein C, fast-type	2015	45	128847	8.2
136	MYPC2	Myosin-binding protein C, fast-type	1838	41	128847	8.2

154	NEBU	Nebulin	964	6	775406	9.5
532	PGM1	Phosphoglucomutase-1	876	37	61696	6.3
1046	PLCB2	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	37	2	134908	5.9
332	PYGM	Glycogen phosphorylase, muscle form	1651	44	97487	6.6
333	PYGM	Glycogen phosphorylase, muscle form	1611	47	97487	6.6
335	PYGM	Glycogen phosphorylase, muscle form	1938	53	97487	6.6
336	PYGM	Glycogen phosphorylase, muscle form	1778	50	97487	6.6
344	PYGM	Glycogen phosphorylase, muscle form	1560	43	97487	6.6
751	TBA4A	Tubulin alpha-4A chain	539	33	50634	4.8
755	TBA4A	Tubulin alpha-4A chain	642	36	50634	4.79
1304	TITIN	Titin	52	1	3843119	6.0
1451	TNNI2	Troponin I, fast skeletal muscle	378	50	21496	9.6
1029	TNNT1	Troponin T, slow skeletal muscle	146	12	32985	5.8
1043	TNNT1	Troponin T, slow skeletal muscle	599	33	32985	5.8
1048	TNNT1	Troponin T, slow skeletal muscle	738	32	32985	5.8
1049	TNNT1	Troponin T, slow skeletal muscle	652	36	32985	5.8
1056	TNNT1	Troponin T, slow skeletal muscle	687	37	32985	5.8
1044	TNNT3	Troponin T, fast skeletal muscle	257	22	31805	5.6
1080	TNNT3	Troponin T, fast skeletal muscle	552	33	31805	5.6
1047	TNNT3	Troponin T, fast skeletal muscle	403	32	31805	5.6
1032	TNNT3	Troponin T, fast skeletal muscle	491	27	31805	5.6
1051	TNNT3	Troponin T, fast skeletal muscle	507	36	31805	5.6
1059	TNNT3	Troponin T, fast skeletal muscle	394	24	31805	5.6
1060	TNNT3	Troponin T, fast skeletal muscle	481	26	31805	5.6
1061	TNNT3	Troponin T, fast skeletal muscle	457	36	31805	5.6
1065	TNNT3	Troponin T, fast skeletal muscle	421	26	31805	5.6
1073	TNNT3	Troponin T, fast skeletal muscle	482	26	31805	5.6
1083	TNNT3	Troponin T, fast skeletal muscle	583	34	31805	5.6
1090	TNNT3	Troponin T, fast skeletal muscle	520	32	31805	5.6

1095	TNNT3	Troponin T, fast skeletal muscle	452	28	31805	5.6
1100	TNNT3	Troponin T, fast skeletal muscle	474	37	31805	5.6
1101	TNNT3	Troponin T, fast skeletal muscle	540	33	31805	5.6
1328	TPIS	Triosephosphate isomerase	597	71	26938	6.6
330	TPM1	Tropomyosin alpha-1 chain	947	47	32746	4.5
1085	TPM1	Tropomyosin alpha-1 chain	1662	70	32746	4.5
330	TPM2	Tropomyosin beta chain	980	42	32945	4.5
1034	TPM2	Tropomyosin beta chain	1450	69	32945	4.5
330	TPM3	Tropomyosin alpha-3 chain	1102	47	32856	4.5
1085	TPM3	Tropomyosin alpha-3 chain	1478	72	32856	4.5
1606	VIME	Vimentin	1861	69	53676	4.9

Data represent proteins were identified by mass spectrometry and database searches, these were categorised further and ranked by score to determine the primary protein typically found in each spot.

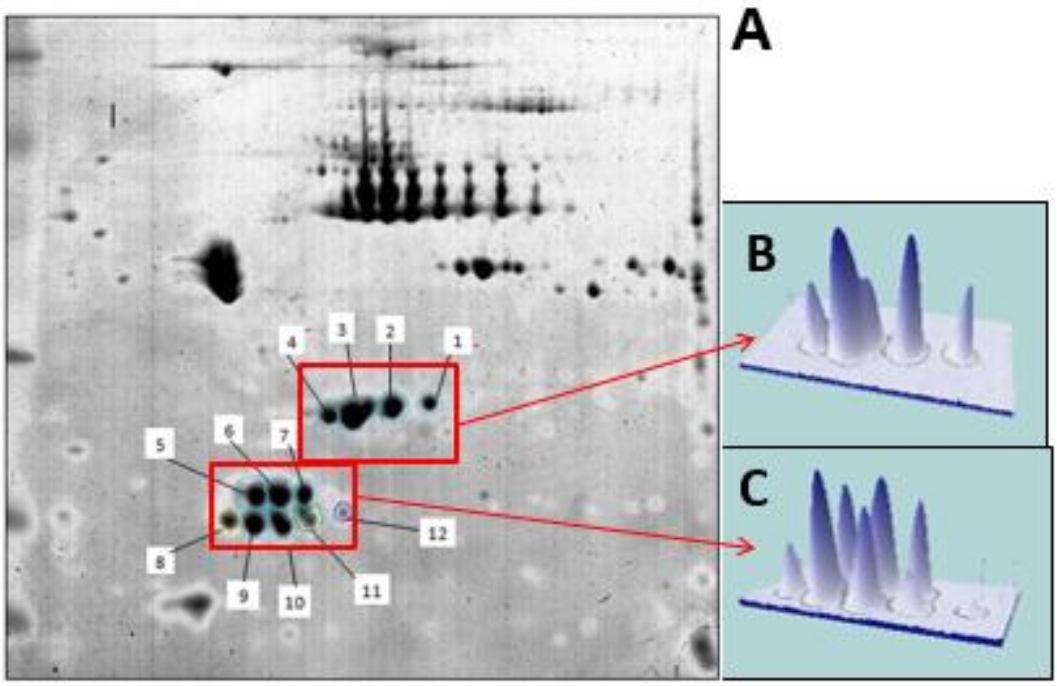


Figure 36. Separation of myosin light chain isoforms by 2DGE. Distinct spots separated based in differences in pH and migration (**A**). Three-dimensional representations of essential myosin light chains MyLC1/3 (**B**) and regulatory myosin light chains MLRV and MLRS spots (**C**).

Table 30. Quantitation of myosin light chain spots presented in Figure 36.

	Spot	Volume (CV) MORNING	Volume (CV) EVENING	T-test (p)
MyLC1/3	1	924,159.287 (54%)	785,715.196 (78%)	0.727
	2	4,426,879.775 (34%)	3,968,444.580 (50%)	0.884
	3	10,942,407.536 (29%)	9,788,719.908 (50%)	0.485
	4	2,378,350.901 (34%)	2,192,077.289 (59%)	0.431
MLRV	5	2,675,675.493 (57%)	2,985,660.793 (73%)	0.498
	6	4,176,636.325 (44%)	3,699,562.525 (59%)	0.459
	7	1,940,942.123 (49%)	1,600,503.123 (63%)	0.381
MLRS	8	1,557,280.702 (66%)	2,206,068.415 (85%)	0.216
	9	4,675,429.898 (34%)	5,214,473.788 (60%)	0.151
	10	4,420,774.648 (26%)	3,762,817.375 (47%)	0.467
	11	1,286,723.923 (42%)	982,627.086 (58%)	0.426
	12	282,739.081 (76%)	-	Unknown

4.3.7 Analysis of two-dimensional gels using SameSpots.

Colloidal coomassie stained gels (total n = 20) were analysed in Same Spots (TotalLab v3.3) and data were exported for analysis in SPSS. Statistical analysis was conducted by repeated measures one-way analysis of variance. The abundance of 8 spots was statistically ($P<0.05$) different between the morning and evening. In the evening greater abundances were observed in myosin binding protein C1 (80 %), glycogen phosphorylase (43 %) and beta enolase (24 %), whereas the abundances of nebulin (-48 %), troponin T slow (-22 %), and 3 spots identified as keratin II (average -30 %) was less abundant in the evening compared to morning. In addition, regression analysis was performed to investigate whether the changes in protein sport abundance correlated significantly with muscle function data such as peak isometric force or rate of force development (Table 32).

Table 31. Proteins in human muscle vastus lateralis detected significant difference in diurnal variations.

#	Protein Accession	Protein Description	Protein MW	Morning Mean ± SD	Evening Mean ± SD	Anova (p)	Fold difference
335	PYGM	Glycogen phosphorylase, muscle	97487	245 ± 44	350 ± 103	0.01549	1.42961
163	MYPC1	Myosin-binding protein C, slow type	129240	34 ± 85	616 ± 356	0.0226	1.79941
703	K2C1	Keratin type II	65678	133 ± 28	113 ± 26	0.03128	-1.17229
758	K2C1	Keratin type II	66170	193±32	160 ± 23	0.03331	-1.20963
874	ENOB	Beta-enolase	47244	814 ± 20	1007 ± 174	0.03348	1.23823
975	K2C1	Keratin type II	66170	172 ± 112	112 ± 32	0.04029	-1.52349
1046	TNNT1	Troponin T, slow skeletal muscle	32985	239 ± 71	196 ± 45	0.04329	-1.22008
154	NEBU	Nebulin	775406	959 ± 648	352 ± 251	0.04347	-1.47945

Data showed the abundance of 8 spots was statistically ($P<0.05$) different from morning to evening. In the evening greater abundances were observed in myosin binding protein C1 (80 %), glycogen phosphorylase (43 %) and beta enolase (24 %), whereas the abundances of nebulin (-48 %), troponin T slow (-22 %), and 3 spots identified as keratin II (average -30 %) was less in the evening compared to morning.

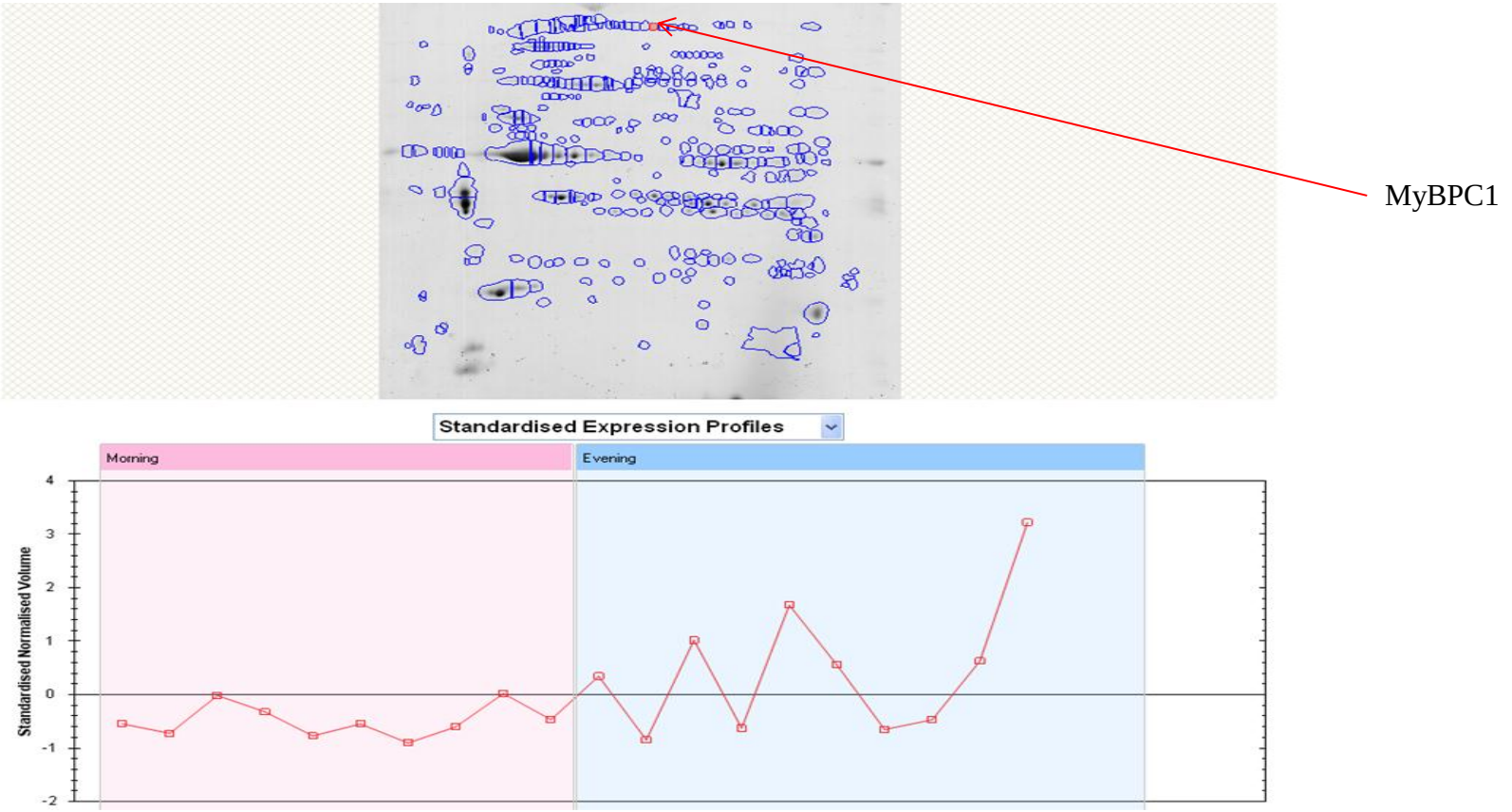


Figure 37. The graph highlighted standardised normalised volume. Three proteins associated with the diurnal variation, that were differentially expressed greater evening than morning (see Table 31) shared patterns of variation

Table 32. Pearson correlations between spots normalised volume and two muscle performance measurement (MVC force and peak RFD).

#	Protein Accession	Protein Description	MVC force	Peak RFD
335	PYGM	Glycogen phosphorylase, muscle	0.478*	0.259
163	MYPC1	Myosin-binding protein C, slow type	0.311	0.219
703	K2C1	Keratin type II	0.320	-0.055
758	K2C1	Keratin type II	-0.121	0.001
874	ENOB	Beta-enolase	0.290	0.120
975	K2C1	Keratin type II	-0.046	-0.283
1046	TNNT1	Troponin T, slow skeletal muscle	0.198	0.021
154	NEBU	Nebulin	-0.114	-0.140

Table I shows the correlation coefficients between the MVC force and peak RFD measures with the normalised volume in all 8 spots. These ranged from r = -0.283 to r = 0.478 but the only significant correlation was between the MVC force with spot number 335 which was Glycogen phosphorylase muscle form OS (r = 0.478, *P* = 0.033).

4.4 DISCUSSION

Muscle Performance testing

Previously, in Chapter 3, we reported peak isometric force, rate of force development and peak torque (measured at 4 velocities using IKD) of the knee extensors each displayed significant ($P<0.05$) time-of-day variation. That is, the performance of the knee extensor muscles was greater in the evening than in the morning. We established that RFD and MVC exhibited the greatest diurnal variation (10.2 % and 18 %, respectively) when compared to IKD (typically < 10 %). Therefore these methods (MVC and RFD) were used as the preferred indicators of diurnal variation in muscle performance for the current biopsy study. In this chapter, percutaneous needle biopsy samples were taken to investigate which modifications occur to the contractile proteins of the skeletal muscle that may affect its ability to produce force at different times of day.

One of the primary aims of this study was to confirm that the previously observed rhythm of muscle force output remained when it was applied to the research design. A diurnal variation in quadriceps MVC was evident with performance being superior in the evening compared to morning without any difference in the level of voluntary activation (consistent with Chapter 3). Indeed, we present comparable force and activation data to previous work from our group (Edwards et al., 2013; Robinson et al., 2013) and suggest participant familiarization and parallels in protocol contribute to similarities. Similarly, an 11 % variation in maximum isometric force is consistent

with literature where diurnal variation is apparent in isometric force output (Coldwells et al., 1994; Guette et al., 2005) and ranges from 6 to 18 %. Contraction velocity (i.e. RFD) is key to time-restricted explosive actions such as kicks, sprints and jumps (Andersen & Aagard, 2006). Consistent with Chapter 3, we confirm RFD is superior (15.7 % improvement) in the evening compared to the morning, and that the magnitude of diurnal variation in RFD is greater than that exhibited by peak isometric force.

Analysis of human muscle proteins

The profile of MyHC isoforms in the vastus lateralis was 42.5 % type I, 48.3 % type IIa and 9.2 % type IIx, and is consistent with literature in this area (e.g. Putman et al., 2004) and the strength-training background of the participants recruited. The fact that biopsy samples were taken in morning/evening in a within participant design enabled us to investigate the reproducibility of this entire method from biopsy through to MyHC isoform analysis. We found MyHC isoform profiling by traditional SDS-PAGE was remarkably precise. The coefficient of variation was 6.4 % for type IIx, 3.2 % for type IIa and 3.9 % for type I, and these values of variation encompass potential differences due to biopsy sampling, muscle processing and SDS-PAGE analysis.

MyHC proteins are highly abundant and we hypothesised that automated profiling of this fraction by mass spectrometry may also be used to resolve the MyHC isoforms. The advantage of the mass spectrometry approach would be time efficiency and the

development of a largely automated technique. Multiple reactions monitoring (MRM) was used to verify differences in the abundance of MyHC. While each MYHC isoform could be readily resolved the final profiling results were not similar to the SDS-PAGE analysis. The exact cause of this difference is not known but is most likely due to ion suppression when the isoform-specific peptides were analysed against a complex background of other myofibrillar proteins. To overcome this issue, greater sample fractionation/separation would be necessary, which would increase the complexity and duration of the analysis to a level equivalent to 1D SDS PAGE. Therefore MRM is not a suitable technique for muscle phenotyping.

Prior to differential analysis of the myofibrillar proteome we performed GeLC-MS/MS mining in order to characterise the entire myofibrillar fraction used in this work. Simple fractionation using a low-salt and mild detergent buffer readily precipitated the myofibrillar apparatus. In total, 193 proteins were identified and of these 33 were know components of the myofibrillar structure. This characterisation work is important because it enables us to interpret and discuss the subsequent profiling data in the context of the entire proteome that was analysed.

As discovered in Chapter 1, LC-MS/MS-based analysis of tryptic peptide mixtures does not resolve proteins to their individual species because protein identification and quantitation are often based on a small selection of proteotypic peptides that often represent small percentage of the entire protein chain. Therefore, based on our experience from Chapter 1, we used large-format 2D gel electrophoresis to perform differential profiling of morning-evening differences in the human myofibrillar

proteome. A weakness of 2DGE is its inability to span a large dynamic range of protein abundances. Indeed, we found that when we optimised the protein load to detect the greatest number of spots on the large-format gels, the abundance of the myosin light chain proteins was so great that their spots had become merged and were there for no longer suitable for quantitation. Rather than reducing the protein load and potentially missing information about low abundance proteins we chose to use small-format narrow-range gels and a low protein load to specifically investigate diurnal differences in the abundance of myosin light chain species. Although post-translational modifications to myosin light chains have previously been reported to alter muscle contractility (e.g. twitch potentiation reported by Ryder et al., 2007) we did not find any significant difference in the abundance of 2DGE spots of the essential or regulatory myosin light chain isoforms.

In addition to GeLC-MS/MS mining we also performed proteome mining of the 2DGE separation. All gel spots were cut and from the 2D gels and processed by in-gel tryptic digestion and LC-ESI-MS/MS analysis. Using this approach, we identified proteins in all 122 spots (Table 29). The majority of proteins are resolved as multiple spots by 2DGE because different post-translational states of a protein migrate to their own specific isoelectric point and relative mass positions (Burniston & Hoffman 2011). Indeed, from these 122 spots, 40 unique proteins were detected in multiple spots. Previous work in our laboratory (Holloway et al., 2009) used similar large-format (13 cm) gels and resolved 256 spots from whole muscle homogenates of human vastus lateralis, whereas studies using larger format (18cm) 2D gels and more sensitive silver staining techniques resolved more than 500 protein spots.This mining work provides a direct comparison of the proteome resolving ability of

GeLC-MS/MS (193 proteins) compared to 2DGE (40 proteins). The much lower number of proteins identified in 2DE is a limitation to the current work and information regarding diurnal differences in other myofibrillar proteins will have been missed.

One of the primary aims of this study was to investigate whether modifications occur to the contractile proteins of the skeletal muscle that effect its ability to produce force at different times of day. As far as we are aware, no previous study has specifically investigated the human myofibrillar sub-proteome. Furthermore, no previous study has used proteomics to investigate diurnal differences in human muscle performance. Proteomic profiling using two-dimensional gel electrophoresis resolved 122 proteins and each of these were unambiguously identified by mass spectrometry. The abundance of 8 spots was statistically ($P<0.05$) different between the morning and evening samples. Three of these spots were identified as keratin type II, 3 spots were known myofibrillar proteins (troponin T, myosin binding protein c and nebulin) and 2 spots were glycolytic enzymes (glycogen phosphorylase and beta-enolase). The greatest diurnal difference was in the abundance of the slow isoform of myosin binding protein C (MyBPC1). This protein was resolved as 5 species and one of these spots was 80 % more abundant in the evening compared to morning, therefore the difference in abundance reported here may represent a change in post-translational modification.

Myosin binding protein C is known to modulate the activity of actin-bound myosin ATPases and MyBPC1 is a substrate for protein kinase C (PKC) phosphorylation

(Ackermann et al., 2011). Therefore the difference in MYBPC1 may also be linked with the changes observed in the cytoskeletal protein, keratin II, which acts as a scaffold for interactions between beta integrin and PKC signalling. The greater isometric force and rate of force development in the evening than morning may be related with MyBPC1 modification because this protein acts as an adaptor to connect the ATP consumer (myosin) with actin. The similarities observed during the present study because of MyBPC1 reported mediates recruitment of muscle-type creatine kinase (CK) to myosin and its role as the regenerator for efficient energy metabolism and homoeostasis (Chen et al., 2011). Our data provide associational evidence of changes in muscle protein modifications and diurnal differences in muscle performance. However, the strength of this association may be relatively weak because there was no correlation between an individual's difference in MyBPC and diurnal variation in muscle performance.

The peak isometric force and rate of force development of human skeletal muscle was significantly greater (~11-16 %) in the evening compared to the morning. This was associated with an 80 % greater abundance of a myosin binding protein C spot, as well as difference in the abundance of 7 other proteins. To determine the strength of association between change in muscle function and the myofibrillar proteome we performed post-hoc regression analysis. Unfortunately we found no correlation between myosin binding protein C abundance and either peak isometric force or the rate of force development. However, we did find a significant ($P=0.033$) but relatively weak correlation between the abundance of glycogen phosphorylase spot 335 and peak isometric force. Similar to MYBPC1, glycogen phosphorylase was

also resolved as numerous protein species and this protein is also known to be regulated by phosphorylation.

This was a ‘discovery’ proteomics experiment designed to generate hypothesis regarding which muscle protein might be responsible for diurnal differences in muscle performance. We discovered 8 spots was statistically ($P<0.05$) different between the morning and evening samples. Three of these spots were identified as keratin type II, 3 spots were known myofibrillar proteins (troponin T, myosin binding protein c and nebulin) and 2 spots were glycolytic enzymes (glycogen phosphorylase and beta-enolase). However our experiment cannot provide causal evidence to prove the involvement of MyBPC, nebulin, glycogen phosphorylase etc. in diurnal variation. Fututre studies are required to specifically test these hypotheses and could use gain or loss of function studies in animal models.

4.5 CONCLUSION

In summary, the evidence provided here supports the previously reported circadian variability of muscle force output. Peak isometric force and the rate of force development each exhibited significant diurnal variation. Proteomic analysis of the myofibrillar fraction profiled the abundance of 122 gel spots and discovered 8 species that differed in abundance between the morning and evening. Post-hoc analysis did not find strong correlations between the changes in protein abundance and muscle function. Nonetheless, the proteins that exhibited diurnal variation could be related to muscle force output through published literature. In particular, our work provides a rationale for future studies on the role of myosin binding protein C in regulating muscle force production.

Synthesis of Findings

SYNTHESIS

The overall aim of this thesis was to combine proteomic analysis of muscle biopsy samples with measurements of muscle performance in humans. Because the proteome is what defines a cell (or tissue) and dictates its functional properties we reasoned that changes in muscle function must be underpinned by changes in the muscle proteome. More specifically, changes in muscle force production will be underpinned by changes to the myofibrillar proteins. To achieve our overall aim we conducted a series of experiments to establish and optimise techniques for proteomic analysis of muscle biopsy samples and techniques for the analysis of human muscle function *in vivo*. The purpose of this chapter is to summarise the findings from each of the foregoing experimental chapters and detail how the main aims of this thesis were met. A secondary purpose of this chapter is to provide recommendations for further research based upon this body of work.

Analysis of the myofibrillar sub-proteome of human muscle

In Chapter 1 the applicability of label-free profiling in skeletal muscle was assessed using rat muscle samples. We found the high enrichment of metabolic enzymes in skeletal muscle to be an asset to high-throughput and time-efficient profiling. Indeed, a relatively simple LC-MS process was able to analyse more than 200 of the most abundant soluble muscle proteins (i.e. metabolic enzymes). We next investigated whether LC-MS profiling could also be used to analyse the myofibrillar sub-proteome. Compared to the soluble protein fraction, the myofibrillar proteome

contained fewer proteins but a more complex array of protein isoforms and splice variants. LC-MS profiling of human muscle was able to identify significant differences in the abundance of myosin light chain isoforms between young and elderly people that were either physically active or habitually sedentary. Moreover, we were able to confirm these findings using multiple reaction monitoring assays.

Because many of the myofibrillar protein isoforms share high degrees of sequence homology we were not able to identify isoform-specific peptides for proteins such as MyHC. Instead, we complimented our proteomic techniques using traditional biochemical analysis of MyHC isoforms. We successfully resolved the 2 isoforms of MyHC (types I and IIa/x) that are expressed in human locomotive muscles and report differences in MyHC profile that correspond with the differences in MyLC measured using LC-MS. This demonstrates that we were able to use the traditional biochemical analysis as a point of comparison for developing proteomic techniques in human muscle samples. To delve deeper in to the myofibrillar proteome we used 1D gel electrophoresis of low-molecular weight (~ 90-10 kDa) proteins. However, this approach was unable to resolve all low molecular weight myofibrillar proteins and many of the 1D gel bands contained numerous different proteins. Therefore we proceeded to a more sophisticated 2D gel electrophoresis method. Mining of the human myofibrillar proteome using 2D gel electrophoresis confirmed that the majority of 1D gel bands are composed of a number of different protein species. The proteome mining work conducted in Chapter 1 using small (7 cm) 2D gels also suggested that future work would require larger format (e.g. 13 cm) separation in order to more clearly resolved myofibrillar proteins.

Measurement of knee extensor force-velocity profile in vivo

This aim was addressed in Chapter 2. Our work began with a preliminary study to find the peak angular velocity of human knee extension during unloaded kicking. Contrary to previous estimations, we found the average peak angular velocity of human knee extension (461 º/s) was within the range (0-500 º/s) of a commercial isokinetic dynamometer. Therefore, we created an IKD protocol that encompassed the entire force-velocity range of human knee extension. Next we investigated the reliability of isolated knee performance measures across the f-v profile of human muscle encompassing a range of velocities using isokinetic dynamometry (IKD). To attempt to relate our work to wider applications in sports performance testing we also assessed the reliability of jump analysis using either a force platform or linear encoder. This enabled us to investigate the strength of correlation between indices of isolated knee performance and a more complex sport-related movement such as jumping. Our knee extension data was consistent with the f-v concept of striated muscle and the reliability of each of the measures of knee extensor performance was acceptable (<15 % CV). The reliability of jump performance measures was also good and we found modest correlations amongst each of the knee measures. Reverse regression analysis found isometric and highest velocity knee kicking on IKD was the strongest predictor of jump performance.

Diurnal variation muscle force production

After investigating proteomic methods in Chapter 1 and muscle function methods in Chapter 2 our next goal was to combine these techniques and apply them to a relevant question in sports physiology. We chose diurnal variation because time-of-

day differences in muscle force and sports performance are widely reported but the mechanisms underpinning this phenomenon are not yet understood. Muscle force production is poorest in the morning and greatest in the evening, and the magnitude of this diurnal variation is large (i.e. ~10 %) in comparison to gains that can be made through sports training in elite athletes. Previous literature reports diurnal variation in a diverse range of measures of sports/ muscle performance (e.g. hand grip-strength, isokinetic dynamometry, vertical jump, time-trial performance etc.) but it was not clear which aspect of muscle output (e.g. high force or high velocity contractions) exhibits the greatest diurnal variation. In Chapter 3 we investigated this question by conducting a battery of muscle performance tests in a population of well-familiarised participants. Our data show that RFD exhibits the greatest diurnal variation (18 %) followed by isometric force (10.2 %). The diurnal variation in IKD data was less robust (range 8.1 - 9.8 %), which may have been due to the lesser precision of this technique compared to MVC and RFD. Therefore MVC and RFD were used in the final study and biopsies were taken from the vastus lateralis to investigate whether modifications occur in the myofibrillar proteome that effect its ability to produce force at different times of day.

Proteomic analysis of diurnal differences in muscle performance

Chapter 4 is the culmination of our work establishing techniques for the analysis of the myofibrillar proteome (Chapter 1) and the output of human knee extensor muscles across their force-velocity range (Chapter 2). Furthermore, the specific experiment design and analysis techniques for investigating diurnal differences in muscle performance were optimised in Chapter 3. In Chapter 4 we re-recruited a

subset of the participants from the experiment reported in Chapter 3 and took biopsy samples of their vastus lateralis in the morning and evening immediately after they had completed the measurement protocols for maximum isometric force and rate of force development. We confirmed that both the rate of force development and maximum isometric force were significantly greater in the evening compared to the morning. Comprehensive analysis of the myofibrillar sub-proteome was conducted, encompassing proteome mining using GeLC-MS/MS and differential proteome profiling using 2D gel electrophoresis. This is the first study to specifically focus on proteomic analysis of the myofibrillar fraction of human skeletal muscle. GeLC-MS/MS mining identified 193 unique proteins, which included a large proportion of the know myofibrillar structure of skeletal muscle. Differential analysis using 2D gel electrophoresis resolved 122 protein species and identified 8 statistically significant in abundance. These may represent candidate biomarkers that could be used to develop and optimise interventions aimed at forestalling morning decrements in muscle performance. Information regarding changes at the protein level was compared with measures of muscle performance to determine the level of association between muscle output and a particular protein modification. For example, a shift in the post-translational state of slow myosin binding protein C (MyBPC1) was detected, and this protein is known to modulate the activity of actin-bound myosin ATPases. However it was weakly correlated with peak isometric force. Meanwhile other protein modifications including glycogen phosphorylase showed a significant correlation with the MVC force.

THESIS STUDY TABLE

1. Establish the new proteomic techniques

Experiment 1.1

- Animal study (HCR & LCR)
- Label-free LCMS → LC-MS profiling → SRM
- Investigation on rapid automated method for profiling major metabolic proteins in skeletal muscle

Key findings:

- Provide protein-level confirmation for earlier transcriptome profiling work
- LC-MS is a viable means of profiling the abundance of almost all major metabolic enzymes of skeletal muscle
- Relatively more time efficient than techniques relying on orthogonal separations
- Demonstrate selection model (SRM) was able to highlight biomarkers

Experiment 1.2

- Human samples (YT, YU, OT & OU)
- 1D SDS-PAGE (8%) → 1D SDS-PAGE (15%) → 2D Gel (7 cm) → LC-MS
- Observation of traditional biomechanical analysis as a point of comparison for developing proteomic techniques in human sample muscle
-

Key findings:

- Average percentages of muscle phenotyping from 1D GeL (8%) showed a similar outcome with existing studies
- 1D GeL (15%) showed significant difference in a band but MS analyses identified multiple proteins within each band
- 2D GeL discovered unique proteins in multiple spot and able to shown distinctive spot in a band e.g MyLC and Troponin C.
- 2D GeL still did not resolve all of the expected abundant myofibrillar proteins

Experiment 1.3

- Observational on solution-based analysis (YT, YU, OT & OU) → LC-MS → MRM

Key findings:

- Number of proteins discover greater than number of protein by 2D GeL
- MyLC showed a significant difference in aging and training
- Demonstrate selection model (MRM) was able to highlight biomarkers

2. Investigate the reliability of method of measurement

Experiment 2.1

- Pilot study
- Investigation the correlation between linear encoder & kinematic measurement
- Observation on IKD & whole body jump performance measurement at high velocities.

Key findings:

- There were correlation between ML and QTM
- Range of velocity were capable at achieving were less than $500^{\circ}.s^{-1}$.

Experiment 2.2

- Investigate the reliability of jump analysis by force platform and force-velocity data produced by isokinetic dynamometry of knee extension and flexion movement
- Observation on IKD performance correlates with whole body jump performance.

Key findings:

- Higher isokinetic velocity tests show better relationships with the vertical jump
- Mean knee extension peak torque data was similar with the f-v relationship concept and consistent with previous reported studies
- 3 time test for muscle performance on force platform and IKD protocol is reliable

Experiment 2.3

- Investigate the reliability of isometric maximal voluntary contraction and the rate of force development.

Key findings:

- 2 time testing (after several familiarisation) for muscle performance on MVC and RFD protocol is reliable

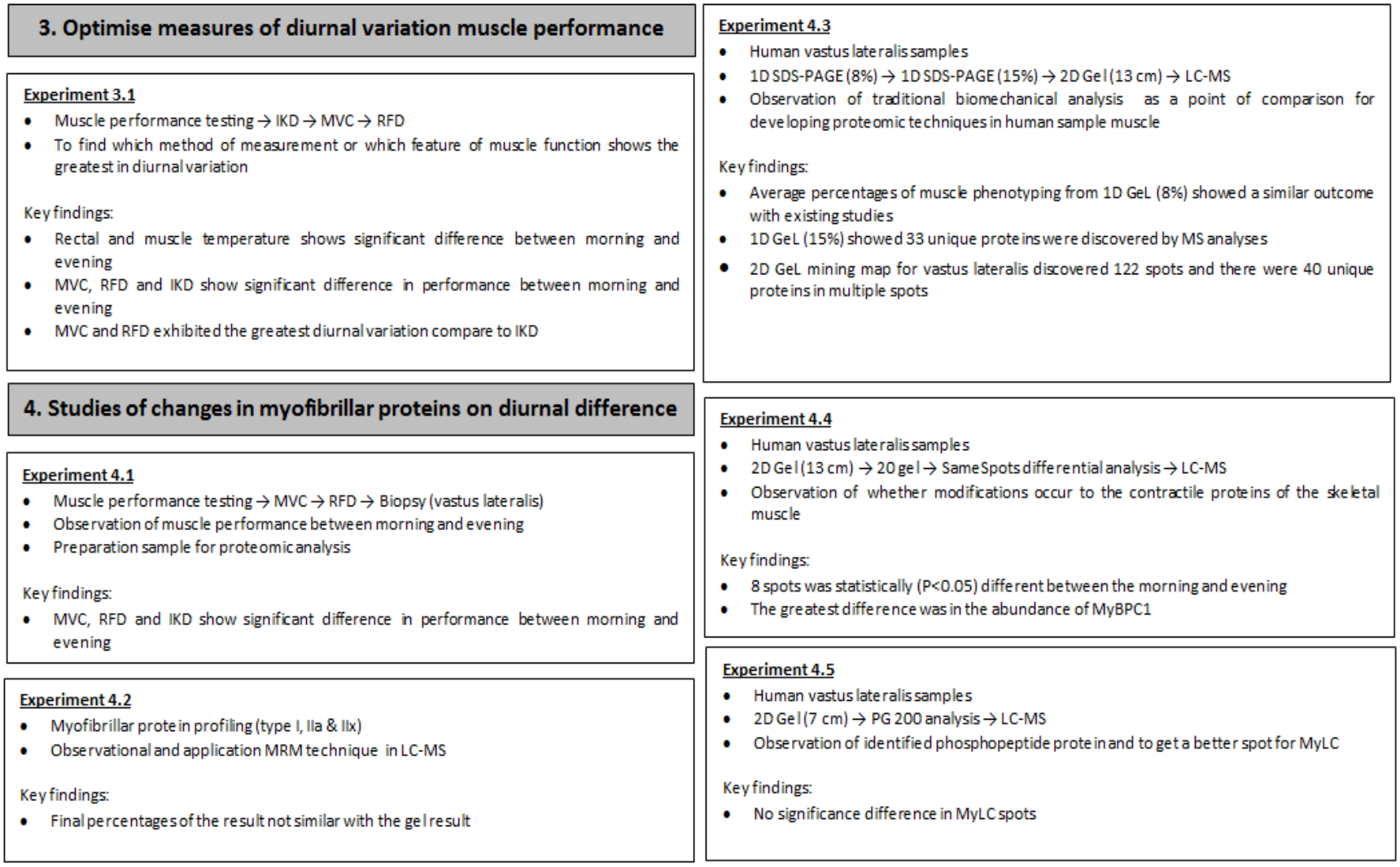


Figure 37. An illustration of the structure, procedures and key findings of the experiment.

Recommendations for future research

The current work investigated the link between the myofibrillar proteome and muscle function in humans in vivo. Because this was the first work of its kind we used robust laboratory measures of isolated limb movements (i.e. knee extension) and took biopsy samples from the vastus lateralis, which is one of the major contributors to this movement. Using this approach we found significant diurnal differences in myofibrillar protein abundance. The most robust difference was myosin binding protein C, but the abundance of this protein did not correlate closely with muscle force output. While the statistical association between myosin binding protein C and muscle force is not strong there was clear corroborating evidence in the literature to suggest a role for myosin binding protein C in modulating force output of myosin-actin cross bridges. Therefore, we recommend future work should characterise the exact post-translational modification that occurs to myosin binding protein C and then use gain or loss of function studies to find the effects of this modification on muscle performance.

The second most prominent difference was glycogen phosphorylase ($r = 0.478$, $P = 0.033$). This is also a viable target for future study because there is evidence for diurnal variation in muscle metabolism. However, the connection between glycogen phosphorylase activity and muscle force output is not direct and our experiment design did not tightly control feeding. That is participants were fast 7h or 4 h in morning versus evening. Therefore more control study is required to confirm this finding.

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Appendices

Register for MPhil WTP PhD

From: "McKeon, Jo" <J.M.McKeon@ljmu.ac.uk>
Subject: Application to Register for MPhil WTP PhD
Date: 5 April 2012 14:26:57 GMT+01:00
To: "Ab Malik, Zulezwan" <Z.Ab-Malik@2011.ljmu.ac.uk>
Cc: "Burniston, Jatin" <J.Burniston@ljmu.ac.uk>

Dear Zulezwan,

Further to the consideration of your Application to Register for MPhil WTP PhD which was considered by the University's Research Degrees Committee (RDC) at it's meeting of Thursday 22nd March 2012.

Please note that your application was approved subject to your project being granted full approval by the LJMU Research Ethics Committee (REC). Therefore, please may I ask that you submit your Ethics application by Thursday 19th April 2012 which is the deadline for agenda items for the REC meeting on Thursday 3rd May 2012.

The Research Ethics website is available at: http://www.ljmu.ac.uk/RGSO/93042.htm

Please call me if you have any queries.

Kind regards,

Jo McKeon
Research Support Officer
Research Support Office
Liverpool John Moores University
Kingsway House
Hatton Garden
Liverpool
L3 2AJ
Tel: (0151) 904 6463
Fax: (0151) 904 6462
Email: j.m.mckeon@ljmu.ac.uk
Website: http://www.ljmu.ac.uk/RGSO/index.htm

Ethical Approval

From: "McKeon, Jo" <J.M.McKeon@ljmu.ac.uk>
Subject: Application for Ethical Approval No.: 12/SPS/013
Date: 9 May 2012 15:28:57 GMT+01:00
To: "Ab Malik, Zulezwan" <Z.Ab-Malik@2011.ljmu.ac.uk>
Cc: "Burniston, Jatin" <J.Burniston@ljmu.ac.uk>

Dear Zulezwan,

With reference to your application for Ethical approval:
Lower-limb muscle performance in athletes from different sport disciplines

Liverpool John Moores University Research Ethics Committee (REC) has reviewed the above application at the meeting held on 3rd May 2012. I am happy to inform you that the Committee are content to give a favourable ethical opinion and recruitment to the study can now commence.

Approval is given on the understanding that:
· any adverse reactions/events which take place during the course of the project will be reported to the Committee immediately;
· any unforeseen ethical issues arising during the course of the project will be reported to the Committee immediately;
· any substantive amendments to the protocol will be reported to the Committee immediately.
· the LJMU logo is used for all documentation relating to participant recruitment and participation eg poster, information sheets, consent forms, questionnaires.

The JMU logo can be accessed at http://www.ljmu.ac.uk/corporatecommunications/60486.htm

For details on how to report adverse events or amendments please refer to the information provided at http://www.ljmu.ac.uk/RGSO/RGSO_Docs/EC8Adverse.pdf

Please note that ethical approval is given for a period of five years from the date granted and therefore the expiry date for this project will be **3rd May 2017**. An application for extension of approval must be submitted if the project continues after this date.

Yours sincerely
PP:

Professor Andrew Young
Chair of the LJMU REC
Tel: 0151 904 6463
E-mail: j.m.mckeon@ljmu.ac.uk

Transfer from MPhil to PhD

Personal & Confidential

Dear Zulezwan,

I am very pleased to confirm that the Chair of the University's Research Degrees Committee has approved your application for transfer of registration from MPhil to PhD. This will be reported to RDC at its next scheduled meeting.

The Chair approved the application on behalf of RDC subject to the Independent Assessor's comments. These have been seen by your Director of Studies to enable you to receive feedback on your application.

Congratulations on your successful transfer from MPhil to PhD.

Yours sincerely

Jo McKeon
Research Support Officer
Tel: (0151) 904 6463
E-mail : j.m.mckeon@ljmu.ac.uk

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Ethical approval

From: Jarvis, Jonathan
Sent: 27 January 2014 10:52
To: Hall, Elliott
Cc: Edwards, Ben
Subject: ethics approval

Full Ethical Approval - ethics number: P13SEC003

Lower-limb muscle performance in diurnal variation conditions

With reference to your application for ethical approval

On behalf of the School of Sport and Exercise Science I can confirm that the study has been approved and participant recruitment can now commence. Approval is given on the understanding that:

1. You check the major project handbook for details of the pre-data collection procedures that need to be satisfied BEFORE you are permitted to commence data collection;

2. You use participant codes to protect the anonymity and confidentiality of the participants:

3. Any adverse reactions/events which take place during the course of the project will be reported to the School Research Ethics Committee immediately (contact Jonathan Jarvis);

4. Any unforeseen ethical issues arising during the course of the project will be reported to the School Research Ethics Committee immediately (contact Jonathan Jarvis);

5. Any substantive amendments to the protocol will be reported to the School Research Ethics Committee immediately (contact Jonathan Jarvis).

6. The LJMU logo is used for all documentation relating to participant recruitment and participation e.g. poster, information sheets, consent forms, questionnaires. The JMU logo can be accessed at http://www.ljmu.ac.uk/corporatecommunications/60486.htm.

Please note that ethical approval is given for a period of one year from the date granted. An application for extension of approval must be submitted if the project continues after this date (contact Jonathan Jarvis).

Yours sincerely

Jonathan Jarvis

LIVERPOOL JOHN MOORES UNIVERSITY
Jonathan Jarvis
Professor, Sport and Exercise Sciences
Tom Reilly Building Byrom Street, Liverpool, L3 3AF
t: 01519046253 e: J.C.Jarvis@ljmu.ac.uk m: 07973247895

Ethical approval

From: **Williams, Mandy** A.F.Williams@ljmu.ac.uk
Subject: Approval for NHS Studies
Date: 21 March 2014 16:39
To: Burniston, Jatin J.Burniston@ljmu.ac.uk

Approval for NHS Studies

With reference to your application for Ethical approval:
14/SPS/015 - Mechanisms underpinning diurnal variation in human muscle (14/WM/0065)

I am pleased to inform you that, following confirmation of full, unconditional ethical approval from your NRES REC, Liverpool John Moores University Research Ethics Committee (REC) is content to endorse this approval.

Approval is given on the understanding that the approving REC, named above, will be made aware of any adverse events or substantive changes in protocol and that LJMU REC will be informed of any such events.

Please note that ethical approval is given for a period in line with that approved by NRES and application for extension of approval must be submitted to the approving REC named above and LJMU.

LIVERPOOL JOHN MOORES UNIVERSITY
Mandy Williams, Research Support Officer
Graduate School, Research and Innovation Services
Kingsway House, Hatton Garden, Liverpool L3 2AJ
t: 01519046467 e: a.f.williams@ljmu.ac.uk

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Appendix

Title of Project: Lower-limb muscle performance in athletes from different sport disciplines.

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in athletes from different sport disciplines.
Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Jos Vanrenterghem
Dear All,

You are invited to take part in a research project investigating lower-limb muscle performance in athletes from different sport disciplines. The project will investigate how muscle strength and speed contribute to maximum power output during a squat jump.

We would like to recruit male and female participants aged between 18-30 years that are experienced in strength training and free of recent musculoskeletal injuries.

You will be required to visit the laboratory on 4 separate occasions, each session lasting approximately 1 and half hour. You will have the opportunity to practice and become familiarised with each performance test before your maximum effort is recorded. The tests include measurement of your one-repetition back squat and your power output whilst squat jumping with 30 %, 60 % or your maximum lift. In addition, you will also be asked to perform a number of knee extension exercises to measure your maximum strength and speed of movement.

This study has been approved by the ethics committee of Liverpool John Moores University. All data collected from you will be identified by a code from which you cannot be identified by simply reading the code. Data collected from you will remain strictly confidential between you and the researcher.

You will be required to undertake at least 4 sessions including 2 muscle strength sessions (isolated knee exercise and squat jump), each session lasting approximately 1 and half hour. The study has received full unconditional ethical approval from LJMU.

Those who wish to take part will gain first-hand experience of the latest sports science techniques. Also, the data obtained from the testing protocol could be of benefit to the individual's in the continuing pursuit of their own fitness goals. Another potential benefit for individual participants is that they will help towards findings that could have a potential breakthrough for future athletes showing benefiting in an increase in overall their performance.

If you are interested in participating in this study then please read the attached participant information sheet and contact:
(Z.Ab-Malik@2011.ljmu.ac.uk)

Regards,
Zulezwan

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in athletes from different sport disciplines.

Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Josh Vanrenterghem.

School: Sport and Exercise Sciences
Contact details: Z.Ab-Malik@2011.ljmu.ac.uk

You are being invited to take part in a student research study. Before you decide it is important that you understand why the research is being done and what it involves. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

1. What is the purpose of the study?

In many sports, success depends on an athlete's peak power output, which is based on their ability to produce both rapid and forceful movements. This project will investigate how the components of maximum strength and maximum speed of movement contribute to the peak power output during a whole-body exercise (i.e. squat jump). To achieve this we intend to investigate lower-limb muscle performance in accomplished athletes from different sport disciplines using a range of different scientific tests. We will use the information collected in these experiments to draw links between muscle performance during specific knee exercises such as knee extension and the more complex squat jump movement.
The knowledge we gain from completing this work will help us to devise optimised training programmes for improving power output.

2. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do you will be given a copy of this information sheet keep and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw will not affect your rights/any future treatment/service you receive.

3. What will happen to me if I take part?

- *The research will take part in the LJMU muscle strength laboratory (Tom Reilly Building).There are specific labs designed to implement the work with specific equipment.*
- *You will be familiarised with the tests then you will need to perform the tests day at least 3 more times over the period of a view weeks.*
- *You will be required to participate in at least four sessions.*

- *The sessions will be spread over two or three weeks, with at least one day between each session.*
- *Each visit should last about one and half hour, so therefore you will be required to spend 6 hours in the laboratory (please note each visit will be on a separate occasion).*
- *You will only be permitted to participate in the study if:*

- You are aged between 18-30 years
- You have history of sport specific training

- *During the first sessions you will be familiarised with the equipment and you will have the opportunity to practice each of the tests. You will be required to wear normal training clothing and abstain from alcohol 24 hours before each session and caffeine 2 hours before each session.*
- *During muscle strength testing you will again be required to wear your usual training clothing and abstain from alcohol and caffeine 2 hours before each session. You will participate in a warm up on a cycle ergometer at 150 W for 5-min before the test.*
- *This tests will involve :-*

- *Visit the laboratory on 4 occasions each separated by at least 24 h*
- *Squat jumps (0%, 30 % and 60 % of 1RM) & isometric squat test.*
- *Isolated knee exercise {Isokinetic dynamometry (Biodex).}*
  - ***1 RM squat test**. During the first session, subjects will complete a 1 repetition maximum (RM) squat test. 1 RM is the maximum weight that they can lift for one repetition. This will consist of a standardise protocol, which will establish their 1RM within 4 attempts. Sufficient rest (3 minutes) will be allocated between each repetition to allow for recovery and to give enough amount of rest to lift with maximum effort for the next trial. The results of the 1RM squat test will then be used to calculate their individual external loads required to represent each of the sub maximal squat jumps. This will enables us to record the force-velocity relationship which is used to calculate your peak power output.*
  - ***Jump squat**. Subjects will be required to perform a maximal isometric strength using an isometric squat test and squat jump across a series of intensities (0%, 30% and 60% of the individual's 1RM). They will be asked to standing on a force plate in a back squat position pushing against an immovable rigid bar for isometric squat test. For jump squat performance, they will be required to do the jump squat while holding a rigid bar across their shoulder.*
  - ***Isokinetic dynamometry flexion and extension.** Subject will be seated on the equipment (BIODEX) and tightly secured to the seat with straps, and their dominant leg will be strapped to the lever arm on the machine. Biodex machine is an equipment that allow your limb move at constant fast and slow speed. You will be asked to push against the lever arm at two different speeds (fast and slow). The subjects were asked to do 4 repetitions for each angular velocity. 1 minute rest was given between each angular velocity.*

- ***DEXA.*** *Subjects will undergo a whole body fan-beam DEXA scan. It is for assess their body composition, lean body mass and to see the cross sectional area (CSA) of the thigh.*
  - *During visit 1 your body mass (kg) and height (m) will be measured using calibrated scales and equipment.*

Other relevant issues

- *You will be required to fill in a physical activity readiness questionnaire (PAR-Q) and each individual's recent training history will be recorded.*

Table 1: List of assessments

SESSIONS	ACTIVITIES
1	QUESTIONNAIRE, FAMILIARISATION TEST, DEXA, 1RM TEST
2, 3 & 4	TESTING FOR JUMP SQUATS AND ISOLATED KNEE EXERCISE.

4. Are there any risks, discomforts, benefits involved?

- *Each of the tests requires you to perform maximal muscle contractions, which may be associated with some tiredness and risk of injury. However, it is unlikely that you will feel any more discomfort than usual high-intensity training session.*
- *The twitch interpolation may at first cause a little discomfort but after familiarisation you should become used to the sensation.*
- *Exercise may result in muscle soreness up to 72 hours after testing if you are unaccustomed to the specific exercise.*
- *There is a small risk of soft tissue injury involved in the testing. Should you become injured you may need to withdraw from the study, however to minimise the risk of injury you will be closely supervised and you will also participate in a warm up before muscle strength testing.*
- *You will be closely supervised throughout the tests and the researcher has ensured that the location of the study is appropriate and any potential risks have been minimised.*

5. Will my taking part in the study be kept confidential?

Data collection

- *No personal data will be collected from you beyond gender and residential status therefore all data will be anonymous.*
- *All data collected from you will be identified by a code from which you cannot be identified by simply reading the code.*
- *Data collected from you will remain strictly confidential between you and the researcher.*

- *To ensure confidentiality please do not include your name or any information that could be used to identify you on this questionnaire.*
- *Personal information will be treated in the strictest confidence with no association been made between your identity and the data observed.*
- *People will not know which answers are yours, apart from me and the other researchers.*

Data storage

- *All information/data will be stored confidentially and only accessed by the researcher. At the end of the study all personal identifiable information will be deleted and the data stored anonymously.*
- *Information linking you to the participant code is stored in a password protected file, which is only accessible by the researcher.*
- *All data will be stored on a password protected computer system.*

Data access

- *No one other than the researcher will have access to the data collected from you as part of this study.*

Breaking confidentiality

- *In accordance with UK law, the researchers are obliged to breach the promise of confidentiality if they have reason to believe that you are in danger from others or are likely to cause danger to others.*
- *It is possible that some participants may disclose other participants' confidential information.*

NB. You must freely volunteer to be a subject and are able to withdraw, without prejudice, at any time. Thank you for your time and interest in the study.

Contact Details of Researcher

Z.Ab-Malik@2011.ljmu.ac.uk

LIVERPOOL JOHN MOORES UNIVERSITY

Project title: Lower-limb muscle performance in athletes from different sport disciplines.

Researcher: Zulezwan Ab Malik
Sport and Exercise Sciences

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐
3. I understand that any personal information collected during the study will be anonymised and remain confidential ☐
4. I agree to take part in the above study *(if appropriate please specify the type of study or particular intervention you are seeking consent for – eg focus group, interview, training programme)* ☐

Name of Participant	Date	Signature
Name of Researcher	Date	Signature
Name of Person taking consent *(if different from researcher)*	Date	Signature

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in athletes from different sport disciplines.

Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Jos Vanrenterghem.
School: Sport and Exercise Sciences
Contact details: Z.Ab-Malik@2011.ljmu.ac.uk

For most people, physical activity should not pose any problem or hazard. The PAR-Q is designed to identify the small number of adults for whom physical activity might be inappropriate or those who should seek medical advice concerning the type of activity most suitable for them.

1. Do you have a muscle, ligament bone or joint problem such as arthritis or previous injuries, which has been aggravated by exercise or might be made worse with exercise?	YES	NO
2. To your knowledge, do you have high blood pressure?	YES	NO
3. To your knowledge, do you have low blood pressure?	YES	NO
4 Do you have Diabetes mellitus or any other metabolic disorder?	YES	NO
5. Has your doctor ever said that you have raised cholesterol (serum level above 6.2mmol/L)?	YES	NO
6. Do you have or ever suffered a heart condition?	YES	NO
7 Have you ever felt pain in your chest when you do physical exercise?	YES	NO
8. Is your doctor currently prescribing you drugs or medication?	YES	NO
9. Have you ever suffered from shortness of breath at rest or with mild exercise?	YES	NO
10. Is there any history of Coronary Heart Disease within your family?	YES	NO
11. Do you ever feel feint, have spells of dizziness or have ever lost consciousness?	YES	NO
12. Do you currently drink more than the average amount of alcohol per week (21units for men and 14 units for women (1 unit = 1/2 pint of beer/cider/larger or 1small glass of wine))	YES	NO
13. Do you currently smoke?	YES	NO
14. Do you NOT currently exercise regularly (at least 3 times per week) and/or work in a job that is physically demanding.	YES	NO
15. Are you, or is there any possibility that you might be pregnant?	YES	NO
16. Do you know of any other reason why you should not participate in a programme of physical activity?	YES	NO

LIVERPOOL JOHN MOORES UNIVERSITY

Training history questionnaire

Title of Project: Lower-limb muscle performance in athletes from different sport disciplines.

These questions are designed to gather information about your recent training that will help us to interpret the results of the exercise tests. Please answer each question to the best of your ability. If you are unsure about any of these questions, do not hesitate to refer to the researcher.

1. What is your primary sport discipline? ……..

2. How many times per week do you perform each of these types of training?

Training mode	Number of days per week						
	1	2	3	4	5	6	7
Heavy resistance exercise							
Metabolic resistance training							
Plyometric jumping							
Calisthenics							
Aerobic endurance exercise							
Sport-specific/ technical training							
Other (please specify)							
Other (please specify)							

3. If you perform resistance training how much of your training is performed at these different intensities?

Intensity/ Typical number of reps to failure	Frequency: Please rank each of these on a continuum from 1 to 10 where 1 indicates that you seldom work at this intensity and 10 indicates that you almost always work at this intensity									
	1	2	3	4	5	6	7	8	9	10
≥ 95 % Max / 1-2 reps										
85 - 95 % Max / 3-6 reps										
75-85 % Max / 6-10 reps										
67-75 % Max / 10-15 reps										
≤67 % Max / >15 reps										

4. If you perform resistance training how much of your training is performed at these different speeds?

Speed	Frequency: Please rank each of these on a continuum from 1 to 10 where 1 indicates that you seldom work at this speed and 10 indicates that you almost always work at this speed									
	1	2	3	4	5	6	7	8	9	10
Slow controlled movements										
Ballistic/ explosive movements										

Appendix

Title of Project: Lower-limb muscle performance in diurnal variation conditions.

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in diurnal variation conditions.

Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Jos Vanrenterghem

Dear All,

You are invited to take part in a research project investigating lower-limb muscle performance in different time of day. The project will investigate how muscle strength and speed of movement contribute to your maximum power output.

We would like to recruit male participants aged between 18-39 years that are experienced in strength training and free of recent musculoskeletal injuries.

You will be required to visit the laboratory on 8 separate occasions, each session lasting approximately 1 h. You will have the opportunity to practice and become familiar with each performance test before your maximum effort is recorded. The tests include measurement of your power output during maximum voluntary contraction and a number of knee extension exercises performed at different speeds.

This study has been approved by the ethics committee of Liverpool John Moores University. All data collected from you will be anonymous and will remain strictly confidential between you and the researcher.

Those who wish to take part will gain first-hand experience of the latest sports science techniques. The data obtained during this work could be of benefit to individuals in their continuing pursuit of performance goals. In addition, we hope the findings from this research will help to advance elite training practices in the future.

If you are interested in participating in this study then please contact:
(Z.Ab-Malik@2011.ljmu.ac.uk)

Regards,
Zulezwan

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in diurnal variation conditions.

Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Jos Vanrenterghem.

School: Sport and Exercise Sciences
Contact details: Z.Ab-Malik@2011.ljmu.ac.uk

You are being invited to take part in a post-graduate student research study. Before you decide whether to take part it is important that you understand why the research is being done and what it involves. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

6. What is the purpose of the study?

In many sports, success depends on an athlete's peak power output, which is based on their ability to produce both rapid and forceful movements. This project will investigate the range of difference in maximum strength at slow, moderate, fast and very fast movement speeds and how this might contribute to the peak power output during a different time of day. (i.e. morning and evening). To achieve this we intend to investigate lower-limb muscle performance using a range of different scientific tests. We will use the information collected in these experiments to draw links between muscle performance during specific knee exercises on morning and evening. The knowledge we gain from completing this work will help us to look further in differences of diurnal for improving power output.

7. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do you will be given a copy of this information sheet keep and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw will not affect your rights/any future treatment/service you receive.

8. What will happen to me if I take part?

To participate in this study you must meet the following criteria:
Aged between 18 and 39 Years.
Greater than 2 years of strength training.
Free from recent serious musculoskeletal injuries.

This study will be conducted at the Research Institute for Sport and Exercise Sciences, Tom Reilly Building, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF. You will be required to visit the Research Institute on 8 occasions consisting of 4 familiarization sessions and 4 experiment sessions (2

morning and 2 evening). Each session is likely to last for less than 1 h and must be interspersed by a rest period of ~48 h. So, it is likely that you will need to attend the laboratory over a duration of 4 weeks. During each session you will need to wear normal gym/training clothing and you should abstain from alcohol for 24 hours and caffeine for 2 hours before each visit.

Introductory session.
During your initial session you will be asked to complete 3 short questionnaires about your health status, chronotype and recent activity and your body mass (kg) and height (m) will be measured. You will then be introduced to the exercise tests and you will have the opportunity to practice each test and become familiar with the equipment and test procedures. You will be familiarised with MVC and isolated knee exercises that will be used in the experiment sessions. These techniques are explained in detail in the paragraph below.

Our aim is to accurately measure lower-limb muscle performance using the state-of-the-art techniques and a robust scientific approach. Therefore you will be required to perform the experiment session described below on 4 separate occasions. This will enable us to determine the differences in each measurement so that we can be certain which differences in performance are due to differences in training status rather errors in measurement.

Experiment Session.
Each experiment session will begin with a general warm up of 5 minutes on a cycle ergometer at a low to moderate intensity of 75 W followed by a specific warm up of 5 sub-maximal jumps.
The power output of the knee extensor muscles (quadriceps) is a key factor in squat jump performance and the second part of the experiment session will focus on measuring your maximum force and speed during knee extension exercise. You will be seated in a specifically designed chair, straps will be used to minimise the movement of your thigh and upper body and the ankle of your dominant leg will be clamped. The test will measure the maximum force that you can produce while your leg is held stationary. You will be asked to push against it quickly and as hard as possible for a period of 4 seconds with and without twitch interpolation. This test will be repeated 3 times with a few minutes rest between each attempt. Next the power output of your leg will be measured using a technique known as isokinetic dynamometry. In this mode, the machine will only allow you to move your leg at a predetermined speed even though you will be asked to push against the resistance of the machine as hard and as fast as possible. You will perform warm up sets of 3 kicks at approximately 50 % maximum effort and then during each test you will be required to kick and pull your leg back 3 times with maximum effort. This test will be conducted at four speeds, i.e. slow, moderate, fast and very fast.

9. Are there any risks, discomforts, benefits involved?

- Each of the tests requires you to perform maximal muscle contractions, which may be associated with some tiredness and risk of injury. However, it is unlikely that you will feel any more discomfort than usual high-intensity training session.
- Exercise may result in muscle soreness up to 72 hours after testing if you are unaccustomed to the specific exercise.
- There is a small risk of soft tissue injury involved in the testing. Should you become injured you may need to withdraw from the study, however to minimise the risk of injury you will be closely supervised and you will also participate in a warm up before muscle strength testing.

- You will be closely supervised throughout the tests and the researcher has ensured that the location of the study is appropriate and any potential risks have been minimised.

10.Will my taking part in the study be kept confidential?

Data collection

- All data collected from you will be identified by a code from which you cannot be identified by simply reading the code.
- Data collected from you will remain strictly confidential between you and the researcher.
- Personal information will be treated in the strictest confidence with no association been made between your identity and the data observed.

Data storage

- All information/data will be stored confidentially and only accessed by the researcher. At the end of the study all personal identifiable information will be deleted and the data stored anonymously.
- Information linking you to the participant code is stored in a password protected file, which is only accessible by the researcher.
- All data will be stored on a password protected computer system.

Data access

- No one other than the researchers will have access to the data collected from you as part of this study.

Breaking confidentiality

- In accordance with UK law, the researchers are obliged to breach the promise of confidentiality if they have reason to believe that you are in danger from others or are likely to cause danger to others.
- It is possible that some participants may disclose other participants' confidential information.

NB. You must freely volunteer to be a subject and are able to withdraw, without prejudice, at any time. Thank you for your time and interest in the study.

Contact Details of Researcher

Z.Ab-Malik@2011.ljmu.ac.uk

LIVERPOOL JOHN MOORES UNIVERSITY

Title of Project: Lower-limb muscle performance in diurnal variation conditions.

Name of Researcher (Student): Zulezwan Ab Malik

Name of Research Supervisors: Dr Jatin Burniston, Dr Ben Edwards & Dr Jos Vanrenterghem.

School: Sport and Exercise Sciences
Contact details: Z.Ab-Malik@2011.ljmu.ac.uk

For most people, physical activity should not pose any problem or hazard. The PAR-Q is designed to identify the small number of adults for whom physical activity might be inappropriate or those who should seek medical advice concerning the type of activity most suitable for them.

1. Do you have a muscle, ligament bone or joint problem such as arthritis or previous injuries, which has been aggravated by exercise or might be made worse with exercise?	YES	NO
2. To your knowledge, do you have high blood pressure?	YES	NO
3. To your knowledge, do you have low blood pressure?	YES	NO
4 Do you have Diabetes mellitus or any other metabolic disorder?	YES	NO
5. Has your doctor ever said that you have raised cholesterol (serum level above 6.2mmol/L)?	YES	NO
6. Do you have or ever suffered a heart condition?	YES	NO
7 Have you ever felt pain in your chest when you do physical exercise?	YES	NO
8. Is your doctor currently prescribing you drugs or medication?	YES	NO
9. Have you ever suffered from shortness of breath at rest or with mild exercise?	YES	NO
10. Is there any history of Coronary Heart Disease within your family?	YES	NO
11. Do you ever feel feint, have spells of dizziness or have ever lost consciousness?	YES	NO
12. Do you currently drink more than the average amount of alcohol per week (21units for men and 14 units for women (1 unit = 1/2 pint of beer/cider/larger or 1small glass of wine))	YES	NO
13. Do you currently smoke?	YES	NO
14. Do you NOT currently exercise regularly (at least 3 times per week) and/or work in a job that is physically demanding.	YES	NO
15. Are you, or is there any possibility that you might be pregnant?	YES	NO
16. Do you know of any other reason why you should not participate in a programme of physical activity?	YES	NO

LIVERPOOL JOHN MOORES UNIVERSITY

Project title: Lower-limb muscle performance in diurnal variation conditions.
Researcher: Zulezwan Ab Malik
Sport and Exercise Sciences

5. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐

6. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐

7. I understand that any personal information collected during the study will be anonymised and remain confidential ☐

8. I agree to take part in the above study *(if appropriate please specify the type of study or particular intervention you are seeking consent for – eg focus group, interview, training programme)* ☐

Name of Participant	Date	Signature
Name of Researcher	Date	Signature
Name of Person taking consent *(if different from researcher)*	Date	Signature

Appendix

Title of Project: Mechanisms underpinning diurnal variation in human muscle performance

Recruitment letter/ email

Mechanisms underpinning diurnal variation in human muscle performance

Study Number: 14/WM/0065

LIVERPOOL JOHN MOORES UNIVERSITY

Zulezwan Ab Malik, BSc, MSc
Research Institute for Sport & Exercise Sciences
Z.Ab-Malik@2011.ljmu.ac.uk
0779 939 6665

Dear potential participant,

My name is Zule, I'm a PhD student in the School of Sport and Exercise Sciences supervised by Dr. Jatin Burniston. I am currently recruiting participants to take part in a research study for my PhD thesis on muscle function and myofibre protein analysis.

I'm looking for males aged 18 – 39 who are physical active and have a background in resistance exercise/ strength training. If you would like to offer your time for this exciting and beneficial research project you will be asked to attend the laboratory on 2 main occasions, one in the morning (7-8 am) and one in the afternoon (5-6 pm) to:

- perform muscle performance tests
- have a muscle biopsy where a ¼ pea-sized amount of muscle will be taken from your outer thigh muscle.

After the data are analysed, each participant will be able to view their muscle performance results and myofibre profile.

If you are interested or would like to chat about further details of the project, please contact me using one of the options listed below. **PLEASE NOTE THAT BY DISPLAYING AN INTEREST YOU ARE IN NO WAY OBLIGED TO PARTAKE IN THIS STUDY.**

Many thanks,

Zulezwan Ab Malik.

Zulezwan Ab Malik. TRB Rm 1.47
Z.Ab-Malik@2011.ljmu.ac.uk
Tel: 0779 939 6665

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Basic and Detailed Protocol

Mechanisms underpinning diurnal variation in human muscle performance

Study Number: 14/WM/0065

Zulezwan Ab Malik, BSc, MSc
Research Institute for Sport & Exercise Sciences
Z.Ab-Malik@2011.ljmu.ac.uk
0779 939 6665

This work will examine whether the difference in muscle performance between morning and evening is due to differences that occur to contractile proteins within muscle. This specific question has not been investigated before and we hope to discover new information about how muscle force production might be regulated by the body-clock. We hypothesise the greater force output in the afternoon is due to short-term modifications to contractile proteins within muscle that enable it to work more efficiently. If we are able to discover which proteins correspond with optimal muscle performance in the afternoon, we will be able to use these as a research tool or 'biomarker.' Such a biomarker could be used to provide objective evidence that pharmacological treatments or exercise interventions are able to prevent the decrement in muscle performance that typically occurs in the morning.

In order to test our hypothesis we aim to recruit 10 active male participants aged between 18 and 39 years. After consenting to take part in the study, these participants will be required to complete a protocol that consists of the following components:

- 2 x skin temperature recordings using adhesive skin thermistors
- 2 x core temperature recordings using a flexible rectal probe
- 4 x muscle temperature measurements using a 3 cm needle thermistor
- 2 x exercise tests, including general warm-up (5 min cyclergometry) and measurement of muscle function using isometric dynamometry (i.e. maximum voluntary contractions; MVC)
- 2 x needle biopsies (about the size of ¼ of a pea) from the muscle on the outside of your thigh

The muscle samples obtained will be analysed using state-of-the-art techniques capable of identifying hundreds of different proteins within muscle and characterising their different states of modification. The protein data will be aligned with the muscle force measurements to discover which proteins or protein modifications might regulate diurnal variations in force production.

Please see overleaf for a detailed version of the protocol

Purpose – To examine whether the difference in muscle performance between morning and evening is due to post-translation modification of human myofibrillar proteins.

Sample Population – Young (aged 18-39) recreationally active males from Liverpool John Moores University or associated sports teams.

Design – Balanced cross-over design where the order of the measures (morning-evening or evening-morning) will be randomised.

Methodology.

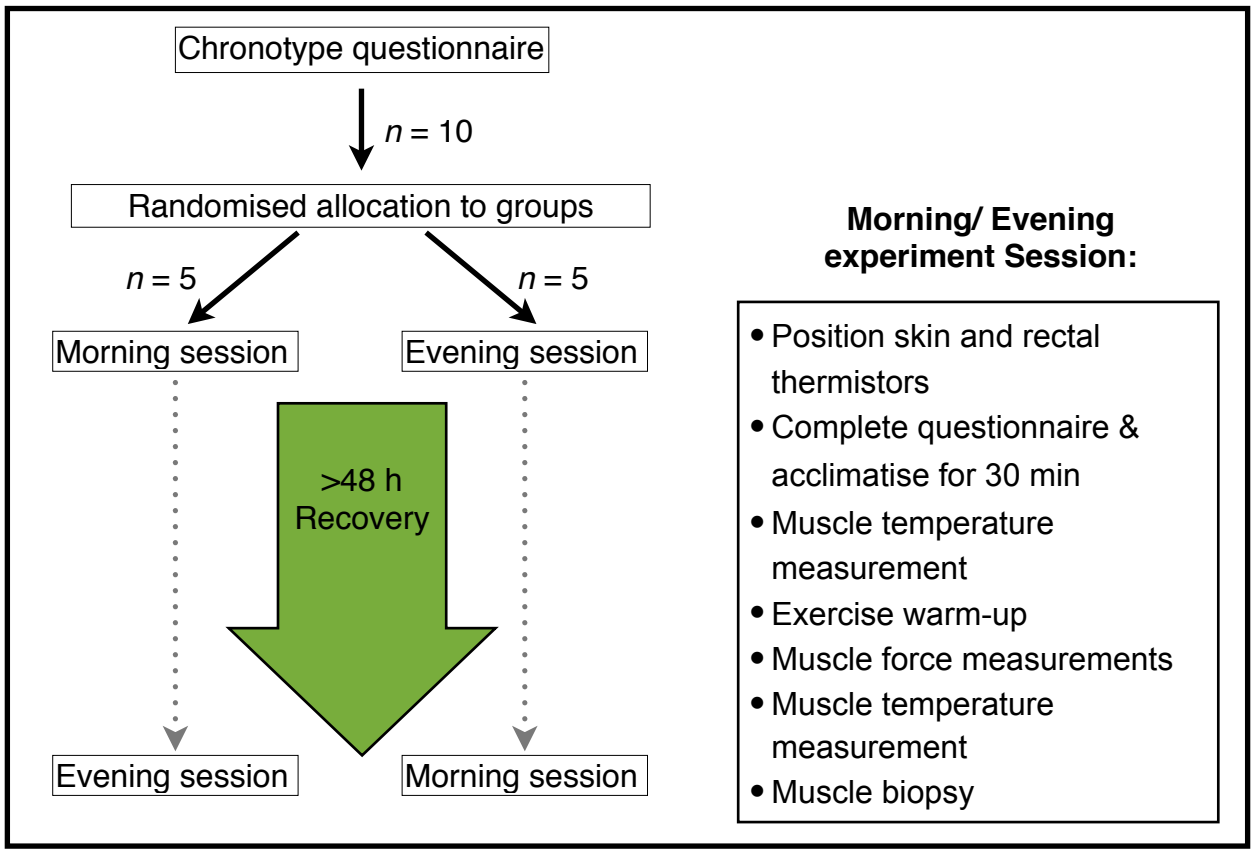

Figure 1 – Schematic representation of the proposed experimental protocol

Scientific Justification.

The process of human ageing from maturation to senescence is associated with marked losses in skeletal muscle mass and function that negatively impact mobility, quality of life and independence. In particular, muscle weakness is associated with a greater risk of falls, which may require hospitalization. The World Health Organisation reports 1 in 3 adults over the age of 65 falls each year, and the incidence of falls increases further with advancing age and muscle frailty. Interventions aimed at maintaining muscle mass are key in the defense against age-associated muscle loss (Sarcopenia). However, indices of muscle performance such as force or power output often decline earlier and more precipitously than the measured changes in muscle volume. Moreover, approximately twice as many falls occur in the morning, when muscle strength is at its lowest, compared

to in the afternoon/ early evening, when muscle performance is optimal. Diurnal variation in muscle performance is also widely reported in young healthy individuals but the mechanisms underpinning this phenomenon are not yet understood.

Day-to-day movements or sporting actives rely on the ability of skeletal muscle to produce force against varying external loads and at different speeds. Indeed, muscle output can be described along a continue from high-velocity low-force movements to low-velocity or stationary contractions. Recently we completed preliminary work using a battery of laboratory tests to determine which aspects of muscle performance show the greatest magnitude of diurnal variation. Twenty young healthy males completed measures spanning maximum force production through to maximum speed of contraction. Values were consistently greater in the evening compared to morning, and the magnitude of diurnal variation ranged from 7.6 % to 13.3 % across the 6 different measures. Rate of force development (RFD) and isometric force during maximal voluntary contraction (MVC) exhibited the greatest diurnal variation (2.89 ± 0.56 - 3.23 ± 0.52 Nm/s, P= 0.009 and 634.98 ± 53.8 - 704.7 ± 73.2 Nm, P=0.002 respectively). The proposed work will use these robust measures of performance and attempt to discover changes within muscle that correlate with the differences in muscle output.

Fluctuations in force production co-occur alongside biological rhythms in core temperature but we have found diurnal variation in muscle performance is not entirely explained by differences in muscle temperature. For example, passive heating in the morning to simulate the warmer afternoon muscle temperature did not bring about a similar elevation in muscle performance. Similarly, passive cooling in the afternoon to replicate the cooler muscle temperature of the morning was not associated with a decrease in performance. Thus intrinsic differences occur in the ability of skeletal muscle to produce force during the course of the day. The parameters of muscle performance (i.e. maximum force or maximum speed) are determined by a collection of contractile proteins. Differences in genetic background, habitual activity or training status effect the relative amounts of each muscle protein and are one of the main reasons for broadly different physiques and physical performances, e.g. sprinters compared to marathon runners. The same contractile proteins can also be rapidly modified to cope with short-term changes in demand. This latter mechanism contributes to the positive effect of warming up prior to strenuous exercise, and similar mechanisms could also be responsible for the greater output of skeletal muscle in the afternoon compared to the morning.

The current study will use state-of-the-art proteomic techniques to simultaneously investigate modifications to numerous contractile proteins in human skeletal muscle. Information regarding changes at the protein level will be compared with measures of muscle performance to determine the level of association between muscle output and a particular protein modification. If successful, this work will provide candidate biomarkers that could be used to develop and optimise interventions aimed at forestalling morning decrements in muscle performance.

Consent form

Study: *Mechanisms underpinning diurnal variation in human muscle performance*

Researchers: Zulezwan Ab Malik, Jatin Burniston, Graeme Close, Ben Edwards

Study Number: 14/WM/0065

Zulezwan Ab Malik, BSc, MSc
Research Institute for Sport & Exercise Sciences
Z.Ab-Malik@2011.ljmu.ac.uk
0779 939 6665

Please either initial or tick each box to acknowledge that you consent/ agree with the individual statements. Please then sign and date the document.

1. I confirm that I have read and understand the information sheet dated 11/03/2014 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that data collected during the study will be recorded and looked at by members of the research team listed above. I give permission for these individuals to have access to these records. ☐

4. I agree to take part in the above study ☐

Name of Participant	Date	Signature

Name of person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher's records

Medical History Questionnaire and Trial Inclusion Criteria

Name:.....

Age:.....

Height:..... (cm) **Weight:**..... (kg)

Telephone:.....

Zulezwan Ab Malik, BSc, MSc
Research Institute for Sport & Exercise Sciences
Z.Ab-Malik@2011.ljmu.ac.uk
0779 939 6665

The following information is required in order for the principal investigator to determine whether you are eligible to take part in the trial. This is to ensure that there is minimal risk to you when taking part in the investigation. Please circle as appropriate when answering.

Are you currently being treated for any medical condition or have been in the last year? If yes to either, please provide further information.

YES / NO

If yes _____

When was your last medical check-up? (if not sure please skip the question)

_____

Are you taking any medication, non-prescription drugs or herbal supplements?

YES / NO

If yes _____

Are you currently taking any dietary supplements including multivitamins, protein supplements, creatine etc? **If yes, state what and how frequently.**

YES / NO

If yes _____

Has there been any change in your health over the past year? If yes please explain.

YES / NO / NOT SURE

If yes _____

Do you have any allergies? If yes, please use the criteria below to tick the appropriate choice.

YES / NO / NOT SURE

(i) Medications

(ii) Rubber/latex products

(iii) Other (food, hayfever etc) please state _____

Have you ever had an adverse reaction to a needle injection?

YES / NO

Do you have or have ever had heart or blood pressure problems? If yes please state what.

YES / NO

If yes _____

Have you ever been hospitalized for any illness or operation? If yes please state what.

YES / NO

If yes _____

Do you have or have ever had any of the following? **Please tick the appropriate boxes.**

☐ Chest pain, angina	☐ Rheumatic fever	☐ Pacemaker	☐ Steroid therapy	☐ Seizures (epilepsy)	☐ Osteoporosis medications (e.g. Fosamax, Actonel
☐ Heart attack	☐ Mitral valve prolapse	☐ Lung disease	☐ Diabetes	☐ Kidney disease	
☐ Stroke	☐ Heart murmur	☐ Tuberculosis	☐ Stomach ulcers	☐ Thyroid disease	
☐ Shortness of breath		☐ Cancer	☐ Arthritis	☐ Drug/alcohol dependency	

Are there any medical conditions not listed above that you have? If yes, please state what.

YES / NO

If yes _____

Are you currently unfit to perform physical activity? If yes, please state why.

YES / NO

If yes _____

Do you have a bleeding problem or bleeding disorder?

YES / NO / NOT SURE

Have you travelled across multiple time zones or undertaken shift work during the past 3 months?

YES / NO

If yes, please provide details _____

_____

How many hours of physical activity (e.g. gym use, playing sport etc) do you engage in on average per week? **Please tick the appropriate choice.**

(i) 0-1

(ii) 1-3

(iii) 3-5

(iv) More than 5 hours

I confirm that the details that I have provided here are correct to the best of my knowledge and understand that withholding medical information can increase the risk of adverse events. I understand that the information that I have provided is confidential and that is for the attention of the principal investigator only.

PRINT NAME: _____

SIGNED: _____ DATE: _____

PI SIGNATURE: _____ DATE: _____