NON-INVASIVE AND INVASIVE ASSESSMENT OF CARBOHYDRATE INTAKES AND MUSCLE GLYCOGEN UTLISATION IN RUGBY LEAGUE AND AFL

Harry E Routledge

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"Success is no accident. It is hard work, perseverance, learning, studying, sacrifice and most of all, love of what you are doing or learning to do"

Pele

Abstract

The importance of muscle glycogen availability for both endurance and high-intensity intermittent exercise performance has been recognised for several decades. The measurement of muscle glycogen in human skeletal muscle has traditionally been performed through the invasive muscle biopsy technique. However, recent data from laboratory-based cycling studies suggests that ultrasound technology (using commercially based software known as MuscleSound) provides a valid and non-invasive assessment tool for which to measure exercise-induced glycogen utilization. Nonetheless, no data currently exist to evaluate the validity of the MuscleSound software in team sport athletes in response to field-based training or match play situations. Accordingly, the aim of the present thesis was to therefore examine the reliability and validity of ultrasound technology (via the MuscleSound system) to non-invasively assess muscle glycogen concentration in skeletal muscle of team sport athletes.

The aim of Study 1 (Chapter 3) was to quantify the test re-test reproducibility of the MuscleSound scoring system between (vastus lateralis, VL; vastus medalis, VM; rectus femoris RF) and within (25, 50 and 75% of the muscle length) muscles. Using a test-retest (60 minutes apart) scanning protocol in 100 recreationally active subjects (78 males, 22 females), it was identified that 50% of the VL muscle provided the most reliable anatomical site for which to obtain reproducible MuscleSound scores, as evidenced from the coefficient of variation (CV) – 5.8, intraclass correlation (ICC) – 0.89 (0.85 - 0.93), and ratio limits of agreement 1.02 (1.17) reliability statistics.

Having identified the optimal anatomical location for which to obtain reproducible MuscleSound scores, the aim of Study 2 (Chapter 4) was to evaluate the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in elite male Rugby League players (n=16) during competitive match play. Although match play depleted absolute muscle glycogen in the VL muscle by approximately 200-250 mmol·kg^{-1} dw, there were no significant differences in MuscleSound scores obtained before and after the game. As such, these data suggest that MuscleSound is not a valid tool to assess muscle glycogen utilisation during the high-intensity intermittent activity profiles that are inherent to team sports. Given that these data are in contrast to previous validation studies conducted with cycling-

based protocols, it was suggested that differences in mechanical loads and fluids shifts between exercise protocols may explain the discrepancies between studies.

To eliminate the potential confounding effects of acute changes in fluid shifts associated with differing exercise stimuli, the aim of Study 3 (Chapter 5) was to assess the validity of the MuscleSound system to detect changes in muscle glycogen re-synthesis across a time-scale in which acute changes in fluid shifts have been restored to pre-exercise values. Using a combination of glycogen depleting cycling-based exercise followed by 36 h of an isoenergetic high CHO or low CHO diet, resting muscle glycogen of the VL muscle (from 16 recreationally active males) was approximately 500 versus 250 mmol·kg^{-1} dw in the high and low CHO trials, respectively. However, no differences were observed in the MuscleSound scores obtained between the high and low trials. When considered with the results of Chapter 4, these data collectively suggest that the MuscleSound system is unable to detect differences in muscle glycogen concentration within the physiological range that typically occurs as a result of exercise-induced glycogen utilisation and/or post-exercise muscle glycogen re-synthesis.

Given that MuscleSound was not deemed a valid measurement tool, it was recommended that evaluations of the daily CHO and muscle glycogen requirements of team sport athletes should therefore be undertaken using traditional assessments of energy intake and muscle biopsies, respectively. Accordingly, the habitual CHO intakes of 44 elite professional male Australian Rules Football (AFL) players were quantified across the weekly micro-cycle in Study 4 (Chapter 6). These data demonstrate that elite AFL players practice elements of daily CHO periodisation in accordance with fluctuations in daily training load and proximity to the match day itself. Nonetheless, such data also highlighted that players do not consume CHO at the recommended dose to achieve glycogen storage that is likely required to facilitate optimal match day physical performance. In Study 5 (Chapter 7), muscle glycogen utilisation in the VL muscle was subsequently quantified using the muscle biopsy technique in two elite professional males during competitive match play. Importantly, players commenced the match after adhering to a 24 h CHO loading protocol of 8 g·kg^{-1} and after consuming a pre-match meal of 2 g·kg^{-1}. Data demonstrate that such a dietary protocol achieves pre-match muscle glycogen

concentration >500 mmol·kg^{-1} dw and that match play can induce an absolute glycogen utilisation >400 mmol·kg^{-1} dw. However, with the different demands observed between positions, total glycogen utlisation may vary. As such, it appears that the metabolic demands of AFL maybe more CHO dependent than other invasive team sports such as soccer and rugby.

In summary, the work undertaken in this thesis has demonstrated that the MuscleSound software is not valid to detect differences in muscle glycogen concentration within the physiological range (i.e. 200-300 mmol·kg^{-1} dw) that typically occurs as a result of exercise-induced glycogen utilisation and/or post-exercise muscle glycogen re-synthesis. For this reason, assessment of the CHO requirements of team sport athletes is likely dependent on the traditional approaches of dietary assessment and muscle biopsy. Using the latter methods, the present data also demonstrate that the CHO requirements of AFL match play is potentially different (though larger sample sizes are required) than other team sports such as soccer and rugby. Further studies are also required to quantify the glycogen requirement of the typical training sessions undertaken by professional players of specific team sports.

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Declaration

I declare that the work in this thesis, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own. Additionally, all attempts have been made to ensure that the work is original and does not to the best of my knowledge breach any copyright laws and has not been taken from the work of others, apart from work that has been fully acknowledged within the text of my work.

Publications and presented abstracts arising from this thesis:

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List of Abbreviations

ADP, Adenosine Diphosphate

AFL, Australian Rules Football

AGL, Amylo – α- 1,6- glucosidase, 4- α- glucanotransferase

AMP, Adenosine Monophosphate

ATP, Adenosine Triphosphate

$Ca2+$, Calcium Ions

cAMP, Cyclic Adenosine Monophosphate

CHO, Carbohydrate

CI, Confidence Interval

CV, Coefficient Variance

DW, Dry Weight

FTa, Fast Twitch a

FTb, Fast Twitch b

G-1-P, Glucose-1-Phosphate

G-6-P, Glucose-6-Phopsphate

GLUTs, Glucose Transporters

GLUT1, Glucose Transporter 1

GLUT2, Glucose Transporter 2

GLUT4, Glucose Transporter 4

GPS, Global Positioning System Device

HCHO, High Carbohydrate

HK, Hexokinase

HR_{Peak}, Heart Rate Peak

IMF, Intermyofribillar

Kg, Kilograms

LCHO, Low Carbohydrate

LDH, Lactate Dehydrogenase

NDP, Nucleoside Diphosphate Glucose

PAS, Periodic Acid-Shiff

PDH, Pyruvate Dehydrogenase

PFK, Phosphofructokinse

PHOS, Phosphorylase

Pi, Phosphate

PKA, Protein Kinase A

PPO, Peak Power Output

RF, Rectus Femoris

RHIE, Repeated High Intensity Efforts

RL, Rugby League

SR, Sarcoplasmic Reticulum

SS, Subsarcolemmal

ST, Slow Twitch

TEM, Transmission Electron Microscopy

UDP, Uridine Diphosphate

US, Ultrasound Technique

UTP, Uridine Triphosphate

VL, Vastus Lateralis

VM, Vastus Medialis

VO_{2max}, maximal oxygen uptake

VO_{2peak}, peak oxygen uptake

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

It is well established that high carbohydrate (CHO) availability improves both endurance and high-intensity intermittent exercise performance (Cermak & van Loon, 2013). As such, the practice of CHO loading to super-compensate muscle glycogen stores in the day(s) immediately prior to competition remains the cornerstone of nutrition practices (Thomas et al., 2016). Depending on the duration of the event, elite athletes may also consume CHO during competition (typically in the form of sports drinks, energy gels and bars) so as to maintain CHO availability late into exercise. The practice of ensuring high CHO availability before and during exercise emerged from the seminal studies of Bergstrom and colleagues (Bergstrom & Hultman, 1966, Bergstrom & Hultman, 1967, Bergstrom et al., 1967, Hermansen et al., 1967) demonstrating that high muscle glycogen availability (as achieved via consumption of a high CHO diet) improved exercise capacity. This work is some of the most highly cited in the field and is referenced accordingly in the most recent sport nutrition guidelines (Thomas et al., 2016).

The emergence of the muscle biopsy technique from Bergstrom and colleagues therefore provided the platform for the following decades of research examining the regulation of glycogen storage and metabolism during exercise. Nonetheless, the technique of removing muscle tissue has associated risks due to potentially endangering neurovascular structures, thus limiting its use to muscles with low vascularity such as the vastus lateralis. For this reason, the invasive nature of muscle biopsy sampling may induce complications for the assessment of glycogen metabolism in elite athletic populations. Indeed, much of our understanding of glycogen metabolism during exercise has arisen from laboratory-based exercise protocols in those subjects typically classified as "recreationally active" or "trained", although the latter population are usually not representative of elite endurance or team sport athletes. With this in mind, there is a definitive need to better understand the CHO and muscle glycogen requirements of the training and competition loads typically experienced in the field settings of elite athletes.

The recent emergence of the ultrasound (US) technique to non-invasively assess muscle glycogen (Hill & Millan, 2014, Nieman et al., 2015) therefore holds much promise for those practitioners working at the coalface of elite sport. This technique

is now available through commercially available software (MuscleSound) and is based on the premise that glycogen storage can be assessed through its association with water. In this way, glycogen depletion within the muscle (and hence loss of intracellular water) can manifest as changes in pixilation intensity upon US imaging. In the initial study in this area, Hill and Millan (2014) observed significant correlations between changes in the MuscleSound scoring system (an arbitrary score ranging from 0-100) and changes in muscle glycogen concentration (as directly assessed from the biopsies obtained from the rectus femoris) before and after 90-minutes of steady-state cycling exercise. Despite similar correlations and associations between glycogen utilisation within the vastus lateralis muscle during cycling and the MuscleSound scoring system (Nieman et al. 2015), there have been no further assessments of reliability or validity of the MuscleSound scoring system in either laboratory or field-based settings. Additionally, an alternative approach to assess the validity of the MuscleSound system to non-invasively assess muscle glycogen concentration is to assess its ability to detect the re-synthesis of muscle glycogen concentration in recovery from glycogen depleting exercise.

The potential feasibility of the MuscleSound system to non-invasively assess muscle glycogen concentration holds particular promise for team sport athletes given the intense training demands and fixture schedules typically experienced by such populations. For example, team sport athletes may train 4-5 times per week as well as partake in 1-3 competitive games (Anderson et al. 2016). Team sports athletes are also likely to encompass a range of CHO requirements owing to the demands of each sport, player position requirements and alterations to daily training loading patterns across the training micro-cycle (Malone et al. 2015). The physiological and metabolic demands of team sports have been typically well quantified for soccer with the aerobic system being high taxed with average and peak heart rates around 85 and 98% of maximal values respectively (Krustrup et al. 2006). Furthermore, soccer players typically cover distances of 10-13 km with around 800m being at high-speed running (<19.7 km/h) and 300m being covered at speeds < 25.1km/h (Bradley et al. 2009), with these high intensity bouts being glycogen dependant (Krustrup et al. 2006). Although considerably less data exists for other invasive team games such as Rugby and Australian Rules Football (AFL), where the game demands (total distance, high speed running) are greater (Coutts et al. 2010). As such, the

application of the non-invasive assessment of muscle glycogen concentration is particularly attractive as a methodological tool for which to better understand the metabolic demands of competitive match play within the aforementioned sports.

1.2 Aims and Objectives

The overall aim of the present thesis is to therefore examine the reliability and validity of ultrasound technology (via the MuscleSound system) to non-invasively assess muscle glycogen concentration in human skeletal muscle. This aim will be achieved via a series of laboratory and field-based studies conducted on recreationally active males and females plus elite male athletes from Rugby League and AFL.

This aim will be achieved by completion of the following objectives:

1. The quantification of the reliability and reproducibility of the MuscleSound scoring system within and between muscles of the quadriceps. This objective will be achieved through the completion of Study 1 (Chapter 3).

2. The assessment of the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in elite male Rugby League players during competitive match play. This objective will be achieved through the completion of Study 2 (Chapter 4).

3. The assessment of the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen re-synthesis within 36 hours following glycogen depleting based exercise. This objective will be achieved through the completion of Study 3 (Chapter 5).

4. The assessment of CHO intakes and muscle glycogen utilization in elite male AFL players. This objective will be achieved through the completion of Study 4 and 5 (Chapter 6 and 7).

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

The importance of high CHO availability to promote optimal exercise performance is supported by almost 100 years of research. For example, Krogh and Lindhard (1920) first reported the efficiency of CHO as fuel source during exercise and also demonstrated that fatigue occurs earlier when subjects consume a diet that is low in CHO. Levine et al. (1924) also observed that runners who completed the 1923 Boston marathon exhibited hypoglycemia immediately post-exercise, thus suggesting that low CHO availability may be linked to fatigue. The field grew considerably in the late 1960s with the introduction of the muscle biopsy technique where it was established that exercise capacity is proportional to the muscle glycogen concentration prior to exercise (Bergstrom & Hultman, 1967). These early studies paved the way for decades of research demonstrating that muscle glycogen stores augment exercise performance across a range of exercise modalities such as running (Bosch et al., 1993), cycling (Maughan & Poole, 1981) and in team sports situations (Balsom et al., 1999).

In the present review, the reader is provided with an overview of muscle glycogen storage and metabolism in order to provide the basis for a discussion on the effects of endogenous and exogenous CHO availability on exercise performance (where specific attention is then given to CHO requirements for team sports). An overview of the methods to assess muscle glycogen in human skeletal muscle is then presented so as to provide a greater rationale for the experimental studies undertaken in the present thesis.

2.2. Carbohydrate Storage and Metabolism in Exercise

2.2.1 Glycogen Storage

Carbohydrate is predominately stored as glycogen in both the muscle and the liver (approximately 400 and 100 grams, respectively) with 5 grams also circulating in the blood stream as glucose. Glycogen is the storage form of glucose, which is a form of CHO that can be mobilized as an energy source for exercise. In skeletal muscle, glycogen is typically expressed as $mmol \cdot kg^{-1}$ of dry muscle where concentrations in whole muscle homogenate can vary from 50 to 800 $mmol.kg^{-1}$ depending upon training, fatigue and dietary CHO intake (see Figure 2.1).
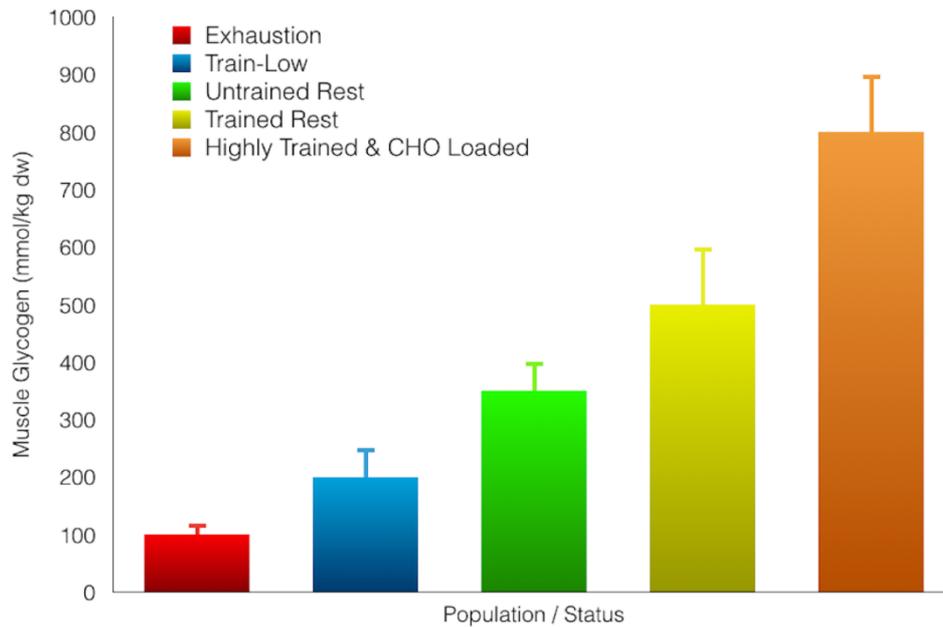
Figure 2.1 – Variations in muscle glycogen storage according to fatigue status, training status and dietary CHO intake (data are compiled from studies including Taylor et al., 2013, Bartlett et al., 2013, Impey et al., 2016, Bussau et al., 2002). "Train-low" refers to training sessions deliberately commenced with low muscle glycogen in an attempt to promote the activation of cell-signaling pathways that regulate oxidative adaptations of human skeletal muscle (Impey et al. 2018).

Glycogen is a large branched polymer of glucose residues that can be broken down to yield glucose molecules when energy is required. The molecular structure of glycogen is essentially a tiered structure of glucose residues that are linked by α-1:4 and 1:6 glycosidic bonds. Glycogen granules are formed on the protein glycogenin and can be as large as 42 mm in diameter as well as having potentially 12 tiers. At its maximal size, the granule can consist of as much as 55000 glucosyl units (Graham et al., 2008). Nonetheless, the majority of glycogen granules in human skeletal muscle are reported to be 25 mm in diameter with approximately 8 tiers (Marchand et al., 2002). Through the use of histochemical techniques it has typically been reported that resting glycogen content is not apparently different between type I and type II fibres (Essen & Henriksson, 1974, Essen et al., 1975, Stellingwerff et al., 2007). Nonetheless, using biochemical quantification (a more quantitative measure) it has been reported that type II fibres may contain 50-100 $mmol \cdot kg^{-1}$ dw more glycogen than type I fibres (Tsintzas et al., 1995). Regardless of method of quantification, glycogen depletion during exercise is dependent on fibre type

recruitment patterns that are dependent upon the specifics of the exercise protocol. This is evident during prolonged steady state type protocols where type I fibres show a preferential depletion whereas during the supra-maximal type activity, type II fibres become recruited and show considerable glycogen depletion (Gollnick et al., 1974). During activities involving high-intensity intermittent exercise, considerable glycogen depletion is observed in both fibre types. Indeed, after a 90 minute soccer match 50% of all fibres are qualitatively classified as empty or partially empty (Krustrup et al., 2006).

In addition to fibre type specific storage and utilisation, the localisation of muscle glycogen has been identified (via electron microcopy) in three distinct subcellular locations: intermyofribillar (IMF) glycogen (located between the myofibrils close to the SR and mitochondria), intramyofibrillar (Intra) glycogen (located in the myofibrils interspersed within the contractile filaments) and finally the subsarcolemmal (SS) glycogen pool (located just beneath the surface of the membrane in close association with the mitochondria, lipids and the nuclei). In terms of deposition of glycogen, the IMF glycogen pool is the main site of glycogen storage consisting of approximately 75% of the cell's total store whereas the Intra and SS glycogen account for 5 to 15% of the total (Nielsen & Ortenblad, 2013). In relation to exercise-induced glycogen depletion, the Intra stores are those that are considered most closely associated with fatigue (Marchand et al., 2007; Nielsen et al., 2011; Ortenblad et al., 2011).

2.2.2 Overview of Glycogen Metabolism

Muscle glycogenolysis (i.e. the breakdown of glycogen to glucose-1-phosphate, G-1-P) is under the control of the enzyme glycogen phosphorylase which in turn can be regulated via hormonal and/or allostric control. During exercise, adrenaline is secreted from the adrenal medulla and binds to its receptor on the muscle cell membrane. This attachment causes an activation of the G-protein, which in turn activates adenylate cyclase converting ATP to cAMP. cAMP then activates the inactive protein kinase A (PKA) resulting in active PKA which in turn activates phopshorylase kinase. Activation of phosphorylase kinase converts glycogen phosphorylase from its inactive *b* form to its more active *a* form, which is the enzyme responsible for cleaving a glucose molecule from glycogen. The activity of glycogen

phosphorylase can also be regulated via allosteric regulation via changes in Ca^{2+} flux, ADP and AMP accumulation. An overview of key regulatory sites of control for CHO metabolism is shown in Figure 2.2 and specific regulatory control points are discussed in relation to key exercise factors in subsequent sections.
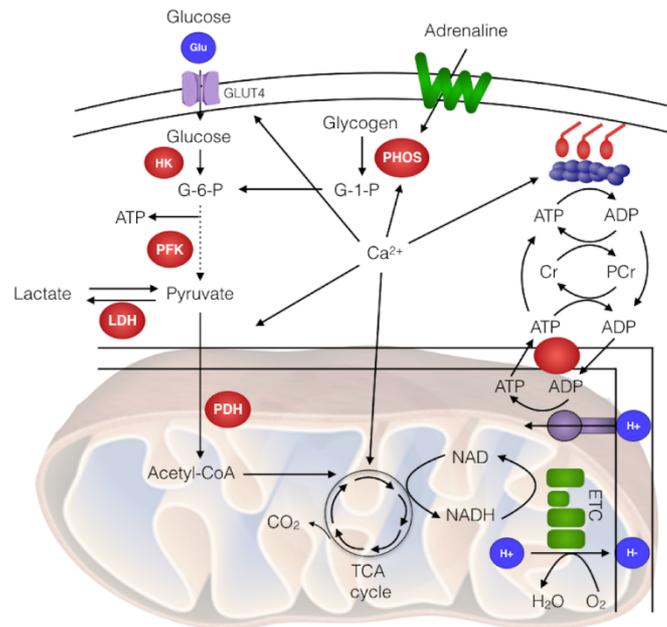
Figure 2.2 - Overview of CHO metabolism and main control points. Key regulatory enzymes are well recognized as phosphorylase (PHOS), hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH). Additionally, the rate of muscle glucose uptake can also determine the flux through glycolysis. ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; Ca^{2+}, calcium; Cr, creatine; ETC, Electron transport chain; G-1-P; Glucose-1-phosphate; G-6-P; Glucose-6-phosphate; Glu, Glucose; PCr, Phosphocreatine.

2.2.3. Effects of Exercise Intensity

As exercise intensity increases to > 85% VO_{2max}, both muscle glycogenolysis and muscle glucose uptake increases such that CHO metabolism predominates (see Figure 2.3). Given that phosphorylase can be transformed via covalent modification (i.e. phosphorylation by phosphorylase kinase) mediated through adrenaline, it would be reasonable to expect that greater phosphorylase transformation from *b* to *a* may be one mechanism to explain increased glycogenolysis evident with increasing exercise intensity. However, the percentage of phosphorylase in the more active *a* form does not appear to be increased with exercise intensity and in actual fact, is

decreased after only 10 minutes of high intensity exercise, which may be related to the reduced pH associated with intense exercise (Howlett et al. 1998). In this regard, vital signals related to the energy status of the cell play a more prominent role. Indeed, as exercise intensity progresses from moderate to high-intensity exercise, the rate of ATP hydrolysis increases so much so that there is a greater accumulation of ADP, AMP and Pi. In this way, the increased accumulation of Pi as a result of increased ATP hydrolysis can increase glycogenolysis as it provides increased substrate required for the reaction. Furthermore, greater accumulations of free ADP and AMP can also subsequently fine tune the activity of phosphorylase *a* through allosteric regulation (Howlett et al. 1998).
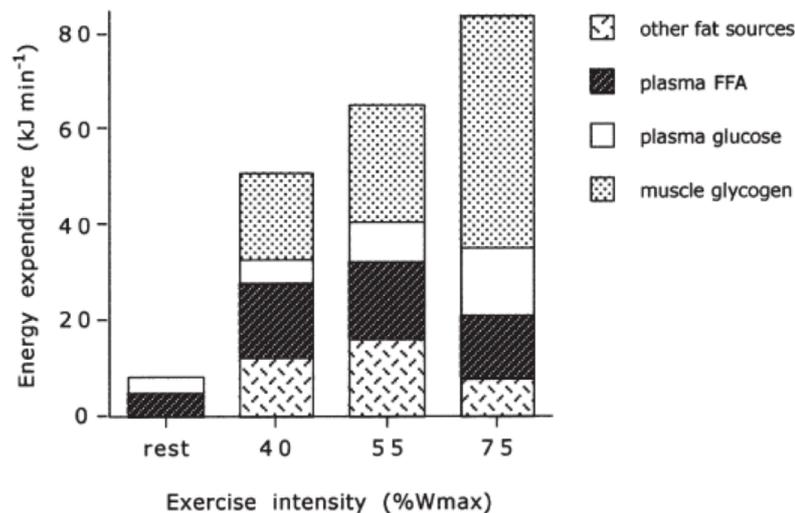
Figure 2.3 – Effects of exercise intensity on substrate utilization during exercise (taken from Van Loon et al., 2001)

2.2.4. Duration

In contrast to exercise intensity, prolonged steady state exercise lasting several hours is characterised by a shift towards increased lipid oxidation and reduced CHO oxidation rates (see Figure 2.4). This shift in oxidation rates is accompanied by an increased contribution of plasma FFA towards energy expenditure and a decreased reliance on both muscle glycogen and intramuscular triglcerides. Studies examining the regulatory mechanisms underpinning this shift in substrate utilisation have suggested that a reduction in muscle glycogen availability (due to progressive

glycogen depletion) and hence a reduced glycolytic flux down-regulate PDH activity thereby leading to reduced CHO oxidation. In addition, progressive increases in plasma FFA availability (due to continual lipolysis in adipose tissue) stimulate lipid oxidation. The down-regulation of PDH activity as exercise duration progresses may be due to reduced pyruvate flux therefore reducing substrate production required for the PDH reaction (Watt et al. 2002). In addition, more recent data demonstrate an up-regulation of PDH kinase activity during exercise which would therefore directly inhibit PDH activity (Watt et al. 2004). Taken together, these data are consistent with the many observations that increasing or decreasing substrate availability is one of the most potent regulators of fuel utilisation patterns during exercise and this concept is discussed in the next section. Given that most competitive endurance events such as marathon running (Leckey et al. 2015) and cycling time-trials are CHO dependent (Torrens et al. 2017), it follows that it is the reduction in CHO availability that is recognised as a major contributing factor that underpins the reduction in race pace that occurs during exercise.
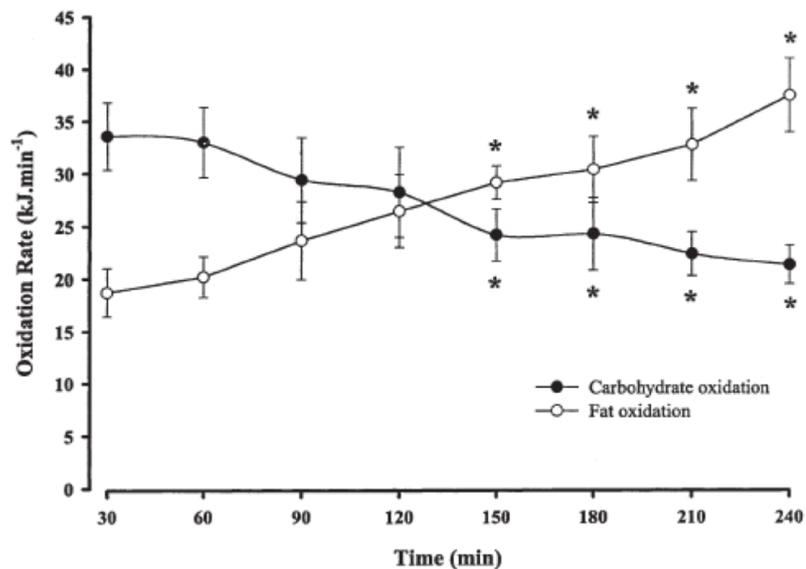
Figure 2.4 – Whole body CHO and lipid oxidation rates during prolonged exercise at 57% VO_{2max} (taken from Watt et al., 2002)

2.2.5. Training Status

Endurance training results in a number of profound physiological and metabolic adaptations which function to reduce the degree of perturbations to homeostasis for a given exercise intensity and ultimately, delay the onset of fatigue (Holloszy & Coyle 1984). Adaptations to endurance training are most recognised functionally by an increase in maximal oxygen uptake as well as a rightward shift in the lactate threshold. From a metabolic perspective, the most prominent adaptation is an increase in the size and number of mitochondria (i.e. mitochondrial biogenesis) which essentially permits a closer matching between ATP requirements and production via oxidative metabolism (Holloszy et al.1967). The adaptive response of muscle mitochondria is also accompanied by increases in capillary density, substrate transport proteins and increased activity of the enzymes involved in the main metabolic pathways. In addition, endurance training increases the capacity for skeletal muscle to store glycogen and triglycerides thereby increasing substrate availability.

In relation to substrate utilisation during exercise following endurance training, the most notable response is a reduction in CHO utilisation with a concomitant increase in lipid oxidation (Henriksson, 1977). For a given exercise intensity, glycogen utilisation is reduced with exercise training (Karlsson et al. 1974), an effect that is confined locally to the actual muscles that were trained (Saltin et al. 1976). The reduced glycogenolysis observed after training is not due to any change in phosphorylation transformation but rather, allosteric mechanisms (Chesley et al. 1996; Le Blanc et al. 2004). Indeed, exercise in the trained state is associated with reduced content of ADP, AMP and Pi thereby providing a mechanism leading to reduced phosphorylase activity. In addition to a reduction in glycogenolysis, muscle glucose uptake is also reduced when exercising at the same absolute workload following a period of endurance training (Bergman et al., 1999). This reduction in CHO metabolism when exercising at the same absolute workload is recognised as the one of the main metabolic adaptations underpinning improved endurance performance that occurs with prolonged endurance training.

2.3. Muscle Glycogen and Carbohydrate Loading Protocols

Given the effects of exercise intensity, duration and training status on muscle glycogen utilization, it follows that glycogen depletion (in both muscle and liver) is a major cause of fatigue in both endurance and high-intensity (intermittent) type activities. As such, traditional nutritional advice for these types of activities (whether it is competitive situations or training sessions) is to ensure high daily CHO intake before, during and after the activity so as to promote both performance and recovery.

The basic principles of CHO loading were developed from the pioneering work of Bergstrom and colleagues in the late 1960's who identified a mechanism that induced a super compensation effect of glycogen storage following a period of exhaustive exercise accompanied by several days of high dietary CHO intake (Bergstrom et al., 1967, Bergstrom & Hultman, 1967). From these early studies, the seven-day CHO loading strategy was formed, whereby untrained individuals perform 3-4 days of hard training with a low CHO intake, followed by a 3-4-day loading phase of a high CHO diet and an exercise taper (Burke, 2007).

In the early 1980s, the CHO loading strategy was later modified for the well-trained athlete. Indeed, it was evidenced that well trained athletes were able to super-compensate their glycogen stores without the need for a severe prior glycogen depletion phase (Sherman et al., 1981). It was observed that a simple exercise taper in conjunction with several days of increased dietary CHO intake was sufficient to increase glycogen storage. In more recent work, it has also been identified that CHO loading can occur with 24 hours of a high CHO intake ($10g \cdot kg^{-1}$ body mass per day) in well trained athletes. Indeed, after one day of the high CHO diet, muscle glycogen levels increased from 90 $mmol.kg^{-1}$ wet weight to 180 $mmol.kg^{-1}$ wet weight (Bussau et al., 2002). After two more days of rest and the CHO rich diet, glycogen levels remained stable, suggesting that well trained athletes are able to increase glycogen storage in both type I and type II fibres with 24-48 hours of an exercise taper and high CHO diet.

In relation to practical application, it also accepted that high glycemic foods are superior to low glycemic foods (Burke et al., 1993) in augmenting glycogen storage in conjunction with dietary intakes of 8-12 $g \cdot kg^{-1}$ per day of CHO (Thomas et al.,

2016). The consensus from the literature over the last 40 years is that CHO loading can improve performance and capacity when the exercise is greater than 90 minutes in duration (Hawley et al., 1997). Indeed, Hawley et al. (1997) cited that CHO loading can improve exercise capacity by approximately 20% and time-trial performance can increase by 2-3%. The enhanced performance effect is likely initially mediated by a delay in the time-point at which energy availability becomes limiting to the maintenance of the desired workload, which in the case of 'race pace' is dependent on sustained and high rates of CHO oxidation (O'Brien et al., 1993, Leckey et al., 2016). In addition to providing substrate for ATP production, it has also been established that glycogen availability in the intramyofibrillar storage pool can directly modulate contractile function given that low glycogen availability reduces Ca^{2+} release from the sarcoplasmic reticulum (Ortenblad et al., 2011, Gejl et al., 2014, Ortenblad et al., 2013). This impaired excitation-contraction coupling is likely to be of particular importance during those situations where higher power outputs and sprint finishes are required in the very late and finishing stages of races. Similarly, this impaired Ca^{2+} release may also explain the reduction in sprint performance that often occurs in the later stages of team sport games such as the second half of soccer matches (Mohr et al. 2003).

In addition to CHO loading, the effects of pre-exercise feeding (3-4 hours before competition) is also advantageous in elevating both muscle glycogen stores (Wee et al., 2005) and liver glycogen content, the latter typically depleted after an overnight fast. This is particularly pertinent given that liver glycogen content is related to exercise capacity (Casey et al., 2000). Sherman et al., (1991) observed that time trial performance (equating to approximately 45 minutes of cycling at 70% VO_{2max}) after a prior 90-minute steady state exercise protocol, also at 70% VO_{2max}, was greater with 150 g of CHO ingestion pre-exercise compared with 75 g of CHO, both of which were greater than no meal. The enhanced performance effect was associated with a maintenance of blood glucose later during the exercise period. Additional work also observed that performance can be further increased when CHO is ingested during exercise in addition to a pre-exercise meal (Wright et al., 1991). As such, current CHO guidelines for pre-exercise feeding advise an intake of 1-4 $g \cdot kg^{-1}$ body mass 3-4 hours prior to exercise (Thomas et al., 2016).

2.4. Exogenous Carbohydrate Intake

In addition to high endogenous pre-exercise muscle glycogen stores, it has also been identified that exogenous CHO feeding during exercise improves physical, cognitive and technical elements of performance (Stellingwerff and Cox, 2014). Initially it was accepted that exogenous CHO oxidation rates were limited at approximately 1 g·min^{-1} due to saturation of intestinal glucose transporters, though it is now known that exogenous CHO oxidation rates can increase to 1.8 g·min^{-1} with the addition of sucrose or fructose to the CHO blend (Jeukendrup, 2014). It is currently thought that CHO feeding during exercise may augment exercise performance via multiple mechanisms consisting of muscle glycogen sparing (Stellingwerff et al., 2007), liver glycogen sparing (Gonzalez et al., 2015) and maintenance of plasma glucose and CHO oxidation rates (Coyle et al., 1986). It is important to note however that the exercise duration, intensity, nutritional status prior to exercise, CHO intake rate and the CHO type consumed during exercise will all have an impact upon the efficacy of these mechanisms, fuel metabolism and performance.

Furthermore, evidence exists to support the notion that exogenous CHO feeding during exercise also improves performance when exercise duration is < 60 minute (i.e glycogen availability is not limiting) (Jeukendrup et al., 1997), an effect that is not apparent when glucose is directly infused to the bloodstream during exercise (Carter et al., 2004). These data suggest that CHO feeding may also improve exercise performance via non-metabolic mechanisms but through effects on the central nervous system (Carter et al., 2004). In this regard, a growing body of literature has demonstrated that simply 'rinsing' CHO in the oral cavity (for 10-second periods every 5-10 minutes during exercise) is also ergogenic to performance (Burke & Maughan, 2015), an effect that is independent of sweetness (Chambers et al., 2009) and that is especially apparent in the absence of a pre-exercise CHO meal (Lane et al., 2015) and low pre-exercise muscle glycogen (Kasper et al., 2016).

Conventional approaches to exogenous CHO fueling strategies are to consume 6-8% CHO beverages. This approach does not allow for flexibility in terms of individual variation in body mass or actual fluid requirements given variations in ambient conditions (Lee et al., 2014). Athletic populations tend to rely on a CHO fueling approach that is based on a combination of solids (e.g bars) semi solids (e.g gels)

and fluids (e.g sports drinks) so as to collectively achieve individualized exogenous CHO targets, typically in the region of 30-90 $g \cdot h^{-1}$ depending upon on exercise duration. Nevertheless, whilst there is negligible differences in the CHO oxidation rates (in fluid matched conditions) between the aforementioned sources (Pfeiffer et al., 2010), it is noteworthy that many athletes experience gastrointestinal discomfort when attempting to hit these targets, possibly related to extreme differences in osmolality between commercially available CHO gels (Zhang et al., 2015) as well as the presence of fibre, fat and protein in energy bars (Pfeiffer et al., 2012). Accordingly, it is now advised that athletes should clearly practice their approach to in-competition fueling during those training sessions of similar intensity and duration as competition. As a general rule, it is suggested that 30-60 $g \cdot h^{-1}$ of CHO is consumed during events lasting < 60-90 minutes whereas in events > 2.5 hours, 60-90 $g \cdot h^{-1}$ (glucose/fructose blends) is the recommended rate (Thomas et al., 2016).

2.5 Carbohydrate Guidelines for Team Sports

Much of the literature reviewed thus far (and indeed that which forms the basis of contemporary sport nutrition guidelines) is based on research conducted in endurance sports. Accordingly, the nutritional guidelines for team sports are largely adopted from this literature base. However, the nutritional requirements of team sports are likely to vary according to the demands of the sport. Furthermore, team sport players represent the full spectrum of body shapes and sizes, thus further highlighting the need for both sport-specific and athlete specific guidelines. The common pattern of play in team sports is 'stop and go' where players perform repeated bouts of brief high-intensity exercise punctuated by lower intensity activity (Williams & Rollo, 2015). The majority of team sports have been estimated to have an average game intensity of 70-80% VO_{2max} (Phillips & Van Loon, 2011) though repetitive periods of anaerobic type activity is well documented through motion analysis studies. Furthermore, team sports such as rugby and Australian Rules Football also involve whole body tackles, scrummaging and wrestling for possession of the ball. Unfortunately, the energy cost of repeated body contact currently is difficult to obtain. Given the high-intensity intermittent activity profiles, the fueling demands for team sports largely focus on advising high CHO availability in order to promote glycogen storage to support training and competition (Burke et al., 2011).

2.5.1 Soccer

Soccer is an intermittent sport in which the anaerobic system is highly taxed with average and peak heart rates around 85-98% of maximum (Bangsbo, 1994, Nielsen et al., 2004, Drust et al., 2000). The physical demands of a soccer match are such that players typically cover distances of 10-14 km per match (Bangsbo et al., 2006, Bloomfield et al., 2007, Di Salvo et al., 2007). The majority of the distance covered in a soccer match has been classified as low-to-moderate intensity at speeds between 0-19.8 km·h^{-1} (Bradley et al., 2009) whereas high-intensity running accounts for ~8% of the total distance completed (Rampinini et al., 2007).

In terms of energy production for soccer performance, muscle glycogen is recognised as the predominant substrate with several studies suggesting that the reduced ability to perform high-intensity exercise towards the end of a game is due in part to glycogen depletion (Mohr et al., 2003). Accordingly, pre-exercise CHO loading elevates physical performance during soccer type exercise protocols and simulations (Balsom et al., 1999, Bangsbo et al., 1992). Saltin (1973) first quantified muscle glycogen use during a soccer match, with pre-game vastus lateralis muscle glycogen values > 400 mmol·kg^{-1} dw showing improved physical performance (e.g. distance ran) when compared with low pre-match muscle glycogen availability (~200 mmol·kg^{-1} dw). Jacobs et al. (1982) also observed post-match glycogen concentration in the vastus lateralis muscle to be approximately 200 mmol·kg^{-1} dw post game (Jacobs et al., 1982). Krustrup et al. (2006) also observed post game glycogen concentrations between 150 – 350 mmol·kg^{-1} dw, although upon analysis of single fibres, glycogen was almost or completely depleted in 50% of all fibres (including both fast and slow twitch fibre types). In this context, an interesting observation on the resynthesis of muscle glycogen following a soccer match is that even 48 h after match play, some players may not have replenished muscle glycogen stores despite consuming a CHO rich diet > 8g·kg^{-1} body mass (Gunnarsson et al., 2013). Accordingly, the nutritional recommendations for optimal match play performance advise high CHO availability before, during and after games (Burke et al., 2011) so as to promote muscle glycogen stores (Balsom et al., 1999, Bangsbo et al., 1992), maintain plasma glucose levels and ensure the ability to perform technical and cognitive skills (Ali & Williams, 2009, Russell & Kingsley, 2014).

In terms of daily CHO intake, recent work from Anderson et al. (2017) identified how elite professional players from the English Premier League appear to adopt elements of CHO periodisation such that relative CHO was greater on match days (6.4 ± 2.2 g·kg^{-1}) compared with training days (4.2 ± 1.4 g·kg^{-1}). Nonetheless, these data demonstrate that elite players do not achieve CHO intakes that are suggestive of match play guidelines in terms of promoting muscle glycogen storage on the day prior to match play.

2.5.2 Rugby

Rugby league (RL) is a high intermittent team sport played over an 80-minute game. RL requires repeated bouts of relatively short, high intensity efforts such as sprints, high impact collisions and sudden changes of direction (Twist et al. 2014). High intensity collision events including tackling, isometric holding, wrestling, hit ups and impacts with the surface, increase the energetic cost of play, similar to that of AFL (Clarke et al., 2017, Gray & Jenkins, 2010). During a typical rugby game players average speeds equate to ~68 m·min^{-1} - ~85 m·min^{-1} (Cahill et al., 2013, Waldron et al., 2011) dependent upon code (Rugby League v Rugby Union). Such speeds are slower than that reported in AFL (~100-120 m·min^{-1}) and soccer (80-100 m·min^{-1} (Varley et al., 2014). Nonetheless, and similar to soccer, it is widely accepted that CHO is the predominant fuel source for rugby match play (Bradley et al., 2015a). Indeed, recent work from Bradley et al. (2016) demonstrated that following a RL match, there is an approximate 40% depletion in muscle glycogen content where pre-exercise values decreased from approximately 450 to 250 mmol.kg^{-1} dw. Such magnitudes of glycogen depletion agree with that observed previously in soccer (Krustrup et al., 2006).

In relation to daily CHO intake, Bradley et al. (2015) also observed that elite rugby union players adopt elements of CHO periodization such that CHO intake on training days equates to 3.5 g·kg^{-1} but increases to > 5 g·kg^{-1} the day before the match. It is noteworthy that rugby players' body mass may be in excess of 120 kg such that in some individuals this could equate to an absolute dose of 600 g of CHO.

2.5.3 Australian Rules Football

Australian Rules Football (AFL) is a field-based collision sport requiring well-developed capacities of strength, power, speed and endurance. Whilst there are large differences between positions, professional AFL players typically cover ~ 11 - 13 km during matches at an average speed of 108-128 m·min^{-1} (Coutts et al., 2015, Sullivan et al., 2014). In addition, AFL players will typically cover up to 1490 m of high-speed running (HSR) (19.8 km·h^{-1} to 25.2 km·h^{-1}) and up to 460 m of sprinting (>25.2 km·h^{-1}) in a game (Wisbey et al., 2010, Coutts et al., 2010, Varley et al., 2014). Further, game data also highlights significant ($P < 0.01$) reductions in total distance and high intensity running from the 1st to the 4th quarter with players also covering a larger amount of total distance and high intensity in the first half (Coutts et al., 2009). Internal load data has shown mean heart rates of 160 bpm and 178 bpm for a back and midfielder respectively. Intense periods of play showed heart rates which reached 170-180bpm (~94% maximum heart rate), with heart rate dropping to ~140 bpm during lengthy recovery periods (Gray & Jenkins, 2010). Such data suggest that AFL match play is more metabolically demanding than both soccer and rugby. However, unlike soccer and rugby, the glycogen requirements of AFL match play have not yet been quantifed.

The habitual nutritional intakes of AFL players are also less well studied than that of soccer and rugby players. Early work from the 1980's (Burke and Read, 1988) reported CHO intakes of 373 ± 94 g (4.5 ± 1.1 g·kg^{-1}) with later work reporting higher daily CHO intakes of 489 ± 88g (5.9 ± 1.1 g·kg^{-1}) (Ebert, 2000, Schokman et al., 1999), a finding that was suggested to be due to an increase in the influence of sport nutrition practitioners. Whilst such studies have reported total daily CHO intake, to the author's knowledge there is yet no study to quantify the potential periodisation of CHO intake across the typical training microcycle. In a recent report, Bilsborough et al. (2016) quantified CHO intake across five time points in the season (start of pre-season, mid pre-season, start of in season, mid-season, end of in season) and observed that players reported daily CHO intakes of 4.1 ± 1.6 g·kg^{-1} were below the nutritional recommendations of 6-10 g.kg^{-1} for training between ~ 1-3 h/day (Mujika & Burke, 2010). Given the suggested elevated energetic demands of AFL compared with both soccer and rugby, it could therefore be suggested that AFL

players perhaps under-consume CHO in relation to the suggested dose that may optimise performance.

2.6. Assessment of Muscle Glycogen in Athletic Populations

2.6.1 Muscle Biopsy Sample

The most commonly used method to assess muscle glycogen is through the use of an invasive muscle biopsy. This procedure is common in sport science research but does have its drawbacks and limitations due to the invasive nature of the procedure. The percutaneous biopsy technique is designed to obtain skeletal muscle tissue from human subjects, with Duchenne (1806-1875) being recognised for the first construction of the first biopsy needle (Shanely et al., 2014). In the 1960's, Bergström developed a similar needle that is still being used today. The latter technique uses the addition of suction to the inner bore of the biopsy needle after the insertion into the subject's muscle (Figure 2.5) (Greene et al., 2017). The suction pulls the surrounding muscle tissue into the needle, taking a large muscle sample (~100 mg) (Evans et al., 1982).

Figure 2.5 - Illustration of an invasive Bergström needle performing a muscle biopsy on the gastrocnemius muscle (taken from Greene et al., 2017).

In addition to the Bergström needle, the Monopty 12 G is a disposable core instrument (BARD, Brighton, UK, Figure 2.6) that is increasingly being used in both the laboratory and field-based studies (Bradley et al., 2016). Once muscle samples

have been obtained, the standard procedure is for the sample to be immediately frozen in liquid nitrogen and stored at -80 °C for later analysis.
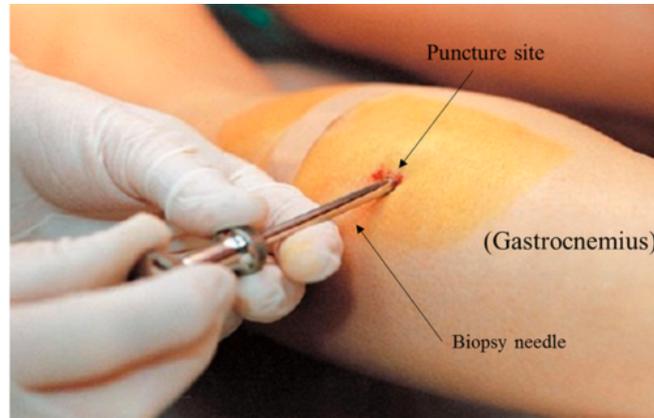
Figure 2.6 - Illustration of the Monotopty 12 G disposable core biopsy instrument sampling muscle tissue from the vastus lateralis (taken from Greene et al., 2017).

2.6.2 Acid Hydrolysis

The most common method to assess muscle glycogen in whole muscle homogenate is the acid hydrolysis approach. After samples are dissected free of blood and other non-muscle fibre materials, samples are freeze dried for 36-48 hours. Samples are then heated in hydrochloric acid for 3 h at 100°C in order to hydrolyze the glycogen to glucosyl units. After neutralizing with a base solution, the supernatant of the sample is then assayed for glucose. Glycogen concentration is then expressed as mmol of glucosyl units per kg of muscle (either in dry weight or wet weight). The acid hydrolysis approach has been used extensively in the literature and also when examining glycogen use in soccer (Saltin, 1973; Jacobs et al. 1982; Krustrup et al. 2006) and rugby (Bradley et al. 2016). Whilst this approach remains the most commonly used method, it does not allow for the assessment of the glycogen concentration within specifc fibres or intracellular pools.

2.6.3 Histochemical Staining

Histochemical staining of muscle samples can also be used to quantify glycogen through Periodic Acid-Shiff (PAS) staining of muscle. The PAS stain (Figure 2.7) is

based on the reaction of periodic acid with the diol functional groups in glucose and other sugars, oxidizing them to form aldehyde, which in turn reacts with the Schiff reagent to give a purple/magenta stain (Prats et al., 2013). The PAS stain is not however a glycogen specific stain, it also stains for glycoproteins. In order to discriminate glycogen from other PAS reactive species, the cryosections can be treated with the glycogenolytic enzyme diastase. Once the staining is complete, the cryosections are imaged through a confocal microscope that is able to identify and quantify fluorescent cellular images. Histochemical methods have been used to identify fiber-type distribution and fibre-type specific glycogen content following soccer match play (Krustrup et al., 2006) (Figure 2.8).
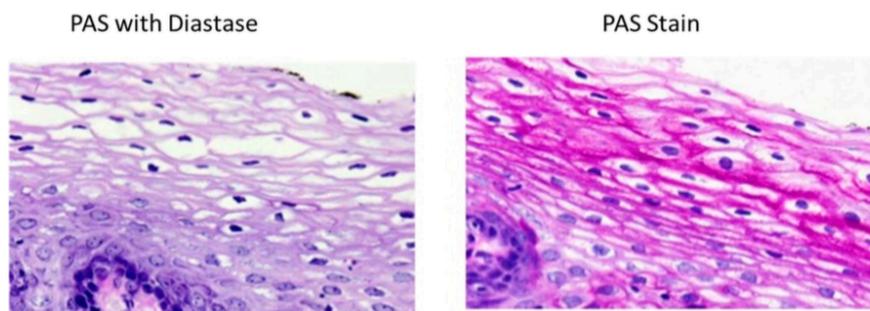
Figure 2.7 - Muscle sample with the presence of PAS staining**.**
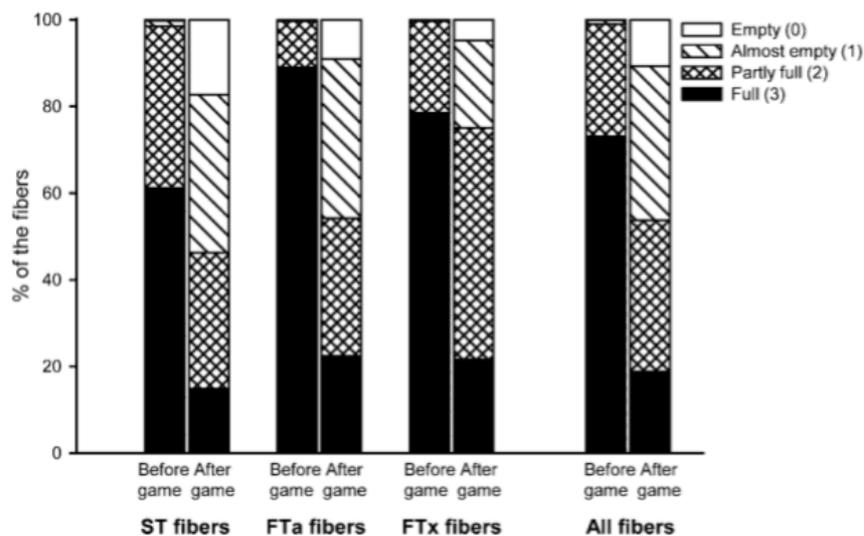
Figure 2.8 – Relative glycogen content in ST, FTa and FTx fibers as well as all fibers before and immediately after a soccer match (taken from Krustrup et al., 2006).

2.6.4 Transmission Electron Microscopy

The technique of transmission electron microscopy (TEM) can also be used to assess muscle glycogen storage within specific intracellular pools (Figure 2.9). Once the muscle sample is taken, it is immediately fixed in 2.0% glutaraldehyde in 0.1% sodium cacodylate buffer. The samples are then post fixed in a solution of 1% osmium tetroxide and 1.5% potassium ferricyanide in 0.1% M sodium cacodylate. The samples are then dehydrated in graded ethanol and infiltrated with graded mixtures of propylene oxide and Spurr's resin. Thin sections (~70nm) are cut and photographed under an electron microscope. Once the images are taken they can be used to examine glycogen within individual muscle fibers and the granule's association with the subsarcolemal organelles, the nuclei and mitochondria (Marchand et al., 2002). This method has been previously utilized in the investigations of glycogen resynthesis during the recovery phase following a soccer match (Nielsen et al. 2012).
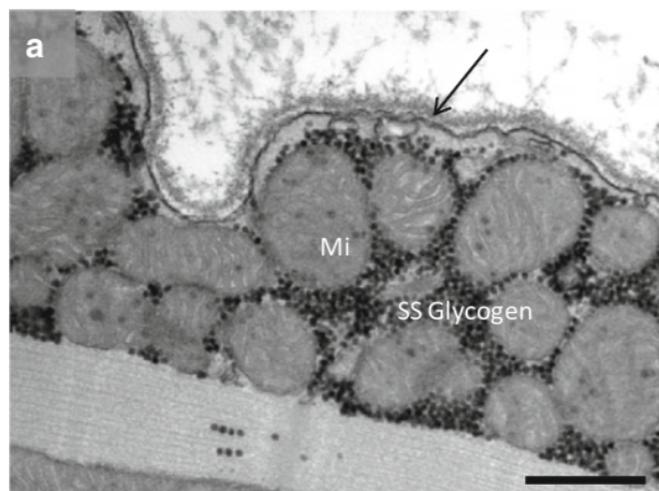
Figure 2.9 – TEM image illustrating three distinct subcellular localizations of muscle glycogen (taken from Nielsen et al., 2012). Black dots represent individual glycogen granules.

2.6.5 Ultrasound Technique

Given that muscle glycogen storage requires water in a 3:1 ratio (Fernandez-Elias et al., 2015b), the use of ultrasound to assess muscle glycogen concentration is based

on the rationale that the measurement of water content via ultrasound may provide non-invasive estimates of muscle glycogen content. This new technology proposes to measure the pixel intensity (greyscale) of a muscle scan through the association of water with the glycogen granule. The commercially available MuscleSound software assumes that the lighter the muscle image, the less fluid is therefore within the muscle, indicating a muscle depleted of glycogen (see Figure 2.10). Once a scan is performed the results can be obtained immediately and involves no invasive procedure.
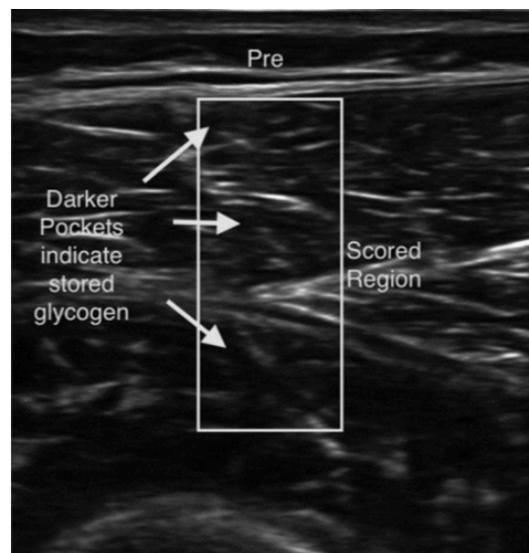
Figure 2.10 – Ultrasonic scan from the vastus lateralis muscle from a male subject with the rectangle area representing where images were segmented to isolate the muscle area under analysis. Images were obtained using a centre crop within the muscle section 25 mm from the top of the muscle sheath (taken from Nieman et al., 2015).

The initial study investigating the validity of this technique was performed in twenty-two trained male cyclists following a 90-minute cycling exercise bout at an intensity equating to lactate threshold (Hill & Millan, 2014). The researchers prescribed a high carbohydrate diet consisting of 8 g·kg^{-1} for 3 days prior to the exercise test. A muscle biopsy was obtained from the rectus femoris muscle and ultrasound measurements were also performed pre-and post the exercise bout. The ultrasound measurement was obtained when the muscle was contracted using a 12 MHz linear transducer and

a standard diagnostic GE LOGIQ-e ultrasound machine. To reduce the possibility for compression artifact caused by inconsistent pressure with the transducer, each subject contracted his quadriceps during the scanning process. At the pre-scanning session, the skin was marked to ensure post-exercise scans were obtained in precisely the same location. Following the collection of the ultrasound muscle images, the images were then wirelessly transmitted to a secure cloud-based web application (MuscleSound). The web application processes high resolution DICOM images of the muscle to create a quantifiable score of muscle glycogen content ranging from 0-100. This method automatically identifies the skin, fat and connective tissue with the muscle. Once the image is processed the mean pixel intensity of the muscle is calculated creating a glycogen score. The researchers observed positive correlations between the MuscleSound glycogen scores and direct assessment of muscle glycogen concentration via biopsies in pre-exercise samples, post-exercise samples and the change scores (see Figure 2.11).
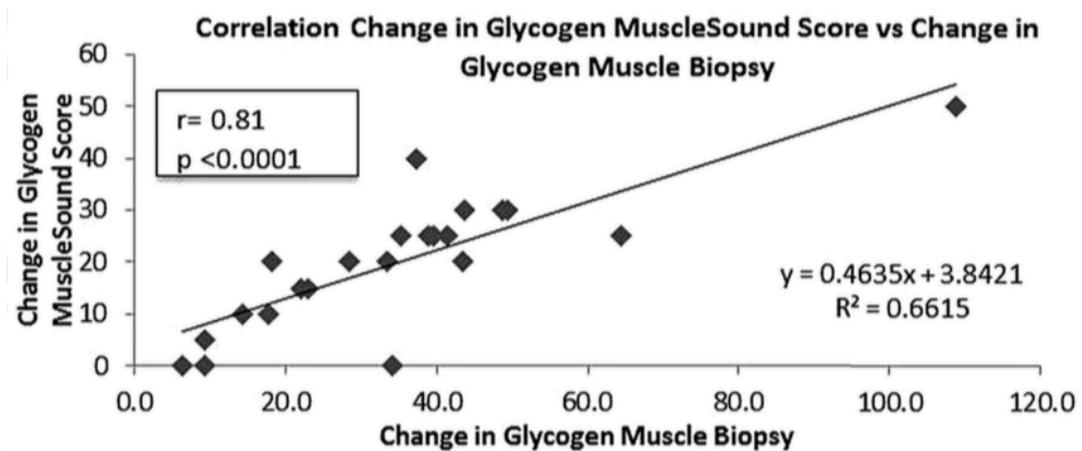
Figure 2.11 - Correlation of the change in rectus femoris MuscleSound glycogen score with change in rectus femoris muscle glycogen concentration (taken from Hill & Milan, 2014).

Glycogen content decreased from pre-exercise levels of 97 ± 34 mmol·kg^{-1} wet weight to 62 ± 22 mmol·kg^{-1} post exercise (P < 0.0001). The MuscleSound ultrasound glycogen score decreased from a score of 59 ± 15 pre-exercise to 39 ± 13. post exercise (P < 0.0001). Correlations between muscle biopsy glycogen

quantification and the MuscleSound scoring system for the change in glycogen from pre-to post exercise was r = 0.81 (P < 0.0001; Figure 2.11). These data highlighted for the first time a method of non-invasive assessment of muscle glycogen that is correlative with biochemical analysis of muscle samples.

In a subsequent study, Nieman et al. (2015) assessed the validity of the MuscleSound software to assess muscle glycogen concentration in the vastus lateralis muscle in 20 trained male cyclists who completed a 75 km time trial (equating to 2-3 hours of exercise). These researchers observed a decrease in muscle glycogen of $77 \pm 17\%$ (Figure 2.12) with an absolute change of 71 ± 23 mmol.kg^{-1} wet weight (P < 0.01), with a large variance of change in muscle glycogen between subjects (32 to 110 mmol.kg^{-1}). The authors also identified a strong correlation in the change in MuscleSound score and muscle glycogen content at the vastus laterlais (r = 0.92, P < 0.01) as shown in Figure 2.13.
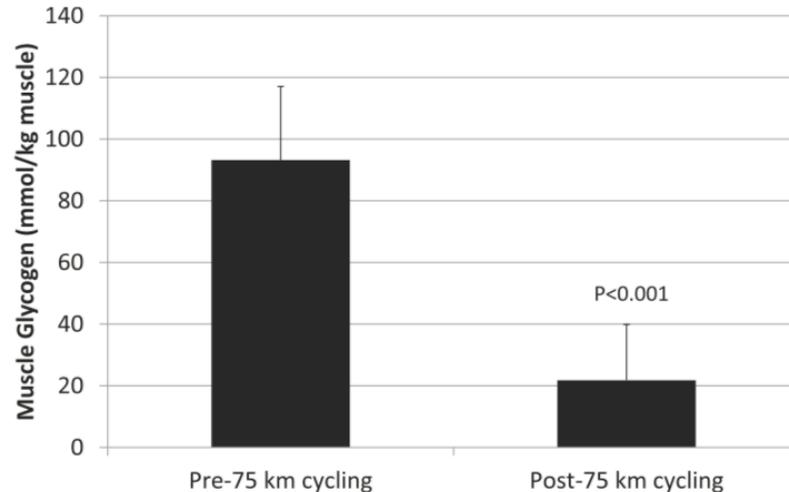
Figure 2.12 –Vastus lateralis muscle glycogen concentration pre- and post-exercise as measured by biochemical assay (taken from Nieman et al., 2015).
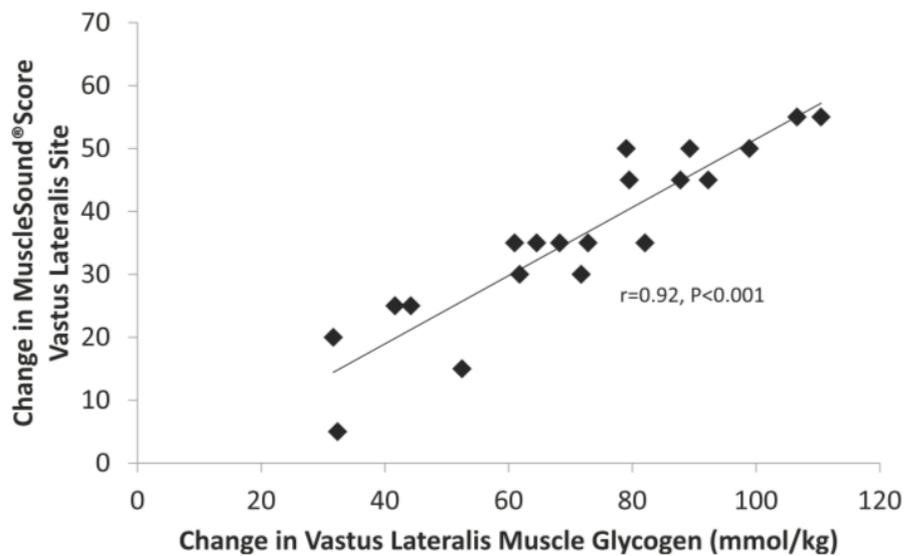
Figure 2.13 – Correlation of the change in vastus lateralis MuscleSound glycogen score with change in vastus lateralis muscle glycogen concentration (taken from Nieman et al., 2015).

When taken together, the above studies suggest that the MuscleSound software is valid to detect changes in muscle glycogen concentration in both the rectus femoris and vastus lateralis muscles of trained male cyclists in response to 2-3 hours of laboratory-based cycling protocols. Such data therefore hold much promise for team sport athletes who have the requirement to train and compete multiple times per week. In this regard, the application of ultrasound has potential to optimise nutritional guidelines for team sport athletes.

2.7 Summary

The importance of muscle glycogen has been recognised for over 50 years and is based on decades of laboratory-based research predominantly focused on endurance-based athletes. Our understanding of glycogen storage and metabolism has also been advanced considerably using the muscle biopsy technique and the measurement of glycogen concentration using traditional biochemical and histological techniques as well as transmission electron microscopy. Nonetheless, the emergence of ultrasound as a measurement tool to non-invasively assess muscle glycogen concentration is especially exciting for the applied sport nutrition practitioner working at the coalface of elite sport, a summary of the methods for assessing muscle glycogen are shown in Table 2.1. Accordingly, the experimental studies undertaken in the following

chapters will aim to further evaluate the reliability and validity of the MuscleSound software where a specific focus is given to team sport athletes.

Table 2.1. Summary of the benefits and limitations of techniques for the assessment of muscle glycogen

Method	Benefit	Limitations
Muscle biopsy technique	- Allows for a sample to be obtained directly from the muscle of choice - Allows for sampling immediately pre and post an exercise bout or game	- Invasive in nature and can take up to 5-7 days for soreness and swelling to subside - Due to the nature of the technique, the muscle fibre the sample is obtained from is hard to determine
Acid Hydrolysis	- Gives glycogen content of the obtained sample relatively simply	- The glycogen content can vary between samples
Periodic Acid-Schiff (PAS)	- Not specific to glycogen it also stains glycoproteins and proteoglycans	- Can be difficult to perform and requires a microscope for analysis
Transmission Electron Microscopy (TEM)	- Allow for the assessment of glycogen in intramyocellular pools and with specific fibre type.	- Expensive to perform and requires a microscope for analysis
Magnetic Resonance Spectroscopy (MRS)	- Measures glycogen through ^{13}C natural abundance levels or ^{13}C atoms incorporated into glycogen - Noninvasive in nature	- Expensive to perform - Usually requires an external trip to the site of an MRI machine
High-Frequency Ultrasound	- Noninvasive in nature - Relatively cheap to perform - Has the potential to give muscle glycogen values rapidly which will help inform practitioners	- There is still limited scientific evidence of the validity of the technique to assess muscle glycogen

CHAPTER 3

THE QUANTIFICATION OF THE RELIABILITY AND REPRODUCIBILITY OF THE MUSCLESOUND SCORING SYSTEM WITHIN AND BETWEEN MUSCLES OF THE QUADRICEPS

3.1 Introduction

The assessment of muscle glycogen through a rapid and non-invasive assessment technique has the potential to have profound implications for applied sport nutrition. In this regard, recent work from Hill and Millan (2014) exhibited a high-frequency-ultrasound method to assess skeletal muscle glycogen content through cloud-based software known as the MuscleSound scoring system. These researchers observed significant changes in glycogen score measured pre- and post a cycling-based exercise bout, with strong correlations observed between biopsy analysis from the rectus femoris (RF) muscle and MuscleSound scores ($r = 0.93$; $P < 0.0001$). More recently, Nieman et al. (2015) reported similar correlations between the decrease in exercise-induced vastus lateralis (VL) muscle glycogen concentration and the associated MuscleSound score ($r = 0.92$, $P < 0.001$). When taken together, these data suggest that the MuscleSound software may be a valid approach to non-invasively quantify the exercise-induced changes in muscle glycogen content.

Despite such observations, however, there is a definitive need to initially report the reliability and in turn the reproducibility of the MuscleSound scoring system and to develop standardized scanning procedures. Reliability can be expressed as relative and absolute. Relative reliability is the degree to which an individual maintains their position in a sample with repeated measures. Absolute reliability is the degree to which repeated measurements vary for individuals. Relative reliability is assessed through a correlation coefficient, with absolute expressed either in actual units are as a proportion of the measured value (dimensionless ratio) (Atkinson and Nevill, 1998). For example, whereas Hill and Millan, (2014) assessed MuscleSound scores in the "contracted" RF muscle, Nieman et al. (2015) quantified MuscleSound scores in the "non-contracted" VL muscle. The former is likely to be problematic given that the strength of contraction is known to affect muscle architecture thereby affecting the image quality that is captured during scanning. Additionally, Nieman et al. (2015) quantified MuscleSound scores from the average of three test scans whereas Hill and Millan (2014) used a single scan time-point. Prior to assessing the validity of the MuscleSound glycogen scoring system in subsequent experimental Chapters, there is the requirement to initially quantify the reproducibility both within and between muscles of the quadriceps as well as to establish standardized scanning procedures.

With this in mind, the aims of the present study were 3-fold: 1) to quantify the test-retest reproducibility of the MuscleSound scoring system within the VL, RF and vastus medialis (VM) muscles, 2) to quantify the variability of the MuscleSound scoring system within different lengths (25, 50 and 75%) of each muscle and 3), to quantify the variability of the MuscleSound scoring system between different muscles. Ultimately, the over-riding aim of this study is to identify the most reliable anatomical site for which to subsequently assess the validity of MuscleSound against direct biochemical determination of muscle glycogen concentration.

3.2 Methods

3.2.1. Subjects

One hundred subjects consisting of both males (n=78; age: 24.5 ± 6.3 years; body mass: 82.1 ± 8.3 kg; height: 183.5 ± 12.3 cm) and females (n=22; age: 20.3 ± 2.1 years; body mass: 65.4 ± 7.4 kg; height: 141.9 ± 7.1 cm) volunteered to participate in the study. None of the subjects had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the course of the study. The study was approved by the Research Ethics Committee of Liverpool John Moores University.

3.2.2. Study Design

Subjects attended the laboratory at 0900 h having not performed any prior structured exercise in the previous 24 hours. In a supine position, the right leg musculature was marked at 25, 50 and 75% percent of the RF and VL at the mid-point of the muscle belly. Following these initial marker points, 50% of the VM was identified. Ultrasound gel was applied to the marked site and the probe placed on the site in a sagittal plane. Pressure was applied to the site to ensure the probe created a straight image from the adipose tissue down through the muscle. One image was taken from each site and these were uploaded to the MuscleSound database. Following the first set of scans, subjects were required to remain in the laboratory in a supine position and were unable to consume food or water. After one hour of inactivity a further set of scans was taken according to the same procedure.

3.2.3. MuscleSound Assessment

Specific locations at 25, 50 and 75% of the muscle length on the vastus lateralis and the rectus femoris were marked with indelible ink (see Figure 3.1-3.3). An ultrasound measurement was then taken at each site using a 12 MHz linear transducer and a standard diagnostic high resolution Terason ultrasound machine (Terason t300, Burlington, USA). After calculating statistics on the color bar to determine the general brightness setting of the machine, images were pre-processed and segmented to isolate the muscle area under analysis using a centre crop within the muscle section 25 mm from the top muscle sheath. As previously identified, muscle with high glycogen stores apparently display darker pixel intensities, with muscle containing lower glycogen stores displaying brighter pixel intensities (Nieman et al. 2015). The pixel intensities of the muscle fibers were measured to quantify the amount of glycogen stores within the region of interest. The cropped images were then scaled (0-100) to create the glycogen score using MuscleSound software, with scores measured every 5 units. All measures were performed by the same researcher.
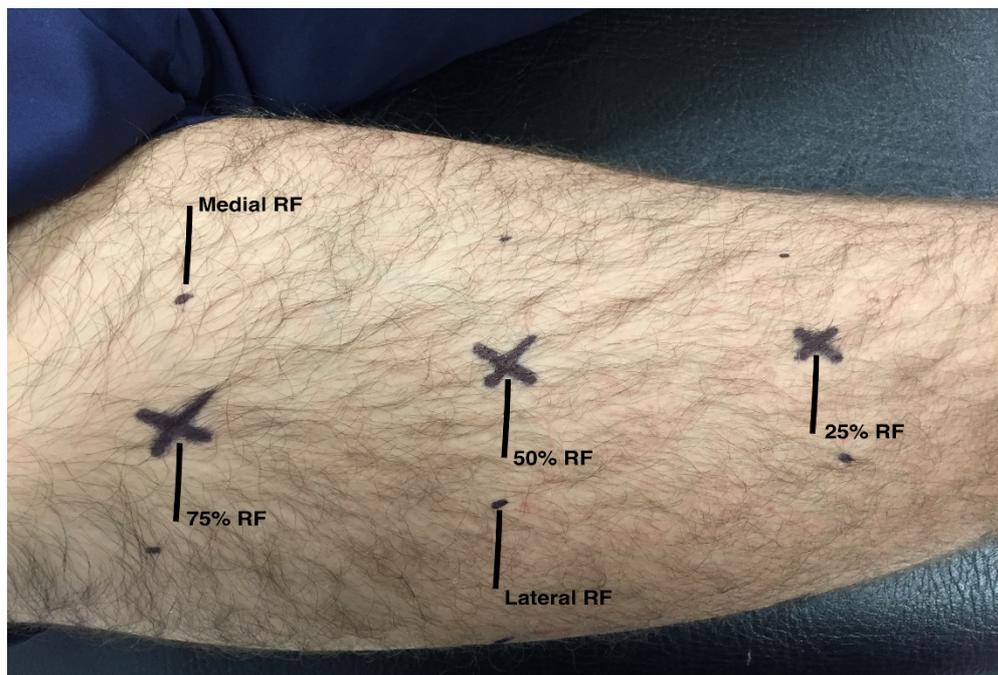
Figure 3.1 – Example of a research subject with the rectus femoris muscle marked at specific sites at 25, 50 and 75% of the muscle length.
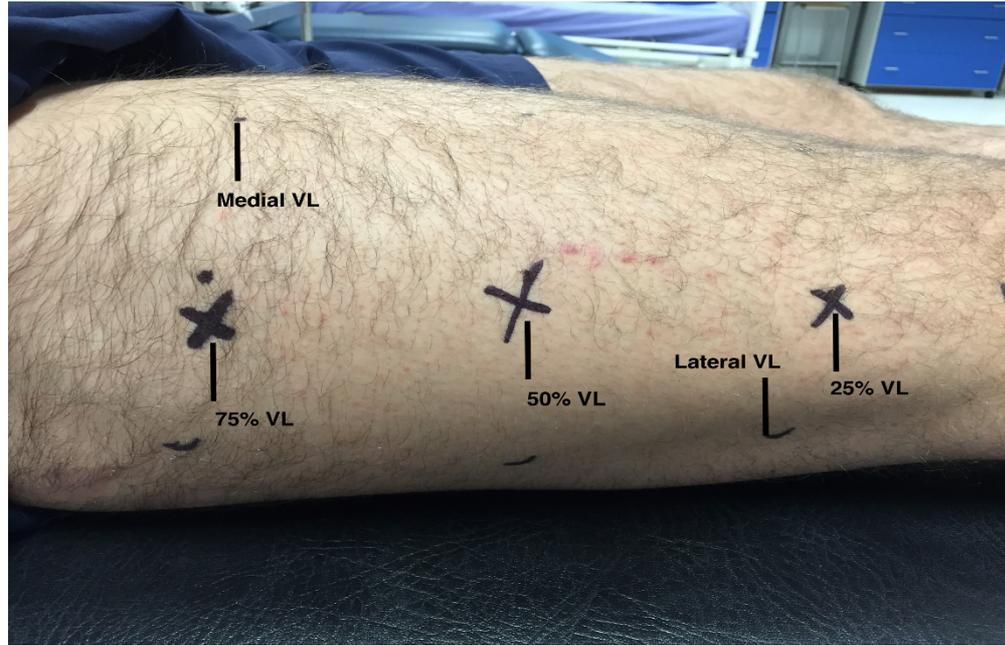
Figure 3.2 - Example of a research subject with vastus lateralis muscle marked at specific sites at 25, 50 and 75% of the muscle length.

Figure 3.3 - Example of a research subject with researcher performing the ultrasound scanning procedure.

3.2.4. Statistical and Data Analysis

MuscleSound scores were recorded for each subject pre-and following one hour of inactivity. Coefficient of variation, intra class correlation and ratio limits of agreement (*/÷) were calculated as a way of assessing the 'repeatability coefficients' (Bland & Altman, 1999). The ICC is a measure of reliability with 0 indicating no reliability, whereas 1.0 indicates perfect reliability (Weir, 2005). To compare between muscles and within muscles, an average of each participant's Trial 1 and 2 data were used for analysis and data were subsequently analysed using a one-way General Linear Model.

3.3 Results

3.3.1 Test Re-Test

The test-retest reproducibility of the MuscleSound glycogen scoring system is presented in Table 3.1 and Figure 3.4 -3.6. Assessment of heteroscedacity through Bland Altman plots are presented in the appendices. There was a strong reproducibility in the average VL score (1.02 */÷ 1.12) with the greatest reproducibility observed at 50% (1.02 */÷ 1.17). A good reproducibility was observed in the average RF score (1.03 */÷ 1.21) with 25% (1.02 */÷ 1.25) being the muscle length indicating the greatest reliability. Fifty % of the VM muscle was the anatomical site that demonstrated poorest reproducibility the (0.97 */÷ 1.46). When taken together, these data suggest that 50% of the VL muscle is the most reproducible site for which to measure MuscleSound scores.

Table 3.1 - CV, ICC (95% CI) and Ratio Limits of agreement (*/÷) for 25, 50 and 75% of the VL and VM muscles and 50% of the RF muscle.

Muscle and Length	Trial 1 Mean (±SD)	Trial 2 Mean (± SD)	CV	ICC (95% CI)	Ratio Limits of Agreement (*/÷)
VL 25%	62.6 (± 11.2)	63.8 (± 12.4)	7.3	0.85 (0.78 – 0.89)	1.02 (1.23)
VL 50%	64.5 (± 10.7)	66.2 (± 10.8)	5.8	0.89 (0.83 – 0.92)	1.02 (1.17)
VL 75%	65.9 (± 12.5)	67.8 (± 12.4)	6.3	0.89 (0.85 – 0.93)	1.03 (1.21)
VL Average	*64.4 (± 10.2)*	*65.9 (± 11.1)*	*4.5*	*0.94 (0.91 – 0.96)*	*1.02 (1.12)*
RF 25%	59.2 (± 11.6)	60.5 (± 10.8)	7.6	0.83 (0.76 – 0.89)	1.02 (1.25)
RF 50%	59.8 (± 12.1)	62.1 (± 11.5)	8.8	0.81 (0.73 -0.87)	1.04 (1.3)
RF 75%	64.5 (± 12.6)	64.9 (± 12.1)	9.0	0.77 (0.67 – 0.85)	1 (1.3)
RF Average	*60.7 (± 11.2)*	*62.1 (± 10.4)*	*6.5*	*0.87 (0.81 – 0.91)*	*1.03 (1.21)*
VM 50%	*62.1 (± 14.5)*	*60.9 (± 15.0)*	*11.1*	*0.79 (0.69 – 0.86)*	*0.97 (1.46)*
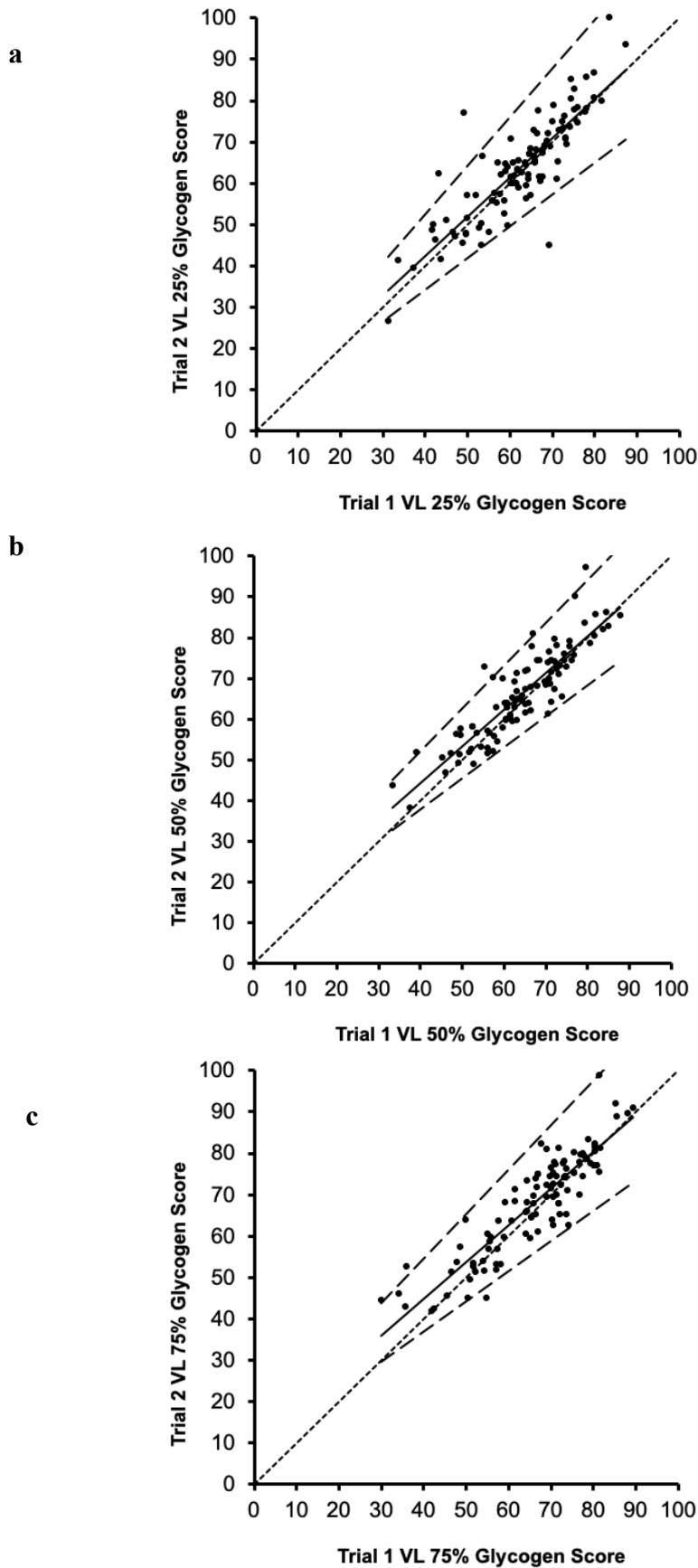
Figure 3.4 – Trial 1 v Trial 2 ratio limits of agreement for the VL at a – 25%, b – 50%, c – 75% of the muscle length. Dotted line indicates line of identity. Black line indicates measurement bias. Dashed line indicates levels of agreement.
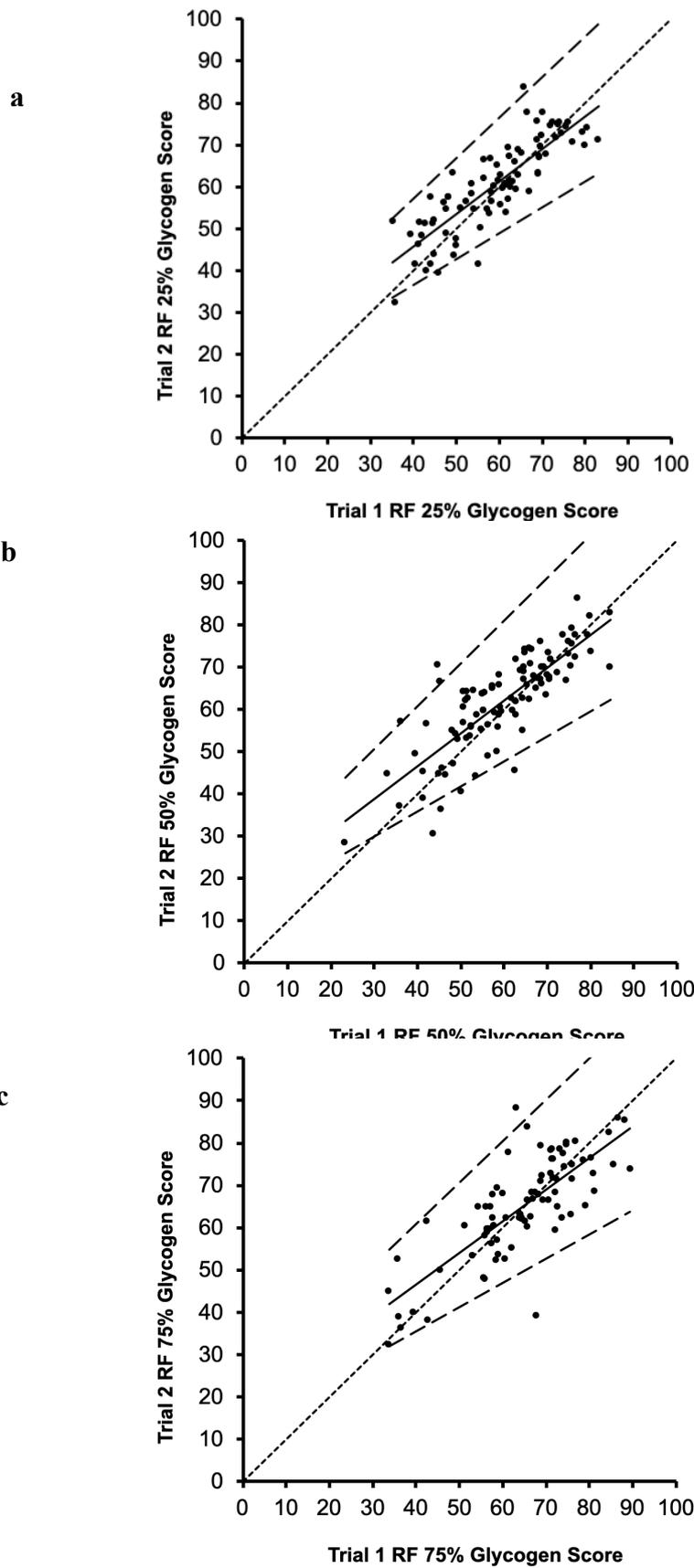
Figure 3.5 – Trial 1 v Trial 2 ratio limits of agreement for the RF at a – 25%, b – 50%, c - 75% of the muscle length. Dotted line indicates line of identity. Black line indicates measurement bias. Dashed line indicates levels of agreement.
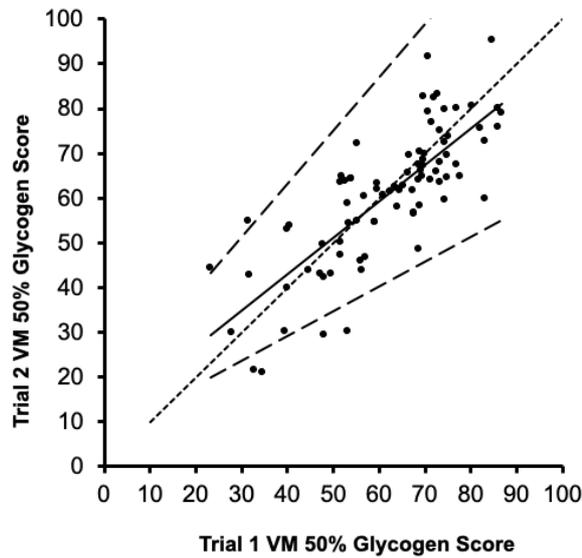
Figure 3.6 – Trial 1 v Trial 2 ratio limits of agreement for the VM at 50% of the muscle length. Dotted line indicates line of identity. Black line indicates measurement bias. Dashed line indicates levels of agreement.

3.3.2 Difference Within Muscles

Differences in the MuscleSound scoring system between different lengths of each muscle is displayed in Table 3.2. Within the vastus lateralis muscle, there was a progressive difference between length, such that 50% was greater than 25% and 75% was greater than both 50 and 25% ($P < 0.05$). Within the rectus femoris muscle, however, differences were only observed between 75 and 50 % of muscle length ($P < 0.05$).

Table 3.2 - Differences in the MuscleSound glycogen scoring system between three muscle lengths (25, 50, 75) of the VL and RF. a, denotes significant difference from 25%, b, denotes significant difference from 50%, $P < 0.05$.

Muscle	Muscle Length Mean ± SD		
	25%	50%	75%
VL	63.2 ± 11.8	65.3 ± 10.8 [a]	66.9 ± 12.4 [a b]
RF	59.8 ± 11.2	60.9 ± 11.8	64.7 ± 12.3 [b]

3.3.3 Difference Between Muscles

In order to compare differences between muscles, we compared differences in the MuscleSound scoring system at 50% of the VL, RF and VM (see Table 3.3). Significant differences were only observed between the VL and the RF/VM muscles ($P < 0.05$). Representative ultrasound images are also displayed in Figure 3.7.

Table 3.3 - Differences in the MuscleSound score between 50% of RF, VL and VM. *, denotes significant difference from RF and VM, $P < 0.05$.

Muscle	Mean ± SD
VL	65.3 ±10.8*
RF	60.9 ± 11.8
VM	61.5 ± 14.7
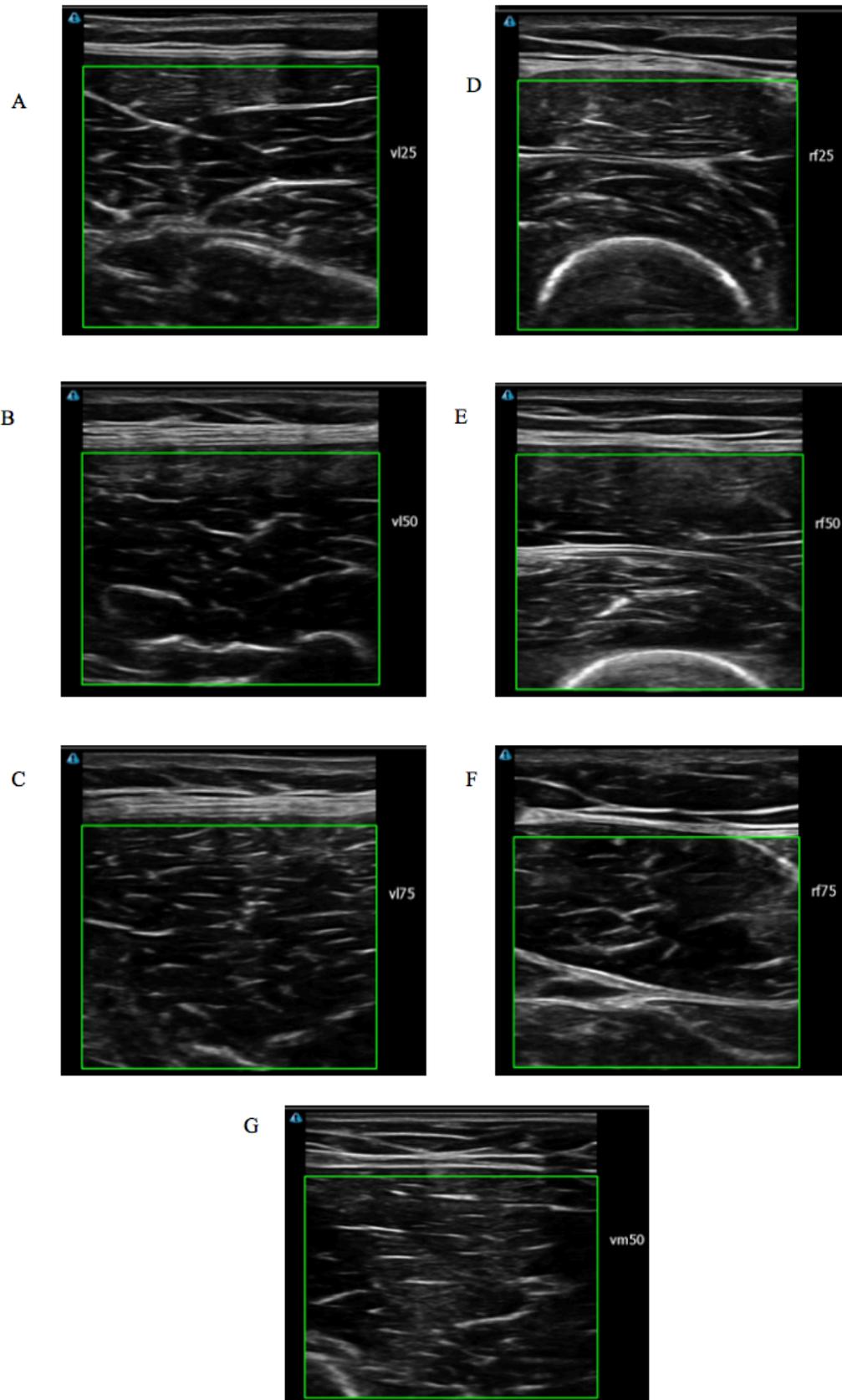
Figure 3.7 - Ultrasound muscle images taken from the MuscleSound software. A-C - VL at 25, 50, 75% muscle length. D-F - RF at 25,50, 75% muscle length. G - VM at 50% muscle length.

3.4 Discussion

Although recent work has reported the validity of the MuscleSound software (Hill & Milan, 2014; Nieman et al. 2015), no data currently exists that examines the reliability of the MuscleSound scoring system both within and between muscles. Accordingly, the aims of the present study were 3-fold: 1) to quantify the test-retest reproducibility of the MuscleSound glycogen scoring system within the vastus lateralis, rectus femoris and vastus medialis muscles, 2) to quantify the variability of the MuscleSound glycogen scoring system within different lengths (25, 50 and 75%) of each muscle and 3), to quantify the variability of the MuscleSound glycogen scoring system between different muscles. In addressing these aims, we hereby provide novel data by demonstrating that 50% of the vastus lateralis muscle is the most reproducible anatomical location for which to obtain MuscleSound scores. As such, this location should be used in future studies that aim to examine the validity of this software against direct biochemical assessment of muscle glycogen concentration.

Initially to identify the appropriate reliability statistics to perform, Bland Altman plots were completed in order to assess for heteroscedacity. These data highlight that Bland Altman plots over and under estimate the reproducibility of absolute values, therefore ratio data was utlised for reproducibility analysis (Atkinson & Nevill, 1998). In order to assess the test retest reproducibility of the MuscleSound scoring, we compared two scans obtained in resting conditions approximately 45-60 minutes apart at 25, 50 and 75% of the VL and RF muscles, alongside 50% of the VM muscle length. We observed distinct differences in the coefficient of variation and related reliability statistics for the reproducibility between sites (see Table 3.1 and Figure 3.4-3.6). In general, the greatest reproducibility was observed in the VL when compared with both the VM and RF muscles. Indeed, a CV score closer to the value of zero indicates a high level of reproducibility and an advanced level of reliability. Of all of the anatomical locations, 50% of the VL showed the smallest CV (5.8%) hence demonstrating the strongest reproducibility. Further, ratio limits agreement analysis also demonstrated greater reproducibility at 50% of the VL with measurement bias (1.02) and levels of agreement (1.12) close to the value of 1. Indeed, 25% (CV 7.3%) and 75% (6.3%) of the muscle length demonstrated reduced reproducibility. Such differences in the reproducibility observed between muscle lengths may be due to the varied muscle architecture and presence of non-muscle

tissue across the muscle length e.g. 25% of the muscle length often presents with increased bone presence in scans (as seen in Figure 3.7 D). The CVs in the RF were higher than in the VL at all muscle lengths (25% - 7.6, 50% - 8.7, 75% - 9) and also showed variations between muscle lengths. The CV in the VM was the highest observed (11%) indicating the poorest level of reproducibility in this muscle. The inferior reliability observed at this site may also be due to the presence of other anatomical features present in the ultrasound images captured. Indeed, due to the anatomical position of the VM in the quadriceps, there is close contact with vessels such as the femoral artery which is often captured during the scanning process. When taken together, these data suggest that 50% of the VL is the most reproducible location for which to obtain MuscleSound scores.

To address our secondary and tertiary aims, we subsequently averaged the scores of the test-retest scans to obtain a single MuscleSound score at each location. When directly comparing differences between lengths within specific muscles, we also observed differences in MuscleSound score, an effect that was especially prevalent in the VL muscle (see Table 3.2). We typically observed a hierarchical scoring system such that 75% > 50% >25%. In contrast, within the RF muscle, differences were only observed at 75% of the muscle length where this site exhibited higher MuscleSound scores than both 50 and 25%. As discussed in the previous section, the differences observed between lengths are likely due to varying muscle architecture and presence of non-muscle tissue at these different sites. For example, as shown in Figure 3.5, images at 25% of the VL have a tendency to contain an increased number of vessels and bone markers effecting the MuscleSound derived score due to the difference in image pixilation, when compared with 50 and 75%. At the distal end of the VL, images at 75% of the muscle length also exhibit increased amounts of adipose tissue that can also affect the MuscleSound score. Similar observations are also apparent within the RF muscle where both 25 and 50% of the RF tend to contain bone in the cropped image. Having quantified the differences in MuscleSound score within muscles, we subsequently compared differences between muscles at 50% of the muscle length for comparative purposes (see Table 3.3). In this analysis, we observed that the VL muscle displayed higher MuscleSound scores than both the RF and VM muscles an effect that may also be due to muscle architecture differences between muscles.

In summary, we provide novel data by providing the first report of the reproducibility of MuscleSound scores within and between muscles. Importantly, we report that in non-contracted muscles, 50% of the VL muscle demonstrates the strongest test-retest reproducibility and also produces higher MuscleSound scores than both VM and RF muscles. As such, practitioners should report the precise location and number of scans that are used to produce the specific MuscleSound score. Furthermore, the one-hour time period used has the potential for practitioners to asses team sport athletes upon arrival at the stadium in order to identify pre-game muscle glycogen levels which may result in a nutritional intervention prior to the start of the game. When taken together, we therefore consider 50% of the VL muscle to be the best site for which to conduct further validity studies (see Chapter 4 and 5) where MuscleSound scores are directly compared against biochemical determination of muscle glycogen concentration.

CHAPTER 4

THE ASSESSMENT OF THE VALIDITY OF THE MUSCLESOUND SCORING SYSTEM TO NON-INVASIVELY ASSESS MUSCLE GLYCOGEN UTLISATION IN ELITE MALE RUGBY LEAGUE PLAYERS DURING COMPETITIVE MATCH PLAY

It is noted that the muscle biopsy data and match analysis data has been published previously by Bradley et al. (2015) and was also part of Dr Warren Bradley's PhD Thesis of which the current candidate contributed to data collection. To allow for the assessment of the validity of the MusclesSound system these data are therefore reproduced here.

4.1 Introduction

It has recently been suggested that the MuscleSound scoring system is a valid method to non-invasively assess exercise-induced changes in muscle glycogen concentration in both the rectus femoris and vastus lateralis muscles of male subjects (Hill & Milan, 2014; Nieman et al. 2015). These studies used laboratory-based cycling protocols consisting of 1.5 hours of steady state intensity below lactate threshold (Hill & Milan, 2014) or a 75-km time trial equating to 2-3 hours of exercise (Nieman et al. 2015). Additionally, in Study 1 (Chapter 3) we demonstrated that scanning at a site equating to 50% of the length of VL (non-contracted) muscle appears the most suitable location for which to obtain reliable test-retest MuscleSound scores.

Despite such observations, no studies have yet examined the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in field-based settings. Indeed, the application of MuscleSound is particularly attractive for field-based team sports given the intense training and fixture schedules that elite team sport athletes typically partake in. In this way, an increased understanding of the muscle glycogen requirements would help inform sport-specific CHO guidelines according to the physical demands of each specific sport. Indeed, whereas the CHO requirements for soccer match play are generally well accepted (as documented from both muscle biopsy and match analysis data; (Krustrup et al., 2006, Anderson et al., 2016, Anderson et al., 2017), the CHO requirements of Rugby League match play remain to be documented. In this case, the formulation of sport-specific CHO guidelines may be warranted owing to the distinct differences in body mass and composition between football codes (Morehen et al., 2015, Milsom et al., 2015) as well as the marked differences in physiological loading between sports e.g. collisions, contacts and damaging activities associated with Rugby League (RL).

Accordingly, the aim of the present study was to therefore assess the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in elite male Rugby League players during competitive match play. To this end, we studied elite male players who partook in a competitive game after adhering to a high (HCHO) or low CHO (LCHO) diet for 36 hours prior to commencing match play.

4.2 Methods

4.2.1 Subjects

Fourteen professional RL players (mean ± SD, age 18.2 ± 0.8 years, body mass 88.4 ± 12.4kg, height 180 ± 8.1cm) from a Super League rugby club academy volunteered to take part in the study. All players competed on the same team and completed a calendared 80-min competitive RL match against a team from the same league, with 16 players giving a muscle biopsy from the VL muscle pre-and post-game. Two players declined to give a post-game muscle biopsy and were withdrawn from this aspect of the study. Therefore, all muscle biopsy data are presented as n=14 whereas match loading data are presented as n=16. Ethics approval for the study was granted by the ethics committee of Liverpool John Moores University and all participants provided written informed consent before starting the study.

4.2.2 Study Design

In a randomised between groups design, players were divided into a HCHO (n = 7) or LCHO (n = 7) group and adhered to a standardized diet for 36 hours prior to match play. A standardized pre-match meal was provided for both HCHO and LCHO groups (~ $1.5g \cdot kg^{-1}$ CHO, ~ $0.2g \cdot kg^{-1}$ protein, ~ $0.1g \cdot kg^{-1}$ fat). Muscle biopsies and MuscleSound scans were obtained from the VL muscle at approximately 60 minutes prior to kick-off and within 40 minutes upon completion of match play. Physical loads during match play were quantified using using GPS technology (Optimeye S5, Catapult Innovation, Melbourne, Australia). Heart rate was also monitored using a code transmitter unit (Polar, Oy, Finland) strapped to the chest with data transmitted and recorded to GPS units for later download and analysis. The environmental temperature of match was $8^{O}C$ ($46^{O}F$).

4.2.3 Diet Manipulation

Individualized pre-game CHO intakes were created for each player to fit into one of five groups according to body mass (70, 80, 90, 100 or 110 kg), which were used to prescribe the CHO content of the individual's diet. Players body mass was recorded, and players were grouped to the closet 10 kg. In a randomised design, players were ranked according to body mass and then block randomised to one of two diet groups

compromising either HCHO (n=7: ~6g kg day^{-1} CHO, ~1.8g kg day^{-1} protein, 0.7g kg day^{-1} fat) or a non isocaloric relatively LCHO (n=7: ~3g kg day^{-1} CHO, ~1.8g kg day^{-1} protein, 0.7g kg day^{-1} fat), with a standardized pre-match meal for both HCHO and LCHO groups (~ 1.5g.kg^{-1} CHO, ~ 0.2g.kg^{-1} protein, ~ 0.1g.kg^{-1} fat). Daily fluid intake was also matched between HCHO and LCHO groups (~3.5L). All foods were selected, purchased and distributed by the club's nutritionist and delivered to players. All players were given strict instructions to follow the diet beginning the day before the match and not to consume any food or liquids than what was provided.

4.2.4 Muscle Biopsy Samples and Muscle Glycogen Analysis

Muscle samples were obtained using a biopsy gun (Monopty 12g, BARD, Brighton, UK) from the lateral portion of the vastus lateralis at 50% of the muscle length using a Bard Montopy Disposable Core Biopsy Instrument (12 gauge x 10cm length, Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained under local anesthesia (0.5% Marcaine, 40-50mg) and immediately frozen in liquid nitrogen for later analysis. Pre- and post-game biopsies were obtained from separate incision points approximately ~2cm apart. All muscle samples were immediately snap frozen in liquid nitrogen and stored at -80°C for later analysis. Muscle glycogen concentration was determined through acid hydrolysis. Approximately 3-5mg of freeze-dried sample was powered, dissected free of all visible non-muscle tissue and subsequently hydrolyzed by incubation in 500 μl of 1M HCI for 3-4h at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 μl 0.12 mol 1^{-1} Tris/2.1 mol 1^{-1} KOH saturated with KC1. Following centrifugation, 150 μl of the supernatant was analyzed in duplicate for glucose concentration according to hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol kg $^{-1}$ dry weight (dw) and intra-assay coefficients of variation was <5%.

4.2.5 MuscleSound Assessment

Prior to the biopsy sample, an ultrasound measurement was then taken at 50% of the length of the VL, following reproducibility established in Chapter 3, using a 12 MHz linear transducer and a standard diagnostic high resolution Terason ultrasound machine (Terason t300, Burlington, USA). After calculating statistics on the color bar to determine the general brightness setting of the machine, images were pre-

processed and segmented to isolate the muscle area under analysis using a center crop within the muscle section 25 mm for the top muscle sheath. As previously identified, muscle with high glycogen concentrations apparently display darker pixel intensities, with muscle containing lower glycogen concentrations displaying brighter pixel intensities (Nieman et al., 2015). The pixel intensities of the muscle were measured to quantify glycogen concentration within the region of interest. The cropped images were then scaled (0-100) to create the glycogen score using MuscleSound software.

4.2.6 Player Load

Global positioning system (GPS) units were used to collect data on total distance (m) and relative distance covered ($m \cdot min^{-1}$) in standing walking (0.3-7.1 $km \cdot h^{-1}$), jogging (7.2-14.3 $km \cdot h^{-1}$), running (14.4-19.7 $km \cdot h^{-1}$) high-speed running (19.8-25.1 $km \cdot h^{-1}$) and sprinting (>25.1 $km \cdot h^{-1}$). Low intensity activity (< 19.7 $km \cdot h^{-1}$), high intensity activity (> 19.8 $km \cdot h^{-1}$) and repeated high intensity efforts (RHIE) (three consecutive efforts, sprint, contact or acceleration, each separated by less than 21s) performed by all players during the match was assessed using GPS technology (Optimeye S5, Catapult Innovation, Melbourne, Australia) as previously described (Bradley et al., 2015a, Bradley et al., 2015b). Heart rate was also monitored using a coded transmitter and recorded to GPS units for later download and analysis.

4.2.7 Statistics

Statistical analysis was conducted using the Statistical Package for Social Sciences software programme (SPSS; version 17, IBM, Armonk, NY). Muscle glycogen concentration and MuscleSound score were analyzed using a two-way mixed design General Linear Model, where the within factor was time (Pre-game vs post-game) and between factor was condition (HCHO vs. LCHO). Correlation analysis were performed using linear regression analysis. All data in text, figures, and tables are presented as means ± SD with *P* values <0.05 indicating statistical significance.

4.3 Results

4.3.1 Player Physical Performance

First and second half movement characteristics are shown in Table 4.1. Low intensity running, RHIE and $m.min^{-1}$ all displayed no significant difference (P > 0.05) between

the 1st to 2nd half. High intensity running and % HR_{peak} were significantly higher in the 1st half than the second half in both HCHO (-4.5m min-1; -0.4% HR_{peak}) and LCHO (-3m min-1; -3.5% HR_{peak}) respectively.

4.3.2 Muscle Glycogen

Muscle glycogen concentration is presented in Figure 4.1. Muscle glycogen decreased ($P < 0.05$) pre-to post game in both HCHO (448 $\pm$ 50 to 212 $\pm$ 94 mmol·kg^{-1} dw) and LCHO conditions (444 $\pm$ 81 to 260 $\pm$ 160 mmol·kg^{-1} dw). There was no significant difference in glycogen concentration between HCHO or LCHO groups (P=0.444) nor was there any interaction effect present (P = 0.320)

4.3.3 MuscleSound

MuscleSound data are presented in Figure 4.2. MuscleSound score showed no significant difference pre-to post game (P = 0.38) or between conditions (P = 0.45) nor was there any interaction effect present (P = 0.07). Correlation data between changes in muscle glycogen and MuscleSound scores is presented in Figure 4.3. The change from pre-to post game in both muscle glycogen and MuscleSound scores showed no significant correlation (R^2 = 0.03, P = 0.55).

Table 4.1 – Mean (SD) match GPS data reported in m.min^{-1} for 6 g.kg^{-1} and 3 g.kg^{-1} dietary conditions. Repeated high intensity effort (RHIE) is defined as a cluster of three user defined high-intensity efforts performed <21 s apart (contacts, accelerations or sprints). % HRpeak = % of peak heart rate. * Difference from 1st half, P < 0.05.

	m.min^{-1}		Low Intensity (m.min^{-1})		High Intensity (m.min^{-1})		RHIE		%HR_{peak}	
Half	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
6g.kg^{-1}	93.7 (12.4)	85.3 (13.1)	78.9 (8.8)	74.9 (9.2)	14.8 (5.4)	10.3* (4.3)	0.4 (0.5)	0.6 (1.2)	82.9 (6.1)	82.5* (7.5)
3g.kg^{-1}	89.4 (9.8)	86.9 (9.7)	76.7 (10.3)	77.2 (9.5)	12.7 (1.9)	9.7* (2.1)	1.5 (1.7)	1.6 (1.9)	81.9 (7.2)	78.4* (10.5)
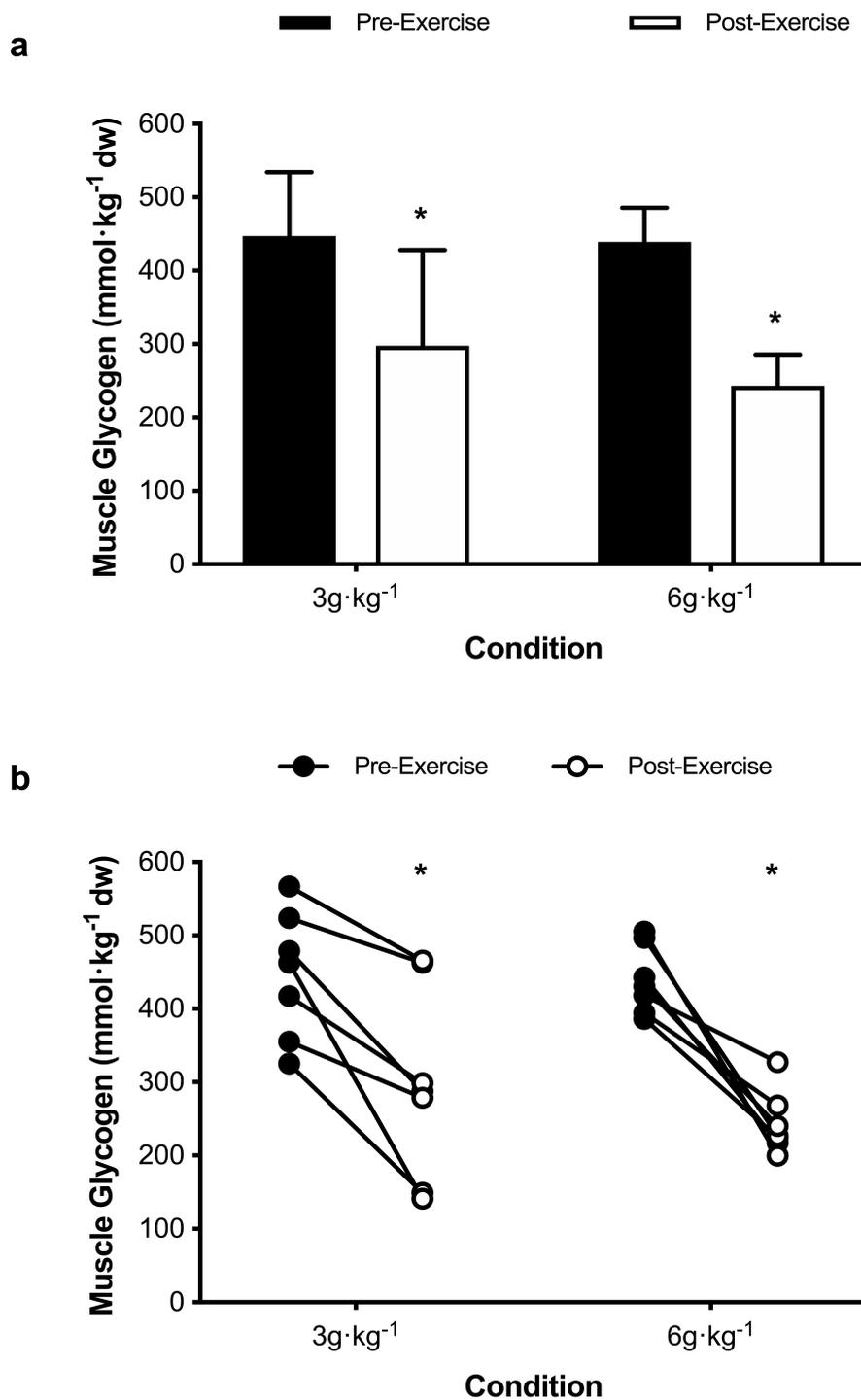
Figure 4.1 – a - Average muscle glycogen concentration between the LCHO (3 g·kg⁻¹) and HCHO (6 g.kg⁻¹) trials pre-and post-game. b - Individual muscle glycogen concentration between the LCHO (3 g·kg⁻¹) and HCHO (6 g·kg⁻¹) trials pre-and post-game. * indicates significance ($P < 0.05$) from pre-to post game.
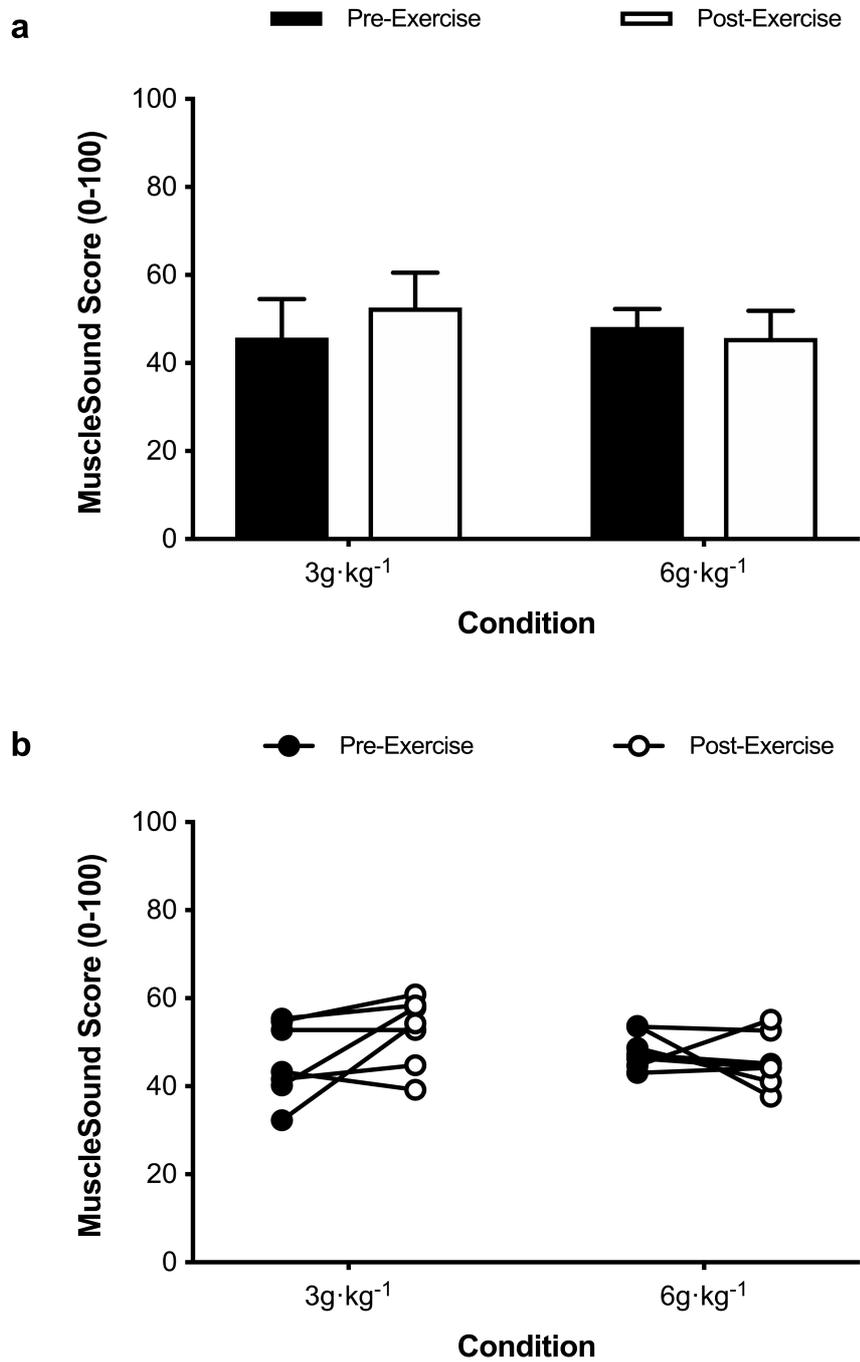
Figure 4.2 – a - Average MuscleSound score (0-100) between the LCHO (3 g·kg⁻¹) and HCHO (6 g·kg⁻¹) trials pre-and post-game. b - Individual MuscleSound score (0-100) between the LCHO (3 g·kg⁻¹) and HCHO (6 g·kg⁻¹) trials pre-and post-game.
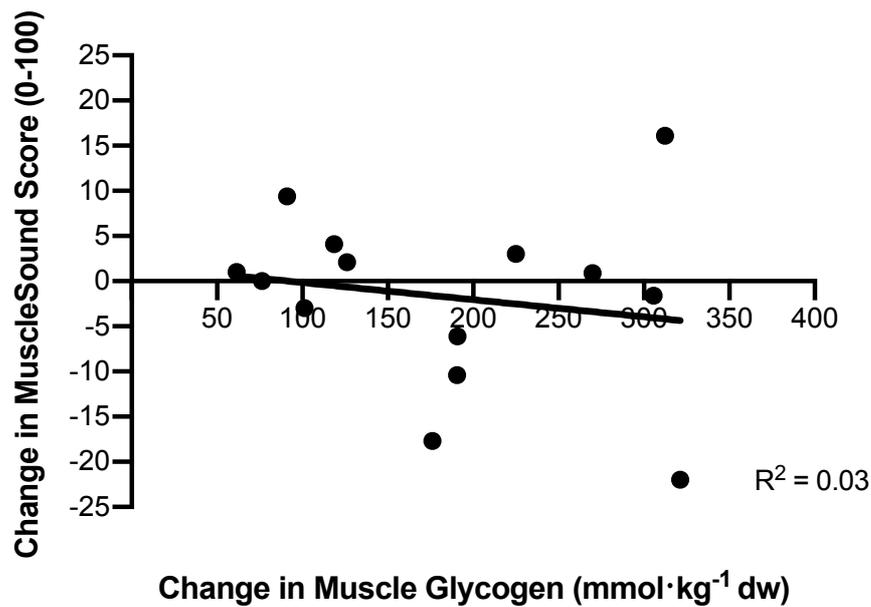
Figure 4.3 - Correlations between change in MuscleSound score (0-100) and change in muscle glycogen concentration (mmol·kg^{-1} dw).

4.4 Discussion

The aim of the present study was to assess the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in elite male Rugby League players during competitive match play. Despite observing that match play depleted muscle glycogen in the vastus lateralis muscle in the magnitude of 40-50% (as evidenced through biochemical assessment), we observed no significant reductions in MuscleSound score in the HCHO group, LCHO group or pooled sample. As such, no significant correlations were present between changes in muscle glycogen concentration and changes in MuscleSound scores. These data suggest that in the field-based setting of elite RL match play, the MuscleSound software is unable to detect absolute changes in muscle glycogen concentration of approximately 200-250 mmol·kg^{-1} dw.

To address our aim, we studied elite male RL players during a competitive match after adhering to a standardized CHO intake equating to high or low CHO

availability. Interestingly, and despite differences in the preceding CHO intake, we observed no differences in pre-match muscle glycogen concentrations between the HCHO and LCHO groups. The finding that no differences were apparent between groups may be explained by the fact that we only controlled and provided CHO intake for the preceding 24-36 h before match play. As such, it is possible that players' habitual CHO intakes and training activity in the days prior to our dietary intervention were already sufficient to induce muscle glycogen concentrations equivalent to approximately 400 mmol·kg^{-1} dw. Unfortunately, we do not have dietary intake data for such days to substantiate this hypothesis. Nonetheless, given that we observed that a prior CHO intake of 6 g·kg^{-1} per day typically induced higher individual pre-game muscle glycogen concentrations (all players presented with values >400 mmol·kg^{-1} dw) than the 3 g·kg^{-1} feeding strategy (four players presented with values <400 mmol·kg^{-1} dw), our data suggest that a CHO intake equating to 6 g·kg^{-1} is more suitable to achieve what would be considered sufficient glycogen storage before match play (Thomas et al., 2016). Further studies are now required to examine the effects of pre-match muscle glycogen availability on sport-specific indices of physical and technical performance, such as completed passes and converted kicks.

Regardless of prior dietary loading protocols, we observed almost comparable relative (40 versus 50% depletion) and absolute glycogen utilisation (200 versus 250 mmol·kg^{-1} dw) in both the LCHO and HCHO groups, respectively. The slightly higher absolute utilisation in the HCHO versus LCHO group is consistent with the well documented finding that elevated resting glycogen concentrations induces higher absolute glycogen utilisation, owing to the regulatory effects on glycogen phosphorylase activity (Hargreaves et al., 1995). Both the relative and absolute glycogen utilisation also appears comparable with that previously observed in professional soccer players (Krustrup et al., 2006), though we acknowledge that examination of individual muscle fibre types may also demonstrate complete depletion in certain fibres (Krustrup et al. 2006). When taken together, our data suggest that absolute CHO intakes of 6 g·kg^{-1} on the day prior to the match is likely sufficient to achieve the absolute muscle glycogen concentrations required to meet the metabolic demands of RL match play.

In contrast to the laboratory-based cycling protocols previously supporting the validation of the MuscleSound software, our data demonstrate no significant changes in MuscleSound scores as a result of match play in either the HCHO or LCHO groups. It is difficult to explain these findings given that the absolute glycogen utilisation observed here (i.e. 200-250 $mmol \cdot kg^{-1}$ dw) is also comparable to that studied previously where mean absolute utilisation of 150 (Hill & Milan, 2014) and 300 $mmol \cdot kg^{-1}$ dw (Nieman et al. 2015) have been observed. On this basis, we suggest that differences in the nature of the contractile stimuli and absolute force production between the intermittent exercise intensities associated with RL match play versus steady-state cycling activities may subsequently affect the image obtained upon ultrasound scanning.

The MuscleSound scoring system methodology is based upon the measurement of water content associated with glycogen in the muscle (Nieman et al., 2015). For example, when glycogen content is high, the ultrasound image is hypoechoic (dark) and with glycogen depletion and water loss, the image is hyperechoic (brighter). During prolonged cycling based models in which the intensity is typically controlled and below lactate threshold (i.e. low force production) it is indeed possible that the muscle loses considerable fluid (Sarvazyan et al., 2005, Fernandez-Elias et al., 2015a, Fernandez-Elias et al., 2015b) in the post-exercise recovery period, an effect that may be especially apparent in those situations in which plasma volume has been considerably reduced due to dehydration. In contrast to prolonged cycling-based protocols studied previously, it is possible that the loading patterns of RL match induces different responses in terms of fluid shifts. Support for this hypothesis is provided by the observation that in response to the high mechanical loads occurring with resistance exercise, muscle actually increases cross-sectional area due to movement in fluid from the vascular space into active muscle (Ploutz-Snyder et al., 1995). Such observations coupled with the environmental temperature ($8^{O}C$, $43^{O}F$) of which the present study was completed, may explain why we observed no actual decrease in pixilation intensity and MuscleSound score in the vastus lateralis muscle of the present participants. Unfortunately, neither we nor previous researchers (Hill & Milan, 2014; Nieman et al. 2015) controlled for hydration status or assessed any parameter of hydration status, total body water content or muscle volume before and after the chosen exercise protocol. Nonetheless, we acknowledge that future studies

examining the validity of the MuscleSound software should at least take into account measurement of muscle water content.

In summary, we report for the first time that the MuscleSound software is unable to detect absolute changes in muscle glycogen concentration of 200-250 mmol·kg^{-1} dw, as occurs with the real-world loading patterns of competitive RL match play. Given that the MuscleSound scoring system is based on the principle of associating changes in glycogen concentration with muscle tissue water content, it is suggested that the differences observed here and in previous validation studies may be due to differences in fluid shifts occurring with the contrasting contractile protocols. To eliminate the potential confounding effects of acute changes in fluid shifts associated with differing exercise stimuli, an alternative approach to assess the validity of the MuscleSound system may be to assess its validity to detect changes in muscle glycogen re-synthesis across a time-scale in which acute changes in fluid shifts have been restored to pre-exercise values.

CHAPTER 5

THE ASSESSMENT OF THE VALIDITY OF THE MUSCLESOUND SCORING SYSTEM TO NON-INVASIVELY ASSESS MUSCLE GLYCOGEN RESYNTHESIS WITHIN 36 HOURS FOLLOWING GLYCOGEN DEPLETING BASED EXERCISE

5.1 Introduction

The validity of the MuscleSound software has previously been assessed using acute cycling-based exercise protocols where significant correlations were observed between changes in MuscleSound scores and changes in muscle glycogen concentration in both the rectus femoris (Hill & Millan, 2014) and vastus lateralis muscles (Nieman et al., 2015). These data lend support for the methodological rationale that changes in glycogen concentration is associated with loss of water from the muscle, the result of which can be used to non-invasively infer exercise-induced changes in muscle glycogen concentration via ultrasound imaging. However, in Chapter 4 we observed that the MuscleSound system is unable to detect exercise-induced reductions of muscle glycogen of 200-250 mmol·kg^{-1} dw within the vastus lateralis muscle, as occurring with 80 minutes of competitive Rugby League match play. We suggested that such differences between studies may be due to differences in fluid shifts associated with the contrasting contractile stimuli and loads induced by steady-state cycling compared with high-intensity intermittent running where higher mechanical loads are experienced.

In an attempt to eliminate the potential confounding effects of acute changes in fluid shifts associated with differing exercise stimuli, an alternative approach to assess the validity of the MuscleSound system may be to assess its validity to detect changes in muscle glycogen re-synthesis across a time-scale in which acute changes in fluid shifts have been restored to pre-exercise values. If this approach proves valid, then MuscleSound is potentially assessing "true" fluctuations in muscle glycogen as opposed to artefacts associated with acute changes in intracellular and extracellular fluid changes.

With this in mind, the aim of the present study was to therefore assess the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen re-synthesis within 36 hours following glycogen depleting based exercise. To this end, we utilised an experimental protocol consisting of an acute cycling-based glycogen depleting exercise protocol followed by 36 h of an isoenergetic high or low CHO diet (that is fluid matched) in order to achieve high and low muscle glycogen concentration.

5.2 Methods

5.2.1 Subjects.

Sixteen recreationally active males volunteered to participate in the study (VO_{2max}: 49.9 ± 7.5 ml.kg^{-1} min^{-1}: age, 21 ± 1.8; body mass, 84 ± 8.3 kg; height, 176 ± 8.4 cm). None of the subjects had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the course of the study. The study was approved by the Research Ethics Committee of Liverpool John Moores University.

5.2.2 Study Design.

In a randomised, repeated measures, cross over design and after an initial assessment of maximal oxygen uptake, subjects completed two 36 h experimental protocols at 7-day intervals in conditions of high (HIGH) or low (LOW) carbohydrate (CHO) availability. Subjects reported to the laboratory on the evening of Day 1 in order to perform a glycogen-depleting bout of intermittent exhaustive cycling. At the cessation of exercise, subjects were provided with either high or low CHO foods (88 g CHO v 2 g CHO) to be consumed over the next three hours. During the LOW trial, subjects also returned to the laboratory on the following morning to perform an additional 45 min bout of steady state cycling in an attempt to induce further muscle glycogen depletion. Upon cessation of exercise, subjects then consumed a standardized diet of low CHO ($2gk \cdot g^{-1}$) for the next 24 h whereas subjects in the HIGH trial did not perform any morning exercise but consumed a high CHO diet (HIGH: $8g \cdot kg^{-1}$ CHO; $2g \cdot kg^{-1}$ Protein; 0.6 g$\cdot$kg^{-1} Fat; 6 litres fluid) or low CHO diet (LOW: $2g \cdot kg^{-1}$ CHO; $2g \cdot kg^{-1}$ Protein; 3.5 g$\cdot$kg^{-1} Fat; 6 litres fluid). In both trials, subjects returned to the laboratory on the morning of Day 3 (0800 h) in a fasted state to have a resting muscle biopsy sampled from the vastus lateralis as well as MuscleSound scans performed. In this way, each subject completed an exercise-dietary intervention that induced a state of high or low muscle glycogen in a fully rested and hydrated state.

5.2.3. Assessment of Peak Oxygen Uptake and Peak Power output

Peak oxygen uptake and peak power output (PPO) were determined using an incremental cycle test to exhaustion on a self-regulated cycle ergometer (Lode

Excalibur, Netherlands). The test commenced with a 5 min warm up at 100 W at a cadence corresponding to 70 revs min^{-1}. Following the warm up phase, power output was increased by 30 W every 120 s until volitional exhaustion. Breath-by-breath measurements were collected throughout the exercise protocol using a CPX Ultima series online gas analysis system (Medgraphics, MN, US). Peak power output was defined as the highest power output that could be maintained for 60 s. Saddle and handle bar position was recorded and replicated for the exercise trials and matched to that of the preliminary testing.

5.2.4. Glycogen Depletion Protocol.

On the evening of Day 1, subjects arrived at the laboratory at 1800 h and underwent an ultrasound scan at 50 % of the VL alongside measurement of body mass and urine osmolality. After a 5 min warm up at 100W, subjects cycled for 2 min at 90% PPO, followed immediately by a 2-min recovery period at 50% PPO. They repeated this work to rest ratio until they could no longer complete 2 min cycling at 90% PPO, determined as an inability to maintain a cadence of 60 revs min. At this point, exercise intensity was lowered to 80% PPO and when participants could no longer cycle for two minutes at 60% PPO the exercise protocol was terminated (Taylor et al., 2013). The intermittent nature of the exercise protocol was designed in order to deplete both type 1 and 2 muscle fibres of glycogen during the exercise. Heart rate and RPE were recorded at the end of each two-minute exercise and recovery phase. Exercise time and activity pattern were recorded and replicated for the second trial. Water intake was consumed ad libitium during the first trial with the pattern of intake replicated in the subsequent trial. Following the completion of the depletion exercise protocol, an additional ultrasound scan was performed alongside measurement of body mass.

5.2.5. Morning Glycogen Depletion Protocol.

In the LOW condition, subjects returned to the laboratory on the following morning at 0700h in a fasted state. Subjects commenced a bout of steady state cycling at 60% PPO for 45 minutes with heart rate and RPE being measured every five minutes. Ultrasound scans were also performed before and after this exercise protocol.

5.2.6. Muscle Biopsy Samples.

Muscle biopsies were obtained the from the lateral portion of the vastus lateralis at 50% of the muscle length on the morning of Day 3 using a Bard Montopy Disposable Core Biopsy Instrument (12 gauge x 10cm length, Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained under local anesthesia (0.5% Marcaine, 40-50mg) and immediately frozen in liquid nitrogen for later analysis.

5.2.7. Muscle Glycogen Concentration

Muscle glycogen concentrations was measured according to Section 4.2.4

5.2.8. Muscle Water Content

Muscle samples were weighed prior to entry into the dry freezer and on exit from the freeze drier after a 48-h period. Muscle water content was calculated using the following equation (wet weight – dry weight)/(dry weight/100) as taken from Costill et al. (1976).

5.2.9. MuscleSound Assessment

MuscleSound assessment was performed according to Section 4.2.5

5.2.10. Statistical Analysis.

Statistical analysis was conducted using the Statistical Package for Social Sciences software program (SPSS; version 17, IBM, Armonk, NY). Differences between muscle glycogen concentration and MuscleSound score between the HIGH and LOW trials were analysed using a students-t test for paired samples. Additionally, differences between physiological responses to exercise during the glycogen depletion protocol were analysed using two-way repeated measures General Linear Model, where the within factor was time (Pre-exercise vs post-exercise) and condition (HIGH vs. LOW). Correlation analysis were performed using linear regression analysis. All data in text, figures, and tables are presented as means ± SD with P values <0.05 indicating statistical significance.

5.3. Results

5.3.1. Physiological and MuscleSound Responses to Evening Glycogen Depletion Protocol.

Subjects cycled for 102 ± 8 min during the evening interval cycling protocol completed in Day 1. There was no significant differences ($P > 0.05$) in average heart rate between the HIGH (168 ± 8 b.min^{-1}) and LOW (170 ± 10 b.min^{-1}) trials (see Figure 5.1a). Similarly, RPE was also not significantly different ($P > 0.05$) between trials (HIGH: 14.8 ± 1.3; LOW: 15 ± 0.5) (see Figure 5.1b). The glycogen depletion protocol induced a significant decrease ($P < 0.05$) in MuscleSound score in both the HIGH (Pre: 67 ± 7; Post: 51 ± 10) and LOW (Pre: 58 ± 11; Post 48 ± 9) trials (Figure 5.1c) though no difference ($P > 0.05$) was observed between conditions. Taken together, these data suggest that the physiological and metabolic responses to the evening glycogen depletion protocol were comparable between trials.
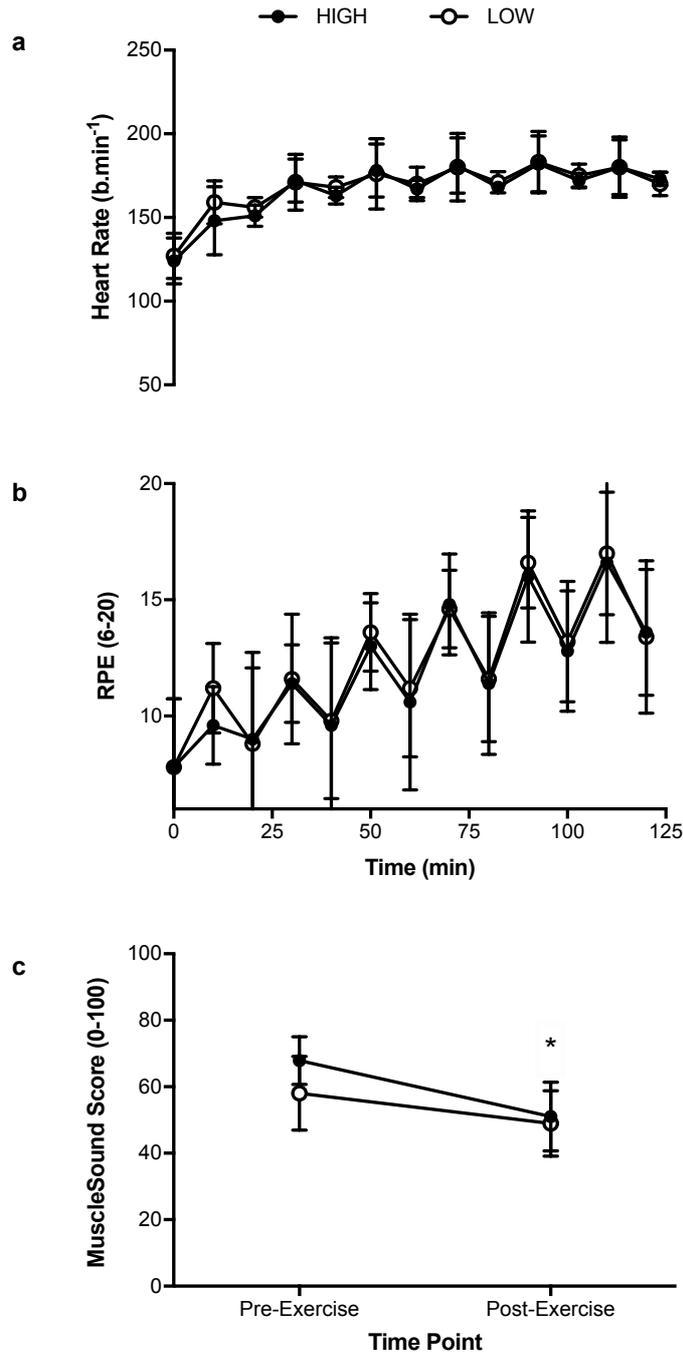
Figure 5.1 – a - Rate of perceived exertion (RPE), b - Heart Rate (b.min⁻¹) and c - MuslceSound score pre-and post the evening depletion protocol for both HIGH and LOW conditions. * denotes significance from pre-to post.

5.3.2. Physiological and MuscleSound responses to Morning Glycogen Depletion Protocol

When subjects completed the LOW trial, they returned to the laboratory on the following morning to complete a further bout of exercise at 60% PPO of in an attempt to further induce muscle glycogen depletion. Subjects' heart rate and RPE are shown in Figure 5.2a and Figure 5.2b respectively. Additionally, this exercise protocol also induced a significant decrease ($P < 0.05$) in MuscleSound score (Pre: 52 ± 7; Post: 45 ± 8) (see Figure 5.2c).
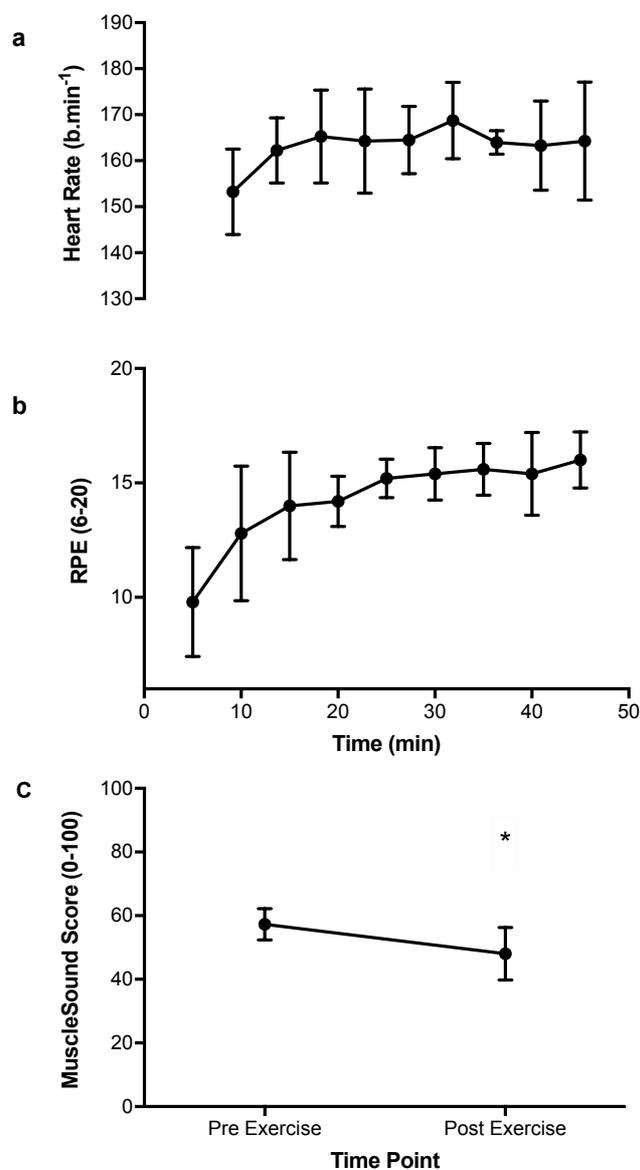
Figure 5.2 – a - Rate of perceived exertion (RPE), b - Heart Rate (b.min^{-1}), c - MuscleSound Score (0-100) pre-and post of the morning steady state protocol * denotes significance from pre-to post-exercise.

5.3.3. Comparison of Muscle Glycogen and MuscleSound.

Following completion of both exercise protocols, subjects then consumed the standardized dietary protocols of HIGH (CHO 8g·kg⁻¹, PRO 2g·kg⁻¹, FAT 0.6g·kg⁻¹) or LOW (CHO 2g·kg⁻¹, PRO 3.5g·kg⁻¹, FAT 4g·kg⁻¹) CHO intake in an attempt to induce HIGH and LOW muscle glycogen concentrations. Subjects then returned to the laboratory on the morning of Day 3 to have a resting muscle biopsy sampled from the vastus lateralis. A comparison of muscle glycogen, muscle water content and MuscleSound scores are shown in Figure 5.3 a-c, respectively. Despite significant differences ($P < 0.05$) in muscle glycogen concentration in HIGH (531 ± 129 mmol·kg⁻¹ dw) versus LOW (252 ± 64 mmol·kg⁻¹ dw), there was no significant difference in muscle water content (HIGH: 265 ± 50 mL/100 g dry mass; LOW: 250 ± 50 mL/100 g dry mass) or MuscleSound score (HIGH: 56 ± 7; LOW: 54 ± 6). There was also no significant correlation between absolute MuscleSound score and muscle glycogen ($R^2 = 0.23$, 95% CI = 0.16 to 0.71) (Figure 5.4a). In addition, there was also no significant correlation between absolute MuscleSound score and muscle water content ($R^2 = 0.002$, 95% CI = -0.392 to 0.304) (Figure 5.4b), aswell as muscle water content and muscle glycogen ($R^2 = 0.028$, 95% CI = -0.192 to 0.488) (Figure 5.4c). Finally, the change between conditions of MuscleSound score and muscle glycogen showed no significant correlation ($R^2 = 0.16$, $P = 0.11$) (Figure 5.5a), nor did MuscleSound score and muscle water content ($R^2 = 0.03$, $P = 0.55$) (Figure 5.5b)
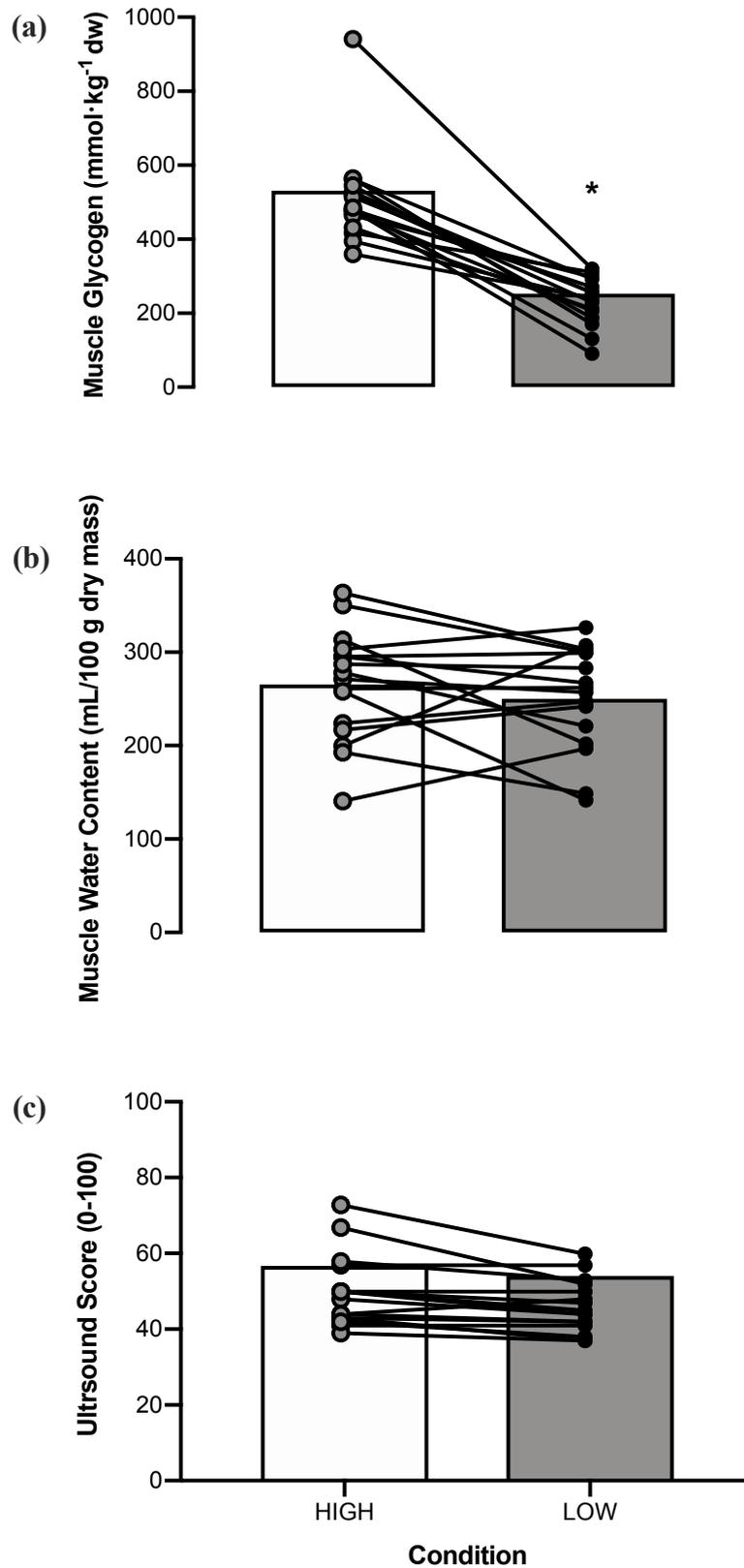
Figure 5.3 – a - Individual muscle glycogen content (mmol·kg⁻¹ dw), b) individual muscle water content (mL/100 g dry mass), c - individual MuscleSound score (0-100) between HIGH and LOW conditions. * significant difference between HIGH and LOW conditions.
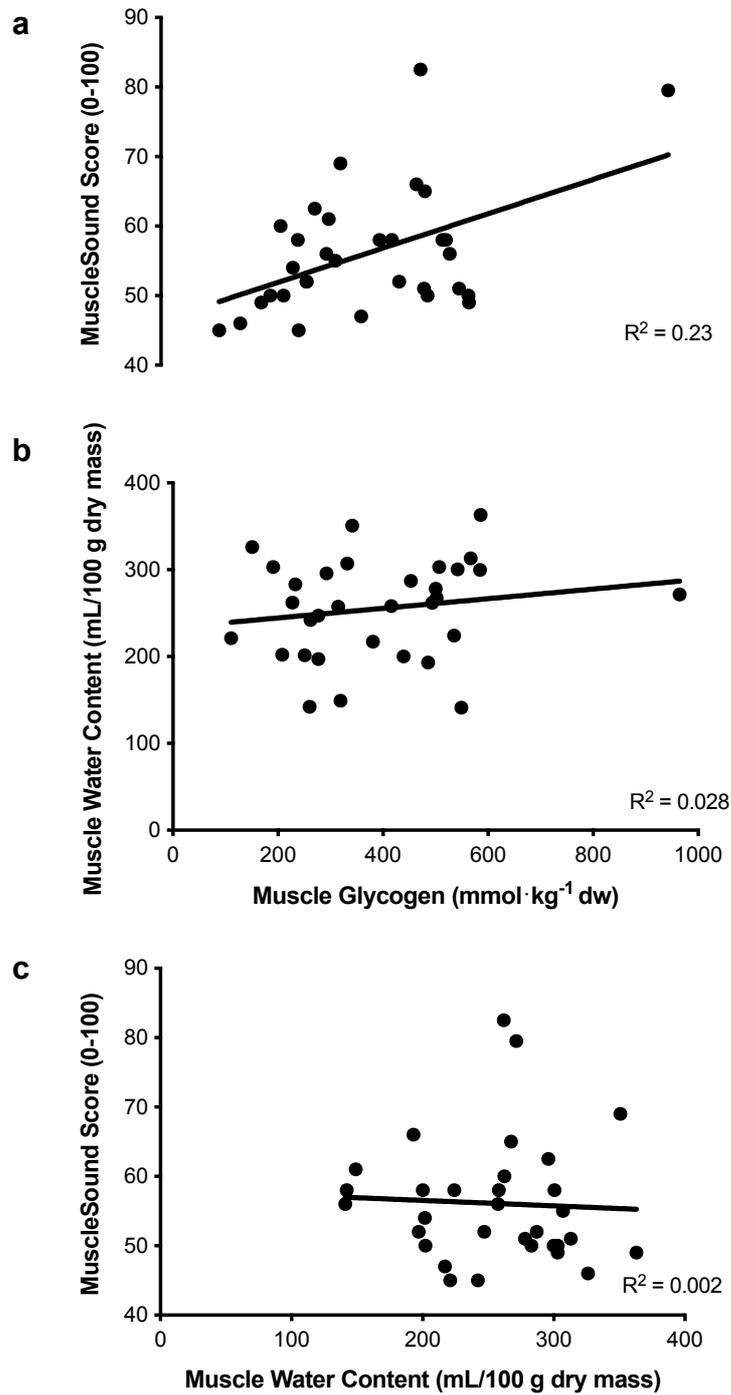
Figure 5.4 – Correlation between, a - muscle glycogen content (mmol·kg^{-1}dw) and MuscleSound score (0-100), b - muscle water content (mL/100 g dry mass) and muscle glycogen (mmol·kg^{-1} dw), c - MuscleSound score (0-100) and muscle water content (mL/100 g dry mass).

(a)

Chnage in Ultrasound score (0-100)

$R^2 = 0.16$

Change in Muscle Glycogen (mmol·kg⁻¹ dw)

(b)

Change in Ultrasound score (0-100)

$R^2 = 0.03$

Change in Muscle Water Content (mL/100 g dry mass)
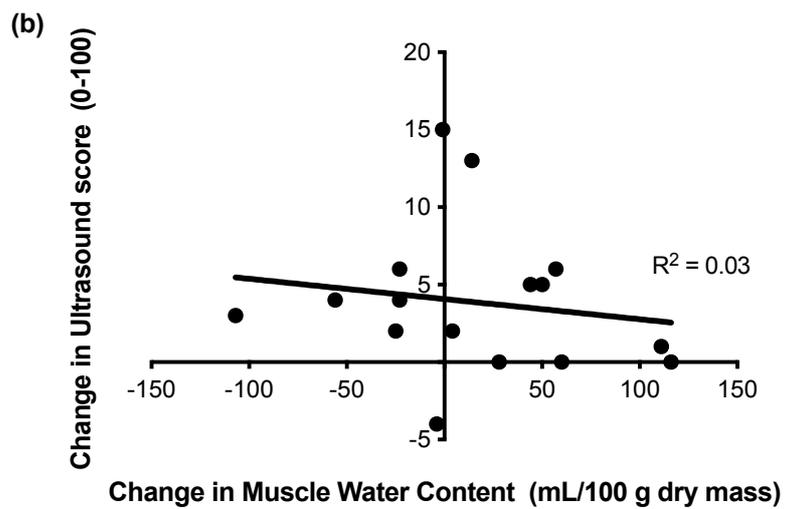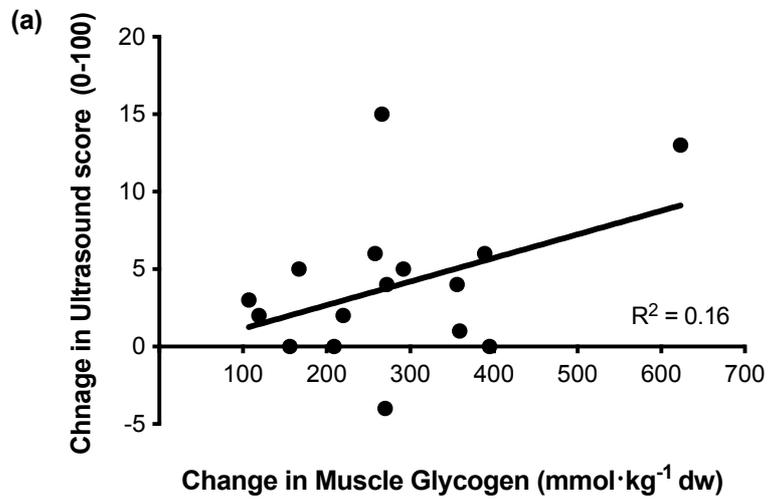
Figure 5.5 – Correlation between a - change in muscle glycogen content (mmol·kg⁻¹dw) and MuscleSound score (0-100), b - change muscle water content (mL/100 g dry mass) and change in MuscleSound score (0-100).

5.4 Discussion

The aim of the present study was to assess the validity of the MuscleSound glycogen scoring system to non-invasively assess muscle glycogen concentration. To this end, we utilised an experimental protocol consisting of a cycling based glycogen depleting exercise protocol followed by 36 h of a high or low CHO diet (that is fluid matched) in order to achieve high and low muscle glycogen concentrations. In using a protocol that examines the recovery of muscle glycogen (as opposed to exercise-induced glycogen depletion studied in Chapter 4), we hoped to minimise the confounding effects that variations in exercise-induced fluid shifts may play in assessing the validity of the MuscleSound system. Nonetheless, despite distinct differences in muscle glycogen concentration between the HIGH and LOW trials (e.g. 500 v 250 mmol·kg^{-1} dw), we observed no differences in corresponding MuscleSound scores where comparable values of 60 were observed. When considered with the results of Chapter 4, these data collectively suggest that the MuscleSound system is unable to detect differences in muscle glycogen concentration within the physiological range that typically occurs as a result of exercise-induced glycogen utilisation and/or post-exercise muscle glycogen re-synthesis.

Previous studies assessing the validity of MuscleSound have utilised cycling based exercise protocols and have observed strong correlations between the changes in biochemical determination of muscle glycogen and associated MuscleSound scores (Hill & Milan, 2014; Nieman et al. 2015). In agreement with these authors, we also observed that acute cycling-based exercise induces significant declines in MuscleSound scores from the vastus lateralis muscle, as evident in both the evening glycogen depleting protocol (see Figure 5.1c) and morning steady state exercise protocol (see Figure 5.2c). Although we did not collect muscle biopsies before and after these protocols, it is possible that we may have also observed positive correlations between changes in muscle glycogen concentration and MuscleSound scores. On the basis of previous data from our laboratory also using glycogen depleting protocols, it is likely that subjects' post depletion muscle glycogen concentration would be <100 mmol·kg^{-1} dw. However, given that we observed no changes in MuscleSound scores as a result of RL match play (see Chapter 4), the present data therefore provide further support for the hypothesis that variations in the mechanical load and alterations in fluid shifts between exercise protocols can

significantly affect the MuscleSound score obtained. Future studies should therefore examine the validity of the MuscleSound system across multiple exercise modalities and between sports. Additionally, further research should also ascertain the role of whole body and muscle tissue fluid status in affecting the MuscleSound scores obtained before and after exercise. Indeed, given that muscle water content has previously been shown to play a minor role in modulating the magnitude of muscle glycogen utilisation during cycling (Fernandez-Elias et al., 2015b), such data further question the rationale that changes in tissue water content can be used to infer subtle but physiologically relevant changes in muscle glycogen concentration.

Having completed the glycogen depleting protocol, the subjects studied here then ingested a high or low CHO diet for 36 h in order to induce distinct differences in muscle glycogen concentration. Accordingly, on the morning of day 3, subjects presented with muscle glycogen concentrations of 531 ± 129 mmol·kg^{-1} dw and 252 ± 64 mmol·kg^{-1} dw when completing the HIGH and LOW trials, respectively. On the basis of subject's training status (e.g. VO$_{2max}$ of 49.9 ± 7.5 ml·kg^{-1} min^{-1}) and absolute CHO intake consumed (e.g. 8 g·kg^{-1}), these data agree well with the normative values of resting glycogen concentration presented in a recent meta-analysis (Areta & Hopkins, 2018). Importantly, the dietary intervention studied here was also matched for fluid intake such that muscle water content was also identical between trials (HIGH: 265 ± 58 mL/100 g dry mass; LOW: 250 ± 50 mL/100 g dry mass). In this way, our data clearly demonstrate that in rested conditions where muscle is matched for water content but contains marked differences in absolute glycogen concentration (>250 mmol·kg^{-1} dw), the MuscleSound scoring system does not provide a valid assessment of muscle glycogen concentration.

In summary, the present data demonstrate that MuscleSound is not a valid measurement tool for which to non-invasively assess muscle glycogen concentration of human skeletal muscle. Rather, our data suggest that MuscleSound is simply measuring acute fluctuations in muscle fluid status as opposed to true changes in the glycogen storage pool. To further address the validity of this technique, future studies could be conducted using research protocols that are designed to independently assess the effects of hydration status and exogenous CHO status on muscle glycogen utilisation and associated MuscleSound scoring. Nonetheless, from a practical

perspective, the data presented in Chapters 3-5 demonstrate that despite good reproducibility of the MuscleSound scoring system (especially at 50% of the length of the vastus lateralis muscle), it does not provide a valid assessment of muscle glycogen concentration. As such, it is recommended that evaluations of the daily CHO and muscle glycogen requirements of team sport athletes should therefore be undertaken using traditional assessments of energy intake and muscle biopsies, respectively.

CHAPTER 6

THE ASSESSMENT OF HABITUAL CARBOHYDRATE INTAKE AND PHYSICAL LOADING ACROSS A WEEKLY MICRO-CYCLE IN ELITE MALE AFL PLAYERS

6.1 Introduction

Elite level Australian Rules Football (AFL) is an intermittent multidirectional contact sport that is completed over match durations of 100+ min (Gray & Jenkins, 2010). The physical demands of AFL match play are now relatively well documented within the literature (Dawson et al., 2004, Coutts et al., 2010, Wisbey et al., 2010). For example, total distance covered during match play is typically in the region of 10-15 km per game where approximately 3 km is comprised of high-speed running (i.e $>$ 14.4 km·h^{-1}) (Aughey, 2011). Interestingly, the physical demands of match play (e.g. total distance, high speed running distance, sprint distance, average speed etc) are greater than that observed in other invasive team sports such as soccer and rugby league (Varley et al., 2014). However, although the physical demands of AFL match play are well documented, there is limited research objectively quantifying the load of individual AFL training sessions across a typical weekly micro-cycle, though assessments of ratings of perceived exertion (RPE) demonstrates that internal load is much greater during match play versus training (Ritchie et al., 2016).

Given the high-intensity intermittent nature of AFL match play and training, it follows that CHO and muscle glycogen is likely the predominant substrate for energy production (Holway & Spriet, 2011). Accordingly, the current CHO recommendations for invasive team-based sports advise high CHO availability ($>$ 6 g·kg^{-1}) in the day prior to match play (Burke et al., 2011) as well as an exogenous intake of 30-60 g·h^{-1} during match play, in order to maximize aspects of physical, technical and cognitive performance (Burke et al., 2011). To achieve the latter, players are likely advised to ingest a mixture of both fluids and gels in accordance with individual preferences. The use of gels is often considered superior to fluids *per se* (usually 6-8% CHO solutions), owing to the flexibility for achieving CHO targets regardless of individual differences in body mass, hydration requirements and ambient temperature etc (Lee et al., 2014). In this regard, it is noteworthy that gels were recently reported as the preferred source of CHO during soccer match play in professional players from the English Premier League (Anderson et al., 2017), though 66% of the sample studied failed to achieve target guidelines of 30-60 g·h^{-1}. Nonetheless, given the apparent differences in match play loading between AFL and soccer, it is possible that both the source and quantity of CHO consumed during match play is different between codes.

In contrast to ensuring high CHO availability during competition, accumulating data over the last decade suggests that deliberately completing carefully selected training sessions with reduced CHO availability may lead to greater adaptive responses of skeletal muscle (Bartlett et al., 2015, Hawley & Morton, 2014, Impey et al., 2018). Indeed, the concept of "fueling for the work required" has recently been suggested as a practical framework for which to apply nutritional periodization strategies to both endurance (Impey et al., 2016, Impey et al., 2018) and team sport athletes (Anderson et al., 2017). Such strategies are intended to concomitantly promote components of training adaptation (e.g. activation of regulatory cell signaling pathways) but yet, also ensure adequate CHO (and energy) availability to promote competitive performance, reduce injury risk and aid recovery (Burke et al., 2011). Indeed, we have recently reported that professional rugby league (Morehen et al., 2016), rugby union (Bradley et al., 2015a) and soccer players (Anderson et al., 2017) habitually adjust both total daily CHO intake and CHO intake during exercise in accordance with the upcoming workload. To the authors' knowledge, however, the habitual CHO feeding strategies of AFL players across two 7-day micro cycles during both game play and training have not yet been reported.

In an attempt to evaluate the potential of CHO periodisation practices, the aim of the present study was to therefore quantify the daily physical loading and habitual daily CHO intakes during two typical weekly in-season micro-cycles (comprising a home and away fixture) of elite level AFL players. Additionally, we also assessed the seasonal quantification of exogenous CHO intake during training (n = 22 sessions) and matches (n = 22).

6.2 Methods

6.2.1 Subjects

Forty-two professional AFL players from an AFL list (mean $\pm$ SD; age 24 $\pm$ 3 years; height: 188 $\pm$ 7.8 cm; body mass: 85 $\pm$ 8.1 kg) volunteered to take part in the study. Players were of four different positions, (Forwards, n=11, Backs, n=13, Midfielders, n = 13, Ruck, n = 5). The study was conducted according to the Declaration of

Helsinki and was approved by the Ethics Committee of Liverpool John Moore's University.

6.2.2. Study Design

Data collection was conducted during the Australian Football League (AFL) 2017 in-season months from March to August. Players continued with their normal in season training, that was prescribed by the clubs coaching staff and players were selected to play upon coaching staff decisions. Within 30 minutes after each main physical weekly training session (n = 22) (game day + 3) and match (n = 22), exogenous CHO intake was quantified according to dietary recall, after prior education on reporting quantities and source. Players had unrestricted access to water, sports drink and CHO gels during both training and match play. The assessment of habitual CHO intake was performed over two weekly in season micro cycles (1 home and 1 away fixture). Following education from club sport science staff, players completed weekly food diaries recording all fluid and food ingestion. In addition, the day after food diary entries, players performed a 24h recall with the lead researcher to cross reference food entries and portion reporting. For each training session and match, physical load was assessed through portable global positioning system (GPS) units. Following each training sessions and match player data was processed by the club's sport scientist.

6.2.3 Quantification of Training and Game Load

For all field-based training sessions and games, players wore GPS devices (Optimeye S5, Catapult Innovations, Australia). Parameters obtained from GPS included total distance (m), high speed running (m) and average speed (m/min). In addition, high speed running was split into three different speed categories running (14.4-19.7 $km·h^{-1}$), high speed running (19.8-25.1 $km·h^{-1}$), and sprinting (>25.1 $km·hr^{-1}$). These speed zones are similar to previously reported in AFL (Coutts et al., 2010) as well as being formerly reported in soccer (Anderson et al., 2016) and shall be referred to for the purpose of this study. Each player wore the same device across the season which was worn inside a custom-made vest supplied by the manufacturer across the upper back between the left and right scapula (Ritchie et al., 2016). All devices were activated 30-minutes prior to data collection to allow acquisition of satellite signal (>8 satellites). The GPS units have a sampling rate of 15 Hz, alongside an internal accelerometer sampling at 100 Hz, with the accuracy of this been shown recently

(Rampinini et al., 2015). Following every training session and game, all GPS derived data were downloaded and analysed by a specialist GPS software package (Sprint 5.1.3, Openfield Version 11.12, Catapult Innovations, Australia). Data was filtered using manufacturers "intelligent motion filter".

6.2.4 Assessment of Habitual Daily Carbohydrate Intake

Habitual CHO intake was assessed from a 7-day player food diaries for all players. The period of 7-days is considered to provide reasonably accurate estimations of energy and nutrient consumptions whilst reducing variability in coding error (Braakhuis et al., 2003). On the day prior to data collection, food diaries were explained to the players by the lead researcher and initial dietary habits questionnaire (24 h food recall) was also performed. These questionnaires were used to establish habitual eating patterns and subsequently allow follow up analysis of food dairies, additionally, they helped to retrieve any potential information that players may have missed in their food diary input. Furthermore, players were provided with educational pictures concealed within the food diary to assist with reporting portion size. In addition, CHO content was also cross-referenced against photos sent to the lead researcher from players. The remote food photographic method (RFPM) allowed for cross referencing of CHO portion size. This type of method has been previously shown to accurately measure energy intake of free-living individuals (Martin et al., 2009). To further enhance reliability, food diaries were cross checked using a 24 h recall with the lead researcher after one day of entries (Thompson & Subar, 2008). To obtain carbohydrate macronutrient composition, professional dietary analysis software was used (Nutritics Ltd, Ireland).

6.2.5 Assessment of Exogenous Carbohydrate Intake

For training sessions (n = 22) and games (n = 22) players performed dietary recall of the quantity and the source of CHO consumed during exercise within 30 minutes of completion of activity. Prior to the data collection period (pre-season), players were instructed on the correct method of recalling exogenous CHO intake. During six pre-season training sessions with the lead researcher present, players had unrestricted access to water, sports drinks (Gatorade, 36g CHO, 33g Sucrose, 3g Glucose) and energy gels (Shotz, 29.8g CHO, 2.9g sugars). Following each session, the lead researcher would demonstrate the correct method to report CHO intake with players

present. This was repeated six times across the pre-season prior to the start of data collection (RD 1 of the 2017 AFL season). For the data collection period following each game and training sessions, players would use a record sheet that was observed by the lead researcher. Furthermore, during matches the lead researcher was present on the team bench and had visible access to cross reference player recall data. The lead researcher was also present for all training sessions and had full access to players to visibly cross reference player recall.

6.2.6 Statistical Analysis

All data are presented as the mean ± standard deviation or 90% confidence intervals (CI). Linear mixed models with random intercepts for individual players were used for data analysis. CHO intake was compared between training and match play by using session type (training/game) as the independent variable in linear mixed models. Additionally, comparison of CHO intake during games from gels versus fluids was performed, by using CHO source (gels/fluids) as the independent variable in linear mixed models. For these comparisons, standardised differences were used as the effect size (ES). ES magnitude was interpreted as trivial (0 to 0.2), small (>0.2 to 0.6), moderate (>0.6 to 1.2), large (>1.2 to 2.0), very large (>2.0 to 4.0), extremely large (>4.0) (Hopkins et al., 2009). All statistical analysis was conducted using R, version 3.3.1 (R Core Team, 2016).

6.3 Results

6.3.1 Quantification of Physical Loading Patters
6.3.1.1 Total Distance

Weekly session time (Figure 6.1) alongside total distance, running, high speed running and sprinting across a home and away fixture are displayed in Figure 6.2. Specifically, total distance (m) displayed significant difference (P = 0.4) of 205m between a home and away weekly micro cycle. Specifically, total distance on game day -4 was significantly higher (P < 0.01) than game day -3 (7124m; ES = 6.98, extremely large) and game day -1 (3555m; ES = 3.48, very large). Training sessions on game day -2 displayed significantly greater total distance than game day -4 (677m; ES = 0.66, moderate), game day -3 (7802m; ES = 7.64, extremely large) and game day -1 (4233m). In addition, game day -1 training sessions were also higher (P >

0.01) in total distance than game day -3 (3569m; ES = 3.50, very large). Total distance covered during a game was significantly higher (P < 0.01) than game day -4, -3, -2, and -1 with mean differences of 6021m, 13146m, 5343m and 9576m respectively and extremely large effect sizes (5.90, 12.88, 5.23 and 9.38 respectively)

6.3.1.2 Running

Running (m) was significantly different between home and away fixture weekly micro cycles with a mean difference of 231m (ES = 0.44, small; P < 0.01). Running in game day -4 training sessions was significantly higher (P < 0.01) than game day -3 (1124m; ES = 2.12, very large) and game day -1 (583m; ES = 1.10, moderate). Running during game day -2 training sessions was significantly greater than game day -3 (1232m; ES = 2.33, very large) and game day -1 (691m; ES = 1.30, large) however there was no significant difference between game day -4 (P = 0.06, 107m). In addition, game day -1 was significantly higher than game day -3 (540m; ES = 1.02, moderate; P < 0.01). Running distance during a game was significantly higher (P < 0.01) than game day -4, -3, -2, and -1 with mean differences of 1491m, 2615m, 1383m, 2074m respectively and very to extremely large effect sizes of 2.82, 4.94, 2.61, 3.92 respectively.

6.3.1.3 High Speed Running

High Speed Running (m) displayed no significant difference between home and away micro cycles (P = 0.8). Game day -4 displayed significantly higher (P < 0.01) high speed (m) compared with game day -3 (514m; ES = 1.70, large) and game day -1 (281m; ES = 0.93, moderate). Furthermore, game day -2 training sessions contained significantly more high-speed running than game day -4, game day -3 and game day -1, with a mean difference of 293m (ES = 0.97, moderate), 808m (ES = 2.68, very large) and 575m (ES = 1.91, large) respectively. The training session on game day -1 also containing significantly more high-speed running than game day -3. High speed running distance during a game was significantly higher (P < 0.01) than game day -4, -3, -2, and -1 with mean differences of 451m (ES = 1.49, large), 966m (ES = 3.20, very large), 157m (ES = 0.52, small) and 732m (ES = 2.40, vey large) respectively.

6.3.1.4 Sprinting

Sprinting distance displayed no significant difference (P = 0.19) between home and away week micro cycles, with a mean difference of 15m. Sprinting distance during game day -4 was significantly greater (P < 0.01) than game day -3 (84m; ES = 0.79, moderate) and game day -1 (61m; ES = 0.58, small). Game day -2 sessions possessed significantly greater (P < 0.01) sprint distance than game day -4 (29m; ES = 0.27, small), game day -3 (113m; ES = 1.07, moderate) and game day -1 (91m, ES = 0.86, moderate). Finally, game day-1 sprint distance was significantly greater than game day -3 (23m; ES = 0.22, small). Sprint distance during a game was significantly higher (P < 0.05) than game day -4, -3, -2, and -1 with mean differences of 73m (ES = 0.69, moderate), 157m (ES = 1.48, large) 44m (ES = 0.42, small) and 134m (ES = 1.27, large) respectively.

6.3.1.5 Session Duration

Sessions duration (minutes) displayed no difference between home and away weekly micro cycles (P = 0.66), with a mean difference of 2 minutes. However, game day -4 sessions were significantly longer than game day -3 and game day -1 with a mean difference of 77 (ES = 8.11, extremely large) and 36 minutes (ES = 3.79, very large). Game day -2 training sessions were also significantly longer than game day -4 (4 minutes, ES = 0.42, small), game day -3 (83 minutes; ES = 8.74, extremely large) and game day -1 (41 minutes; ES = 4.32, extremely large). Session duration (minutes) of a game significantly higher (P < 0.05) than game day -4, -3, -2, and -1 training sessions with mean differences of 29 minutes, 108 minutes, 25 minutes and 67 minutes respectively, and very to extremely large effect sizes of 3.05, 11.37, 2.63 and 7.05 respectively.
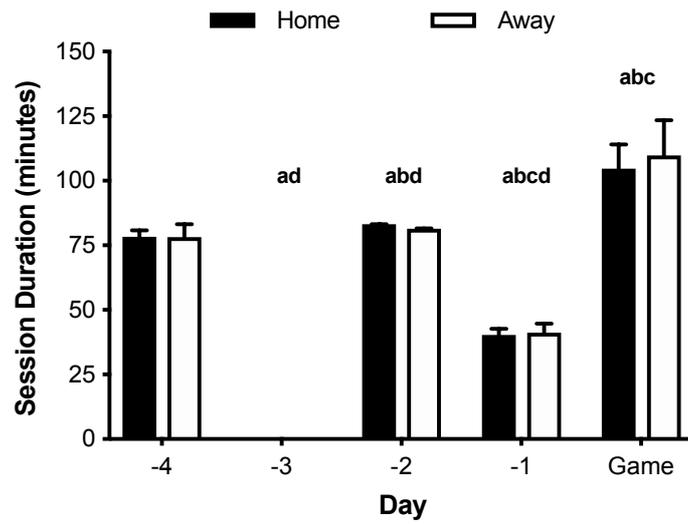
Figure 6.1. Weekly session duration (minutes) for a Home and Away AFL fixture. a denotes difference from -4, b denotes difference from -3, c denotes difference from -2, d denotes difference from game, all $P < 0.01$.
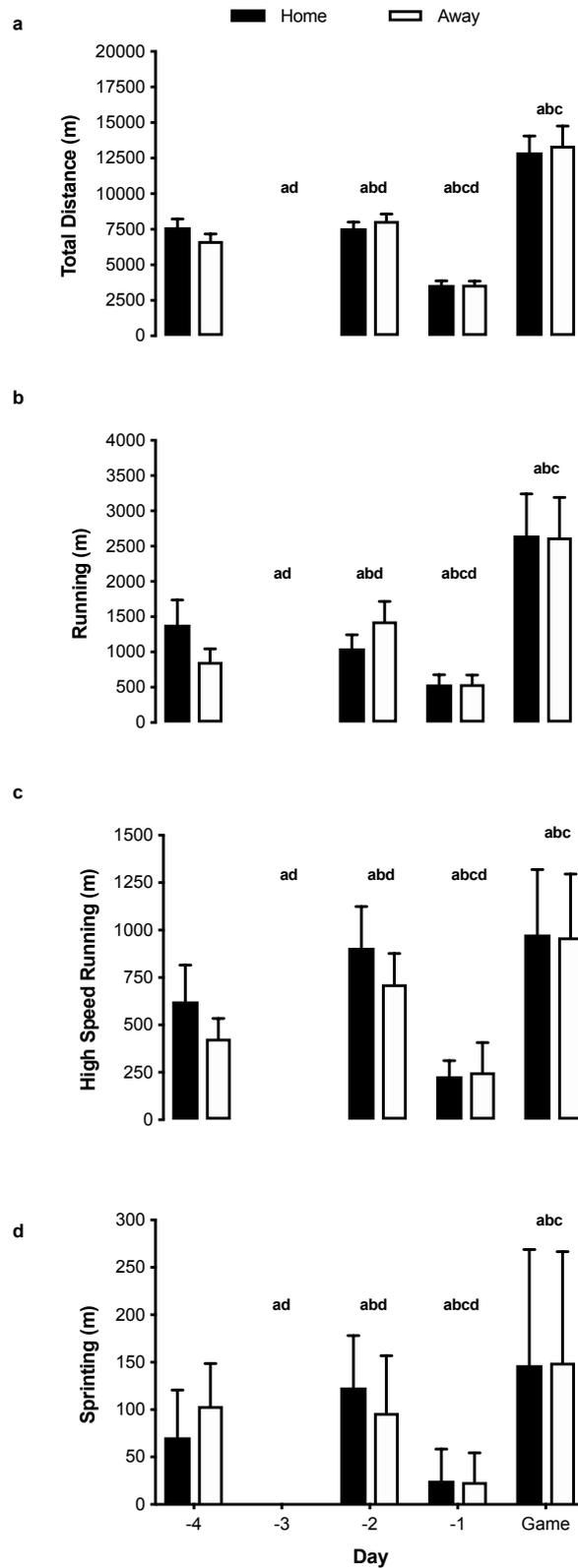
Figure 6.2. a - Total Distance, b - running, c – high speed running and d -sprinting for a weekly micro cycle leading into a Home and Away AFL fixture. a denotes difference from -4, b denotes difference from -3, c denotes difference from -2, d denotes difference from game, all P < 0.01.

6.3.2 Quantification of Daily Carbohydrate Intake and Pre-Game meal

6.3.2.1 Carbohydrate (g)

Daily CHO ingestion across weekly micro cycle in home and away fixtures are displayed in Figure 6.3. Total CHO (g) displayed no significant difference (P = 0.9) between home and away fixture weeks with a mean difference of 1g. Total CHO ingestion on game day -4 was significantly higher than game -3 (P < 0.01) with a mean difference of 44g CHO (ES = 0.83, moderate). The ingestion of CHO on game day -2 was significantly greater than game day -4 and game day -3 with differences of 50g (ES = 0.96, moderate) and 94g (ES = 1.80, large). Furthermore, CHO ingestion was at its greatest on game day -1 with significantly higher CHO's being consumed compared with game day -4 (235g; ES = 4.50, extremely large), game day -3 (279g; ES = 5.34, extremely large) and game day -2 (185g; ES = 3.54, very large)

6.3.2.2 Carbohydrate (g·kg^{-1})

Carbohydrate ingestion in grams per kg (g·kg^{-1}) displayed no significant difference between home and away fixtures (P = 0.8). Game day -4 CHO ingestion was significantly greater than game day -3 (P<0.01) with a mean difference of 0.5 g·kg^{-1} (ES = 0.89, moderate). CHO ingestion (g·kg^{-1}) on game day -2 was significantly greater than game day -4 (0.6 g·kg^{-1}; ES = 1.07, moderate), game day -3 (1.1 g·kg^{-1}; ES = 1.96, large). In addition, CHO (g·kg^{-1}) ingestion on game day -1 was significantly greater than game day -4 and game day -2 with a mean difference of 2.6 g·kg^{-1} (ES = 4.64, extremely large) and 2.1 g·kg^{-1} (ES = 3.75, very large) respectively.

6.3.2.3 Pre-Game Carbohydrate Ingestion

Total pre-game carbohydrate ingestion in g and g.kg^{-1} prior to a home and away AFL fixture is presented in figure 6.4. There was no significant difference of total pre-game CHO (g) intake (P = 0.8) between home (207 ± 59 g) and away fixture (253 ± 80 g). There was also no significant difference of total pre-game CHO (g.kg^{-1}) intake (P = 0.7) between home (2.3 ± 0.6 g·kg^{-1}) and away (2.8 ± 0.9 g·kg^{-1}) fixtures.
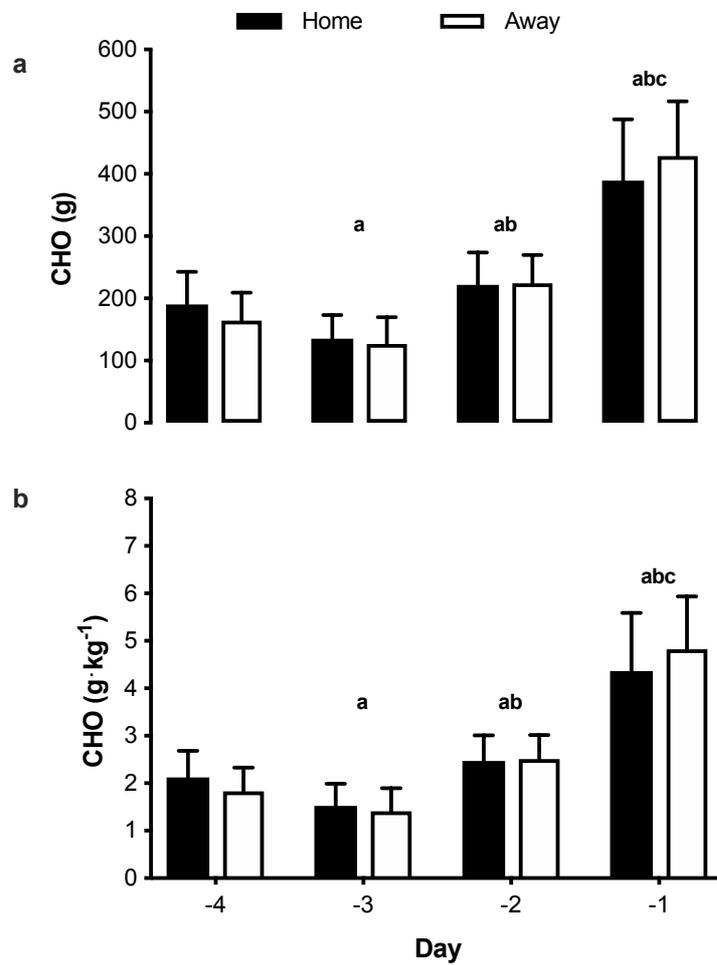
Figure 6.3. Total CHO ingestion a - CHO (g) and b - CHO (g·kg⁻¹) (b), in the weekly micro cycle prior to a Home and Away AFL fixture. a denotes difference from -4, b denotes difference from -3, c denotes difference from -2, all P < 0.01.
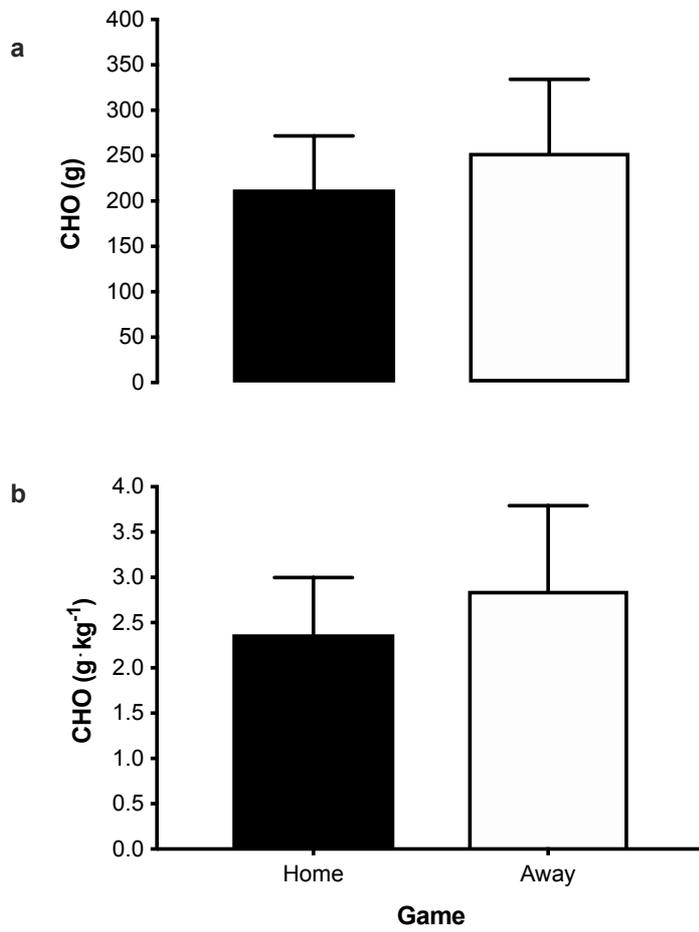
Figure 6.4. Pre-Game CHO ingestion a – g and b – g·kg^{-1} prior to a Home and Away AFL fixture.

6.3.3. Quantification of Exogenous Carbohydrate Intake during Training and Games

The estimated mean (90% CI) total exogenous CHO (Figure 6.5a) consumed during game play (59.3 ± 19.1 g, range: 57.1 to 61.5 g) was higher than consumed during training sessions (0.7 ± 1.1 g, range -3.4 to 4.4) (ES = 4.34, extremely large). Accordingly, CHO feeding when expressed as $g \cdot h^{-1}$ (33.5 ± 11 $g \cdot h^{-1}$, range 32.2 to 34.9 vs 0.0 ± 0.8 $g \cdot h^{-1}$, -2.2 to 2.2; ES – 4.75, extremely large) (Figure 6.5b) and $g.min^{-1}$ (0.55 ± 0.19 $g \cdot min^{-1}$,0.47 to 0.62 vs 0.07, ± 0.01 $g \cdot min^{-1}$, range 0.10 to 0.25; ES = 4.77, extremely large) (Figure 6.5c) were also significantly higher during game play compared to training. In the context of game play, 53.2% of the sample achieved CHO intakes in the recommended range of 30-60 $g \cdot h^{-1}$. In relation to source of CHO consumed during game play, a greater amount of CHO was consumed from fluids versus gels when expressed as total CHO (37.5 ± 18.3 g, range, 34.7 to 40.2 versus 22.2, 17.3 to 27g; ES = 2.04, very large), (21.1 ± 24.1g, range 19.4 to 22.8 versus 13.0 ± 13.8 $g \cdot h^{-1}$, range 10.2 to 15.9 $g \cdot h^{-1}$; ES = 1.67, very large) and $g \cdot min^{-1}$ (0.35 ± 0.16 $g \cdot min^{-1}$, range 0.32 to 0.38 versus 0.22 ± 0.18 $g \cdot min^{-1}$, 0.17 to 0.26 $g \cdot min^{-1}$; ES = 1.70, very large).
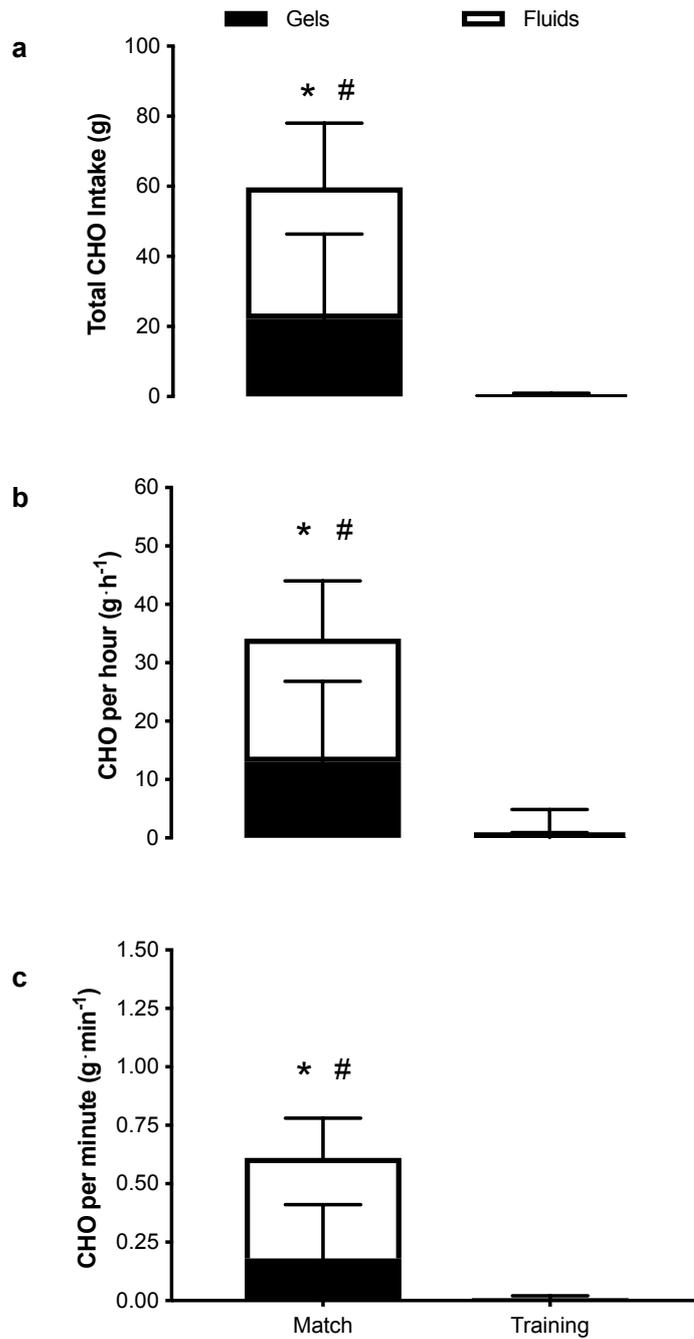
Figure 6.5 - Total CHO (a), CHO $g \cdot h^{-1}$ (b), CHO $g \cdot min^{-1}$ (c) between training and match with total (g), broken down into fluids and gels, * ($P<0.05$) indicates significant difference between match and training with # indicating significant differences between gels and fluids.

6.4 Discussion

The aim of the present study was to quantify the periodisation of physical loading and CHO intake across a weekly micro cycle in addition to highlighting the quantity and source of CHO consumed during AFL game play and training, Using a seasonal long analysis, we report for the first time that 1), "in-season" AFL training sessions exhibit marked reductions in physical load compared with game play, 2) CHO ingestion appears to follow a similar periodisation model to 'in-season' training load 3) players consume greater amounts of CHO (predominantly from fluid sources) during game play versus training, with most players adhering to current CHO feeding guidelines (e.g 30-60 g/h) for team sports. When taken together, these data appear in accordance with the principle of "fuel for the work required" in that players habitually adjust their CHO intake in accordance with the physical load. However, evaluation of habitual CHO intake suggest that players are likely under-consuming CHO, especially in relation to the day prior to the game when considered in the context of CHO loading for match play.

To the authors' knowledge, we are the first group to quantify physical loading of single sessions across a weekly AFL micro cycle. Indeed, although Ritchie et al. (2016) also reported training load using GPS metrics, these data were expressed as weekly accumulative load and hence it is therefore difficult to determine the physical load (and associated CHO requirement) of single sessions. We report herein that physical load of training sessions (-4, -3, -2, -1) was significantly less than game play, as quantified according to total distance in both home (7644m, 0m, 7568m, 3588m v 12900m) and away (6667m, 0m, 8084m, 3603m v 13372m) fixture weeks. Training session data is in conjunction with previous work from (Bartlett et al., 2016) who highlighted training session distance to be 6389 ± 3315m. Running, high speed running and sprinting metrics were all lower during training than a game in both home and away micro cycles. Nonetheless the distances covered during running (1400m v 500m) and high-speed running (550m v 100m) in AFL training appear to be greater than the absolute values previously observed during soccer (Anderson et al., 2016). Such differences between codes are perhaps a reflection of the greater duration of training sessions in AFL versus soccer (i.e 81v 60 minutes), although we acknowledge this hypothesis is limited to the data provided for only two professional

clubs. Nonetheless, these data therefore give evidence to a training periodisation model across an AFL week leading into a game.

Through 7-day food diary analysis, CHO intake was estimated across a two weekly micro-cycle comprising both a home and away fixture. In accordance with the clear evidence of training periodisation between days, our data appear to highlight practices of nutritional periodisation whereby a greater amount of CHO (5 g·kg^{-1}) is consumed on the day prior to game play whereas lower absolute CHO intakes (i.e. < 3 g·kg^{-1}) are consumed on training days. It is, of course, difficult to ascertain if such CHO intakes are detrimental or facilitative of optimal training adaptations. This finding is in conjunction with previous data, where AFL players reported consuming an average of $4.1\text{g} \pm 1.6 \text{ g·kg}^{-1}$ CHO per day, a dose that is below the recommended amount when training between 1-3 h per day (Bilsborough et al., 2016). Nonetheless, and even when considering potential under-reporting, our data suggest that players are under-consuming CHO on the day prior to the match when considering with what is considered as optimal for muscle glycogen loading protocols (Thomas et al., 2016). In relation to quantity of CHO consumed during exercise, we observed marked differences between training and match play (0.7 ± 1.1 g v 59.3 ± 19.1 g respectively). This apparent difference between CHO intake during each activity is also similar to that recently reported by professional soccer players of the English Premier League where values of 3.1 ± 4.4 and $32.3 \pm 21.9 \text{ g·h}^{-1}$ were reported in training and match play, respectively (Anderson et al., 2017).

In terms of promoting game day physical and technical performance in team sports, current guidelines advise CHO feeding at a rate of $30\text{-}60 \text{ g·h}^{-1}$ (Burke et al., 2011). Such a feeding strategy is intended to promote physical performance via multiple mechanisms potentially consisting of maintaining plasma glucose levels and CHO oxidation rates (Coyle et al., 1986), liver and/or muscle glycogen sparing (Stellingwerff et al., 2007, Gonzalez et al., 2015) and finally, direct effects on the CNS (Carter et al., 2004). Of the sample population studied here, 53.2% of the sample achieved CHO intakes in the recommended range of $30\text{-}60 \text{ g·h}^{-1}$. This finding is different to that recently reported from professional soccer players where only 33% of the sample studied consumed $>30 \text{ g·h}^{-1}$ (Anderson et al., 2017). Furthermore, unlike Anderson et al. (2017) who reported that 35% of the CHO consumed was from

gel sources, the athletes studied here consumed 75% of the CHO from fluid sources and only 25% from gel sources. Such differences between studies are most likely related to higher ambient temperatures observed during AFL game play (as opposed to that typically observed during the annual English Premier League) and suggest that players were consciously practicing a CHO feeding strategy that was simultaneously intended to promote hydration. In contrast, the choice of gels may offer a superior strategy to English Premier League soccer players as opposed to fluids per se (usually 6-8% CHO solutions), owing to the flexibility for achieving CHO targets regardless of individual differences in body mass, hydration requirements and difference in interchange number of AFL.

It is difficult to accurately ascertain if such differences in daily CHO intake between days and exogenous CHO intake between training and match play were a deliberate choice of the player and/or coach led (sport scientist) practice or alternatively, an unconscious decision. Nonetheless, given the apparent differences in physical load between training and match play, our data suggest that players practice elements of CHO periodisation within the weekly micro-cycle. Such practices appear in accordance with the principle of "fuel for the work required" that suggests component of training adaptations (e.g. activation of regulatory cell signaling pathways) may be enhanced when training is deliberately completed with reduced CHO availability (Hawley and Morton, 2014, Bartlett et al., 2015, Impey et al., 2016). Indeed, when considered with our previous observations of professional rugby league (Morehen et al., 2016), rugby union (Bradley et al., 2015a) and soccer players (Anderson et al., 2017), the present data provide further evidence that team sport athletes habitually adjust both total daily CHO intake and CHO intake during exercise in accordance with the upcoming workload.

In summary, we simultaneously quantified for the first time the daily physical loading and habitual daily CHO intakes during two weekly micro-cycles of elite level AFL players. When such data is considered in conjunction with the seasonal quantification of exogenous CHO intake during training and matches, our data demonstrate that AFL players appear to practice elements of CHO periodization whereby CHO intake is adjusted in accordance with the upcoming workload. Although it is difficult to ascertain if the habitual CHO intakes reported here are facilitative of optimal training

adaptation, it is likely that players do not consume sufficient CHO intake in the days prior to match play in order to ensure optimal muscle glycogen availability for match play. Future studies should therefore directly (i.e. using the muscle biopsy technique) assess the muscle glycogen cost of elite match play.

CHAPTER 7

CASE STUDY: ASSESSMENT OF MUSCLE GLYCOGEN UTLISATION IN ELITE MALE PLAYERS DURING COMPETITIVE AFL GAME PLAY

7.1 Introduction

The physiological demands of team sports such as soccer (Anderson et al., 2016, Varley et al., 2014) rugby league (Varley et al., 2014; Chapter 4) and Australian Rules Football (AFL) (Varley et al., 2014; Coutts et al., 2015; Chapter 6) are characterised by high-intensity intermittent activity profiles. Given the duration of activity (i.e. 80-120 minutes) and high-intensity intermittent profiles, muscle glycogen is considered as the predominant energy substrate to support the metabolic demands of match play (Mujika & Burke, 2010, Gunnarsson et al., 2013). In relation to soccer, it has been reported that match-play in elite Danish soccer players (Gunnarsson et al., 2013) depletes muscle glycogen concentration by a magnitude of 50% and requires a glycogen cost of approximately 200 mmol·kg^{-1} dw. We also observed similar absolute glycogen utilisation and relative depletion rates in English professional rugby league players during competitive match play (Bradley et al., 2016; Chapter 5). Nonetheless, in the absence of a controlled CHO loading protocol, it is noteworthy that pre-match muscle glycogen concentrations in team sport athletes may range from 300-600 mmol·kg^{-1} dw (Krustrup et al., 2006) and that approximately 50% of muscle fibres are classified as empty or partially empty after soccer match play, thus having potential implications for high-intensity physical performance late in the match. Indeed, in relation to physical performance, it was recognised as early as the 1970s (Saltin., 1973) that commencing match play with reduced pre-exercise muscle concentration (i.e. < 200 mmol·kg^{-1} dw) reduces total distance covered by 25% when compared with higher pre-match muscle glycogen availability (i.e. > 400 mmol·kg^{-1} dw).

Given the longer duration and greater proportion of time spent at high-intensity workloads in AFL match play (Varley et al., 2014; Coutts et al., 2015; Chapter 6) versus both soccer (Anderson et al., 2016; Varley et al., 2014) and rugby (Varley et al., 2014; Chapter 5), it could be suggested that the CHO requirements for AFL players are increased accordingly. Indeed, high velocity running was reported to be significantly greater in AFL (1322m) versus rugby league (327m) and soccer (517m) (Varley et al., 2014). Nonetheless, despite the definitive requirement for high CHO availability for match play, the data presented in Chapter 6 suggest that elite male players do not appear to consume an absolute dose of CHO intake (i.e. >6 g·kg^{-1}) that is likely to achieve optimal muscle glycogen storage for match play. However, no

data are currently available to quantify the effects of CHO loading on muscle glycogen storage in elite AFL players nor do we currently know the glycogen requirement for competitive match play.

The aim of the present case-study was to therefore quantify muscle glycogen utilisation in the vastus lateralis muscles from two elite male AFL players after participation in a South Australian Football League game. Given the apparent insufficient CHO loading practices reported in Chapter 6, we studied two elite players after adhering to a CHO loading protocol and pre-match intake of 8 and 2 $g \cdot kg^{-1}$, respectively. Furthermore, given the inability of the MuscleSound system to non-invasively assess muscle glycogen use during Rugby league match play, we quantified muscle glycogen utilisation using the traditional Bergstrom muscle biopsy technique.

7.2 Methods

7.2.1 Subjects

Two male forward players from a South Australian Football League (SANFL) list (see Table 1) volunteered to take part in the study. The study was conducted according to the Declaration of Helsinki and was approved by the Human Research Ethics Committee of Australian Catholic University Melbourne.

7.2.2 Study Design

In a case-study design, muscle biopsies were obtained from *m. vastus lateralis* before and after participation in a competitive SANFL league game undertaken in August 2017. Quantification of external loading during match play was assessed via global positioning system (GPS) technology.

7.2.3 Diet Manipulation

In the day prior to game play, each player consumed a prescribed CHO loading diet providing CHO, protein and fat intakes corresponding to 8 $g \cdot kg^{-1}$, 2 $g \cdot kg^{-1}$ and 1 $g \cdot kg^{-1}$ body mass, respectively, based on contemporary guidelines of Thomas and colleagues (Thomas et al., 2016). At 4 hours prior to match play, each player also consumed a pre-match meal providing 2 $g \cdot kg^{-1}$ CHO, 0.2 $g \cdot kg^{-1}$ protein and 0.3 $g \cdot kg^{-1}$ fat. During match play, players were able to consume fluids and sports foods at

opportunities that were both predictable (between quarters) and random (rotation on the bench, access to trainer on the ground when the ball is not in play). On such occasions; Player A consumed water only whereas Player B also consumed an additional 88 g of CHO in the form of four isotonic CHO gels (SiS GO Gels, UK) consumed at the quarter time breaks (~20-minute intervals during the game).

7.2.4 Muscle Biopsy Samples.

Muscle biopsies (100 mg) were sampled from *m. vastus lateralis* at 60 minutes prior to the beginning of the game (i.e. prior to the warm-up period) and within 10 minutes of completion of match play participation. Biopsies were obtained under local anesthesia (1% Xylocaine) using a Bergstrom biopsy needle and immediately frozen in liquid nitrogen for later analysis. Approximately 3 mg of freeze-dried muscle tissue was powdered and dissected free of all visible non-muscle tissue. Powered muscle tissue was then mixed with 250 µl of 2 M HCl, incubated at 95°C for 2 hours (agitated gently every 20 min), and then neutralized with 750µl of 0.66 M NaOH. Glycogen concentration was subsequently assayed in triplicate via enzymatic analysis with flurometric detection. Muscle glycogen values were expressed as millimoles per kilograms dry weight (mmol.kg^{-1} dw).

7.2.5 Quantification of Game Load

Both players wore a global positioning system device (GPS) (Optimeye S5, Catapult Innovations, Australia) which recorded activity profile data. The portable device was worn inside a purpose-built elastic vest, positioned across the upper back between the scapula. Each device was activated 30-minutes prior to the start of the game to allow acquisition of satellite signal and lock (>8 satellites). Satellite data sampled at 10 Hz provided measures of duration (min), total distance (m), average speed (m/min) and distance covered within four specific velocity bands corresponding to: jogging (7.2-14.3 km·h^{-1}), running (14.4-19.7 km·h^{-1}), high-speed running (19.8-25.1 km·h^{-1}) and sprinting (>25.1 km·h^{-1}). These speed zones are similar to those previously reported in soccer[1] and allow for direct comparisons between football codes. At the conclusion of the match, data were downloaded and analyzed using (Openfield version 11.12, Catapult Innovations, Australia).

7.3 Results

Subject characteristics, external workload, muscle glycogen utilisation and CHO intake are presented in Table 1. Individual data are presented for both Player A and B.

Table 7.1 Subject characteristics, external workload, muscle glycogen utilisation and in-game CHO intake in Player A and Player B

	Player A	Player B
Playing Position	Forward	Forward
Age (years)	22	27
Body Mass (kg)	87.2	87.6
Height (m)	1.88	1.82
Warm Up Duration (min)	12	12
Distance (m)	1478	1501
High Speed Running (m) 19.8-25.1 km·h⁻¹	156	159
Sprinting (m) >25.1 km·h⁻¹	23	31
Match Play Duration (min)	115	98
Number of Rotations	2	4
Average Speed (m/min)	106	114
Total Distance (m)	12229	11182
Walking (m) 0.3-7.1 km·h⁻¹	3801	2801
Jogging (m) 7.2-14.3 km·h⁻¹	5231	4718
Running (m) 14.4-19.7 km·h⁻¹	2180	2396
High Speed Running (m) 19.8-25.1 km·h⁻¹	837	1070
Sprinting (m) >25.1 km·h⁻¹	135	138
Pre-Match Glycogen (mmol·kg⁻¹ dw)	656	544
Post-Match Glycogen (mmol·kg⁻¹ dw)	223	416
Total Glycogen Utilisation (mmol·kg⁻¹ dw)	433	138
Total Exogenous CHO Consumed (g)	0	88
Exogenous CHO (g·h⁻¹)	0	54
Exogenous CHO (g·min⁻¹)	0	0.9

7.4 Discussion

The aim of the present case-study was to quantify muscle glycogen utilisation during AFL game play. We studied two elite male AFL players during a competitive match from the South Australian Football League. Our data demonstrate that a standardised one-day dietary CHO loading protocol of 8 g·kg^{-1} and 2 g·kg^{-1} in the pre-game meal elevates muscle glycogen concentration (i.e. >500 mmol·kg^{-1} dw) to a magnitude that is sufficient to fuel the metabolic demands of AF match play.

In relation to the external loads reported here, we observed similar loading profiles to that previously reported in Chapter 6 (total distance 13136m, running 2638m, high-speed running 969m, sprinting 148m) alongside those previously reported in elite AFL match play, albeit with a larger sample sizes (e.g. 39 players) (Coutts et al., 2015). This is the case for parameters such as total distance, average speed, high-speed running and sprinting. The between player differences in such parameters are also similar to those previously reported (Coutts et al., 2015). Despite only studying two forward players, we are confident that the external loads reported here are therefore representative of the customary loads experienced in AFL match play from a wider sample of teams and players, though we acknowledge that load differences between playing positions are to be expected (Coutts et al., 2015).

In accordance with a higher workload, our data suggest that the glycogen cost of AFL game play (e.g. Player A utilised 433 mmol·kg^{-1} dw) may be greater than that reported in soccer (Gunnarsson et al., 2013, Krustrup et al., 2006) and rugby match play (Bradley et al., 2016; Chapter 4) where approximately 200 mmol·kg^{-1} dw was utilised in both instances. Such differences in absolute glycogen cost are likely due to greater duration of activity and time spent in higher intensity threshold zones. In contrast to Player A, Player B experienced less total glycogen use (138 mmol·kg^{-1} dw). While such inter-individual variation in glycogen use may be due to differences in total distance covered, duration, pre-match muscle glycogen concentrations, training status and muscle oxidative capacity (Hargreaves et al., 1995), it is noteworthy that Player B also consumed an additional 88 g of CHO during match play. As such, differences in glycogen use between players may also be due, in part, to a potential muscle glycogen sparing effect of CHO feeding, an effect that has been reported previously in *m. vastus lateralis* during running (Tsintzas et al., 1995).

Indeed, the game characteristics of AFL support a more aggressive approach to CHO intake during play than is reported by elite soccer players (Anderson et al. 2017), with opportunities for fuel replacement during scheduled breaks between quarters and time spent on the interchange bench. Nonetheless, we acknowledge that randomised control trials incorporating larger sample sizes are now required to verify any potential metabolic or ergogenic effect of CHO feeding during competitive match play. Additionally, glycogen use in specific muscles (e.g. gastrocnemius versus vastus lateralis), muscle fibre types and intra-cellular storage pools could also be quantified using transmission electron microscopy. We also acknowledge that the glycogen utilisation observed here is also reflective of those activities undertaken during the warm-up period. As such, future studies could also sample biopsies in the minutes prior to match play and at the end of each quarter to further characterise both the total absolute use and rates of glycogen use as the match progresses.

From a practical perspective, our data suggest that pre-match muscle glycogen concentration > 500 mmol·kg^{-1} dw (as achieved via CHO loading of 8 g·kg^{-1}) is sufficient to fuel the physical demands of elite forward AF match play. Given the apparently greater muscle glycogen cost of AFL match-play compared to soccer and rugby, sport physiologists and nutritionists should ensure that AFL players consume sufficient dietary CHO intake (likely > 6 g·kg^{-1}) in the 24 hours prior to participation in match play.

In summary, we provide novel data by reporting muscle glycogen utilisation in *m. vastus lateralis* from two elite male AFL forward players during competitive game play. Our data suggest that total glycogen use is greater than that reported in elite players from other invasive team sports, such as soccer and rugby. Additionally, these data suggest that CHO loading with 8 g·kg^{-1} body mass is sufficient to meet the metabolic demands of AFL game play and that previous suggestions of 10-12 g·kg^{-1} body (for athletes involved in intermittent exercise > 90 minutes) mass may not be necessary for this population. Further studies are now required to quantify the inter-individual variability of glycogen use as well as examine any potential metabolic and ergogenic effects of CHO feedings during match play. Finally, we further demonstrate that the use of the muscle biopsy technique for glycogen assessment

remains the most accurate and valid method for the quantification of glycogen utlisation in athletic populations.

CHAPTER 8

SYNTHESIS OF FINDINGS

8.1 Synthesis of Findings

This Chapter will present an overview of the experimental findings of this thesis in relation to the original aims and objectives outlined in Chapter 1. A general discussion is then presented where initial discussion focuses on the critical evaluation of the non-invasive assessment of muscle glycogen. Additionally, specific attention is also given to the CHO intake and requirements for team sport athletes. Finally, the Chapter closes by outlining potential directions for future research.

8.2 Achievement of the Aims and Objectives

The overall aim of the present thesis was to examine the reliability and validity of ultrasound technology (via the MuscleSound system) to non-invasively assess muscle glycogen concentration in human skeletal muscle. In this way, it was hoped that such a measurement tool could be used to better inform sport-specific CHO guidelines for invasive team sports. To achieve this aim, a series of laboratory and field-based studies were conducted on recreationally active males and elite male athletes from Rugby League and AFL. An overview of specific objectives is provided below.

Objective 1: To quantify the reliability and reproducibility of the MuscleSound scoring system within and between muscles of the quadriceps (Study 1, Chapter 3).

Using a test-retest scanning protocol, this study examined the reliability of the MuscleSound scoring system within and between muscles of the quadriceps in a sample of 100 participants. Data demonstrate that 50% of the length of the vastus lateralis muscle (non-contracted) was the most suitable anatomical site for which to obtain reproducible MuscleSound scores. As such, this site was chosen for the subsequent experimental chapters in order to assess the validity of the MuscleSound system to non-invasively assess muscle glycogen concentration.

Objective 2: To assess the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen utilisation in elite male Rugby League players during competitive match play (Study 2, Chapter 4).

Data presented in this study demonstrated that although Rugby League match play induces absolute glycogen depletion of 200-250 mmol·kg^{-1} dw within the vastus lateralis muscle, there was no significant differences in MuscleSound scores obtained

before and after match play. As such, these data suggest that MuscleSound is not a valid tool to assess muscle glycogen utilisation during the high-intensity intermittent activity profiles that are inherent to team sports. Given that these data are in contrast to previous validation studies conducted with cycling based protocols, it was suggested that differences in mechanical loads and fluids shifts between exercise protocols may explain the discrepancies between studies.

Objective 3: To assess the validity of the MuscleSound scoring system to non-invasively assess muscle glycogen re-synthesis within 36 hours following glycogen depleting based exercise (Study 3, Chapter 5).

To eliminate the potential confounding effects of acute changes in fluid shifts associated with differing exercise stimuli, it was deemed that an alternative approach to assess the validity of the MuscleSound system was to assess its validity to detect changes in muscle glycogen re-synthesis across a time-scale in which acute changes in fluid shifts have been restored to pre-exercise values. Using a combination of glycogen depleting cycling based exercise followed by 36 h of a high CHO or low CHO diet, we achieved differences in resting muscle glycogen concentration of 250 mmol·kg^{-1} dw. Nonetheless, despite such differences in glycogen concentration, no differences were reported in MuscleSound scores. When considered with the results of Chapter 4, these data collectively suggest that the MuscleSound system is unable to detect differences in muscle glycogen concentration within the physiological range that typically occurs as a result of exercise-induced glycogen utilisation and/or post-exercise muscle glycogen re-synthesis.

Objective 4: To quantify the CHO intakes and muscle glycogen utilisation during match play in elite male AFL players (Study 4 and 5, Chapter 6 and 7).

Given that MuscleSound was not deemed a valid measurement tool, it was recommended that evaluations of the daily CHO and muscle glycogen requirements of team sport athletes should therefore be undertaken using traditional assessments of energy intake and muscle biopsies, respectively. Accordingly, we studied habitual CHO intakes in elite male AFL players across the weekly micro-cycle in Study 4 (Chapter 5). These data demonstrate that elite AFL players practice elements of daily CHO periodisation in accordance with fluctuations in daily training load and proximity to the match day itself. Nonetheless, such data also highlighted that such

players do not consume CHO at the recommended dose to achieve glycogen storage that is likely required to facilitate optimal match day physical performance. In Study 5, we subsequently quantified muscle glycogen utilisation in the vastus lateralis muscle in two elite males during competitive match play. Importantly, players commenced the match after adhering to a CHO loading protocol of 8 $g \cdot kg^{-1}$ and after consuming a pre-match meal of 2 $g \cdot kg^{-1}$. Data demonstrate that such a dietary protocol achieves pre-match muscle glycogen concentration >500 $mmol \cdot kg^{-1}$ dw and that match play can induce an absolute glycogen utilisation >400 $mmol \cdot kg^{-1}$ dw. As such, it appears that the metabolic demands of AFL may be more CHO dependent than other invasive team sports such as soccer and rugby.

8.3 General Discussion of the Findings

8.3.1 Overview of the MuscleSound Ultrasound Technique for the Assessment of Muscle Glycogen

Carbohydrate availability has been recognized as a key contributor to exercise performance since the pioneering work of Christensen and Hansen in the late 1930's and the muscle biopsy studies of Bergstrom and colleagues in the late 1960s. More specifically, an understanding of the mechanisms by which muscle glycogen availability improves exercise performance has been established (Bergstrom et al., 1967, Bergstrom et al., 1972, Coyle et al., 1986, Sherman et al., 1981) through the muscle biopsy technique. Due to the invasive nature of biopsies, the development of the non-invasive ultrasound technique (Hill & Milan, 2014) was particularly attractive for practitioners working at the coalface of elite sport. The rationale for this approach is based on the premise that the measurement of the pixel intensity (greyscale) of a muscle through the association of water with the glycogen granule can generate a 'glycogen score'. As such, the MuscleSound software assumes that a lighter muscle image is indicative of less muscle fluid and hence, a lower absolute glycogen concentration.

Initial research studies proved promising and highlighted a positive relationship between exercise-induced changes in MuscleSound scoring system and muscle glycogen obtained from both the rectus femroris and vastus lateralis muscles.

(Nieman et al., 2015; Hill & Millan, 2014). In Chapter 3 of this thesis, we examined the reproducibility and reliability of the MuscleSound scoring system within and between muscles of the quadriceps. We assessed the test re-test capabilities on one hundred subjects who refrained from exercise, fluid or food ingestion. Our findings demonstrate that 50% of the muscle length of the vastus lateralis was the anatomical site whereby the MuscleSound software produced the most reliable and reproducible results. Additionally, we identified the vastus medialis and rectus femoris to be those sites with poor test re-test scores. As such, these data question the use of rectus femoris as the optimal site for which to conduct laboratory-based validation studies.

With the MuscleSound scoring system leading to a potential application to elite sporting athletes, a validation of its use in a 'real world' setting was absent from the literature. In Chapter 4 of this thesis, we examined the relationship between MuscleSound score and the corresponding muscle glycogen concentration prior to and following a rugby league game. As expected, competitive rugby league match play elicits an absolute glycogen use within the vastus lateralis muscle of 200-250 mmol·kg^{-1} dw, though no significant changes were evident in MuscleSound scores obtained before and after the game. Importantly, these data therefore question the validity of the MuscleSound system as a measurement tool to assess glycogen use in response to the typical exercise modalities and intermittent activity patterns inherent to team sport athletes.

In order to better understand the methodology behind the measurement of the MuscleSound scoring system, we examined its validity in a controlled laboratory setting in response to post-exercise feeding with a high or low CHO diet. In Chapter 4, we therefore obtained biopsy samples from the vastus lateralis muscle in conditions of both high and low muscle glycogen availability. We observed muscle glycogen values of 531 ± 129 mmol·kg^{-1} dw in the high condition and 252 ± 64 mmol·kg^{-1} dw in the low condition, though the corresponding MuscleSound scores displayed no differences between the HIGH (66 ± 11) or LOW (60 ± 6) condition. Given that the MuscleSound system is based on the premise of making inferences on the basis of muscle water content, we also directly assessed muscle water content and observed no significant differences between the high (265 ± 58 g·kg^{-1} dw or low condition (250 ± 50 g·kg^{-1} dw). As such, the data obtained from the experimental work undertaken

in Chapters 3, 4 and 5, clearly demonstrate that although the MuscleSound system possesses good reproducibility, it is not valid to detect differences in absolute muscle glycogen concentration across the physiological range associated with exercise. For this reason, it is recommended that practitioners working in team sports should focus their efforts in evaluating training load (through both internal and external loading metrics) and energy intake data in order to help formulate sport-specific nutritional guidelines.

8.3.2 Assessment of Training Load and Carbohydrate intakes in AFL players: Implications for Sport-Specific Nutritional Guidelines

8.3.2.1 Player Load During Weekly AFL

Whilst the simultaneous quantification of training load and energy intakes across the weekly microcycle has recently been undertaken in professional soccer (Anderson et al., 2017) and rugby union (Bradley et al., 2015b), at the time of conducting this thesis no such data was apparent in professional AFL players. In order to develop specific daily nutritional strategies, it was pertinent to first understand the periodisation of training load across the weekly microcycle. To this end, the data obtained in Chapter 5 demonstrate a clear periodisation of training load with training sessions conducted on match day -4, -3, -2 and -1 exhibiting fluctuations in load and of course, being significantly less than match play. This is perhaps best evidenced for traditional training load markers such as total distance in both home (7644, 0, 7568, 3588 v 12900 m on match day) and away (6667, 0, 8084, 3603 v 13372) fixture weeks, absolute distances that are similar to that previously reported in elite AFL players (Ritchie et al., 2016, Bartlett et al., 2016). Whilst the distribution of training load across the training week are indicative of periodisation and a tapering of loading in proximity to match play, it is noteworthy that the absolute distance typically completed in training sessions are greater than that occurring in elite soccer and rugby players (Anderson et al., 2016, Anderson et al., 2017, Bradley et al., 2015b). A summary of the typical training load data and session duration between football codes is shown in Table 8.1. When taken together, it could be suggested that the metabolic demands of AFL training and match play are potentially more CHO dependent than soccer and rugby.

Table 8.1 - Summary of typical training load data for soccer, rugby and AFL. [*] indicates high speed running that also includes sprinting.

	Soccer	**Rugby**	**AFL**
Session Duration (minutes)	60 ± 28 Anderson et al., 2017	35.9 ± 16.9 Lovell et al., 2013	59 ± 25 Bartlett et al., 2016
			67 ± 2 Chapter 6
			59 ± 14 Gallo et al., 2015
Training Load			
Total Distance (m)	3543 ± 1038 Gaudino et al., 2015	1061 ± 61 Costello et al., 2018	5105 ± 1524 Gallo et al., 2015
	2865 ± 1494 Anderson et al., 2017	2801 ± 1578 Lovell et al., 2013	6389 ± 3315 Bartlett et al., 2016
			7826 ± 467 Chapter 6
Running (m) 14.4-19.7 km.h^{-1}	171 ± 122 Anderson et al., 2017		993 ± 226 Chapter 6
High Speed Running (m) 19.8-25.1 km.h^{-1}	426 ± 218[*] Gaudino et al., 2015	116 ± 31 Costello et al., 2018	586 ± 163 Chapter 6
	27 ± 25 Anderson et al., 2017	392 ± 316[*] Lovell et al., 2013	1631 ± 1222[*] Bartlett et al., 2016
			933 ± 367[*] Gallo et al., 2015
Sprinting (m) >25.1 km.h^{-1}	2 ± 4 Anderson et al., 2017		73 ± 46 Chapter 6
	10 ± 6 Gaudino et al., 2015		

8.3.2.2 Carbohydrate Intake in AFL

Carbohydrate ingestion for team sports is a key component of performance to ensure that players are appropriately fueled for training and games in order to maximise their capabilities to perform technical and cognitive skills (Ali & Williams, 2009; Russell & Kingsley, 2014). Given that MuscleSound proved invalid, in order to assess the current nutritional practices of AFL players the traditional method of dietary assessment was utilized in Chapter 6. Using a combination of dietary recalls, food diaries and the remote food photographic method, we estimated CHO intake across two weekly microcycles consisting of both a home and away game. In accordance with fluctuations in daily training load, we also observed that players appear to adjust their daily CHO intake such that absolute CHO intakes are lower on those days with reduced training load and are highest on those days with increased training load or on the day before the match whereby preparation for match play is the goal. However, it is noteworthy that the absolute daily CHO intakes across the 5 days prior

to match play are still considered low to moderate whereby < 3-4 g·kg^{-1} is typically consumed on training days and only 5 g·kg^{-1} on the day prior to the match. Such patterns of CHO periodization (i.e. differences between specific training days) are different to that observed in professional soccer players where specific training days were not different from one another and only match day -1 intakes were significantly different from training days (Anderson et al. 2017). Additionally, the absolute daily CHO intakes also appear lower than that of soccer players and do not appear commensurate with that typically advised for the training loads of team sports (i.e. 3-6 g·kg^{-1}) or to CHO load (6-8 g·kg^{-1}) on the day prior to match play (Thomas et al. 2016). Nonetheless, AFL players do appear to readily meet CHO requirements of the pre-match meal whereby mean intakes of 2.5 g·kg^{-1} were reported on both home and away games. Finally, only 50% of the sample reported achieving CHO intakes during the game within the recommended range of 30-60g per hour. Whilst we acknowledge potential limitations associated with dietary under-reporting and also the fact that these data are obtained from one team only, they also suggest that AFL players potentially under-consume CHO in relation to the metabolic demands of both training and game play.

8.3.2.3 Glycogen Cost of AFL Play

In order to assess the glycogen cost of competitive AFL match play, in Chapter 7 we obtained muscle biopsies from the vastus lateralis muscle before and after match play in two elite male players. On the basis of suboptimal CHO intakes reported in Chapter 6, players completed this match after CHO loading on the day before the match with 8 g·kg^{-1}, consumed 2 g·kg^{-1} in the pre-match meal and also consumed 88 g of CHO during the match itself. Using such best practice protocols, we observed that such CHO loading regimens ensures match play is commenced with absolute glycogen concentration ranging from 550-650 mmol·kg^{-1} dw and that absolute utilization during the match play may be in excess of 400 mmol·kg^{-1} dw in certain individuals. Whilst we acknowledge that this Chapter was a case-study of two players who also differed in their exogenous CHO consumed during match play (e.g. 88 v 0 g), such data suggest that the AFL match play is more CHO dependent than both soccer (Krustrup et al. 2006) and rugby (Bradley et al. 2015). When coupled with the sub-optimal dietary practices reported in Chapter 6, these data suggest that AFL players may benefit from specific dietary coaching on achieving sufficient CHO intakes. A

summary of studies reporting differences in daily CHO intakes in training, match play, exogenous CHO intakes and muscle glycogen utilization is shown in Table 8.2.

Table 8.2 - Summary of CHO intake in training and match play between soccer, rugby and AFL. * indicates unpublished work. NDA indicates no data available

	Soccer	Rugby	AFL
Daily CHO (g·kg^{-1})			
Training Days	4.2 ± 1.4	3.5 ± 0.8	2.4 ± 0.8
	Anderson et al., 2017	Bradley et al., 2015a	Jenner et al., 2018
	2.9 ± 1.1	3.6 ± 2.4	2.1 ± 0.6
	Devlin et al., 2017	Burrows et al., 2016	Chapter 6
			4.5 ± 1.1
			Burke and Read, 1988[*]
			4.7 ± 1.4
			Wray et al., 1994[*]
			5.9 ± 1.2
			Graham et al., 1998[*]
			4.8 ± 0.2
			Schokman et al., 1999[*]
			4.1 ± 1.6
			Bilsborough et al., 2016
			4.6 ± 1.5
			Devlin et al., 2017
Match Day -1	4.2 ± 1.8	5.1 ± 1.5	4.6 ± 1.2
	Anderson et al., 2017	Bradley et al., 2015a	Chapter 6
Match Day	6.4 ± 1.4	NDA	NDA
	Anderson et al., 2017		
CHO Feeding During (g·h^{-1})			
Training Days	3.1 ± 4.4	NDA	0.0 ± 0.8
	Anderson et al., 2017		Chapter 6
Match Day	32.3 ± 21.9	NDA	33.5 ± 11
	Anderson et al., 2017		Chapter 6
Glycogen Utilisation (mmol·kg^{-1} dw)			
Training Session	NDA	NDA	NDA
Match Days	260 ± 26	210 ± 62	280 ± 140
	Krustrup et al., 2006	Bradley et al., 2016	Chapter 7

8.4 Limitations

Chapter 4: Although this chapter demonstrates the use of the MuscleSound software pre and post a rugby league game, intravenous blood sampling would have allowed for a more detailed measurement of CHO utlisation alongside plasma markers of muscle damage. In addition, the assessment of urine osmolality to monitor hydration

status would have given further context to the findings, potentially demonstrating differences in extracellular fluid shifts.

Chapter 5: While this chapter holds methodological rigor for the assessment of the MuscleSound scoring system and muscle biopsy sampling in a rested muscle, the exercise modality did not target optimal muscle fibre recruitment. Although the *vastus lateralis* muscle was highlighted as the most reproducible anatomical location for MuscleSound assessment, running type exercise has been shown to recruit the gastrocnemius to a greater level (Morton et al. 2009). As such, we acknowledge that a running based exercise protocol would have replicated Chapter 4 more closely. Further, urine osmolality assessment would have given further evidence as to hydration status during the MuscleSound and muscle biopsy sampling.

Chapter 6: Here we present a study design that allows for the comparison of weekly external loads and concurrent CHO intakes. Although this chapter demonstrates the use of food diary and 24-hour food recall, we acknowledge the limitations of this method. In order to attempt to eliminate the issues that are associated with food diary analysis the remote food photography method RFPM was initiated having been previously shown to have acceptable levels of validity (Martin et al. 2009).

Chapter 7: The principle limitation to this chapter is the subject number, with only two subjects of the same playing position being recruited for this case study. With a n=2 we are not able to suggest that the findings here are relatable to a whole AFL squad or different playing positions.

8.5 Recommendations for Future Research

Much of our current understanding of team sport nutrition is based on 50 years of research predominantly focused on endurance sports (Burke et al. 2018). It was hoped that the use of non-invasive ultrasound technology to assess muscle glycogen utilisation would allow practitioners to better understand the CHO requirements of competition and specific training sessions. However, given that the MuscleSound software proved to be invalid, it has been identified that the CHO requirements of team sport athletes would have to be identified via traditional practices of dietary assessment and muscle biopsies. To this end, the areas of future research identified

below will still focus on the general theme of quantifying the CHO cost of habitual activities completed by team sport athletes:

1. **Direct assessment of muscle glycogen utilisation during competitive match play of elite soccer, rugby and AFL players in males and females.**

   Despite several published reports (Krustrup et al. 2006, Bradley et al. 2016; Chapter 4, 6), there is a definitive need to better understand the glycogen requirement of match play within specific sports using larger sample sizes and multiple games at relevant points during the season. Glycogen utilisation could also be quantified within specific intracellular pools using transmission electron microscopy. Additionally, given that running is the exercise modality of these sports, glycogen use should also be quantified in the gastrocnemius muscle as opposed to the vastus lateralis muscle given the greater recruitment patterns of the former muscle (Morton et al. 2009). Finally, such studies should also be completed under conditions of high and low CHO availability (from both CHO loading and exogenous CHO feeding) so as to assess the effects of CHO availability on glycogen storage, metabolism and subsequent performance effects.

2. **Direct assessment of muscle glycogen utilisation during training sessions.**

   In addition to the CHO requirements of match play, there is also the requirement to quantify the glycogen cost associated with the typical training sessions completed by the above athletes. An understanding of such metabolic demands would help inform the daily CHO requirements for team sport athletes over and above that which is estimated on the basis of dietary recall studies.

3. **Quantification of muscle glycogen availability and utilisation across the weekly micro-cycle where muscle biopsies are obtained on multiple days to examine the effects of players' habitual CHO intake on glycogen availability leading into training and the subsequent game.**

Given that team sport athletes typically train 4-5 times per week, quantification of glycogen availability at multiple time-points across the weekly microcycle would allow practitioners to better formulate guidelines to ensure sufficient CHO availability is achieved for specific training sessions. Such studies could also be completed in microcycles consisting of high and low CHO diets so as to also examine the effects of daily CHO availability on indices of physical performance that occur during training. Indeed, given that the training loads of team sports athletes are not near that of endurance athletes, it is possible that low daily CHO intakes (e.g. < 3-4 $g \cdot kg^{-1}$) do not manifest as reductions in training intensity.

4. **Examination of the effects of high and low CHO availability on molecular regulators of training adaptation.**

   Given the development of the train-low paradigm whereby endurance training adaptations can be modulated by low CHO availability (Impey et al. 2018), there is also a requirement to assess if such modulation of training adaptation can translate to team sport athletes. As a related question to point 3 and 4, the effects of high and low daily CHO availability (as well as muscle glycogen) on molecular regulators and markers of training adaptation could also be quantified in response to an acute training session or short-term period of training.

In summary, the work undertaken in this thesis has demonstrated that the MuscleSound software is not valid to detect differences in muscle glycogen concentration within the physiological range (i.e. 200-300 $mmol \cdot kg^{-1}$ dw) that typically occurs as a result of exercise-induced glycogen utilisation and/or post-exercise muscle glycogen re-synthesis. For this reason, assessment of the CHO requirements of team sport athletes is likely dependent on the traditional approaches of dietary assessment and muscle biopsy. Using the latter methods, the present data also demonstrate that the CHO requirements of AFL match play is potentially different (though larger sample sizes are required) than other invasive team sports such as soccer and rugby. Further studies are also required to quantify the glycogen requirement of the typical training sessions undertaken by professional players of

specific team sports. Such studies would lead to a greater theoretical understanding and development of sport-specific nutritional periodisation strategies.

CHAPTER 9
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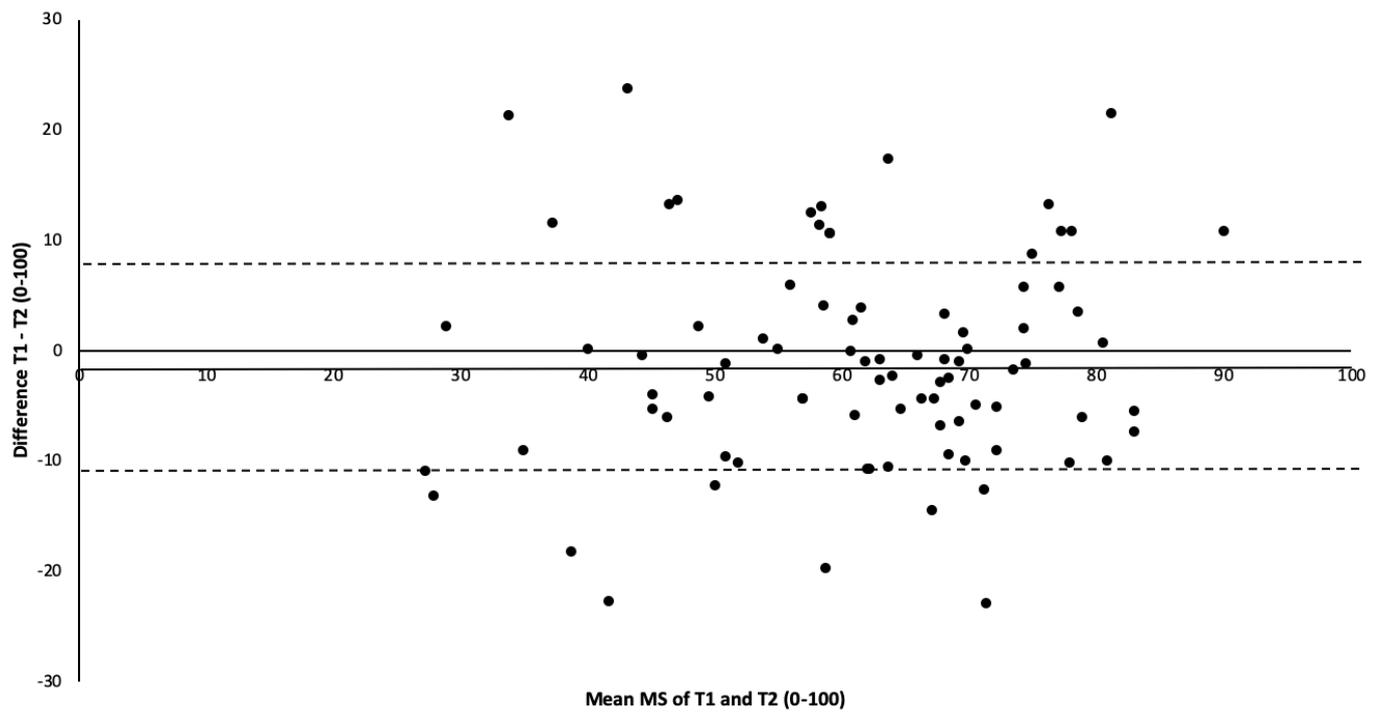
APPENDICES

Appendix 1 -Bland Altman Plot
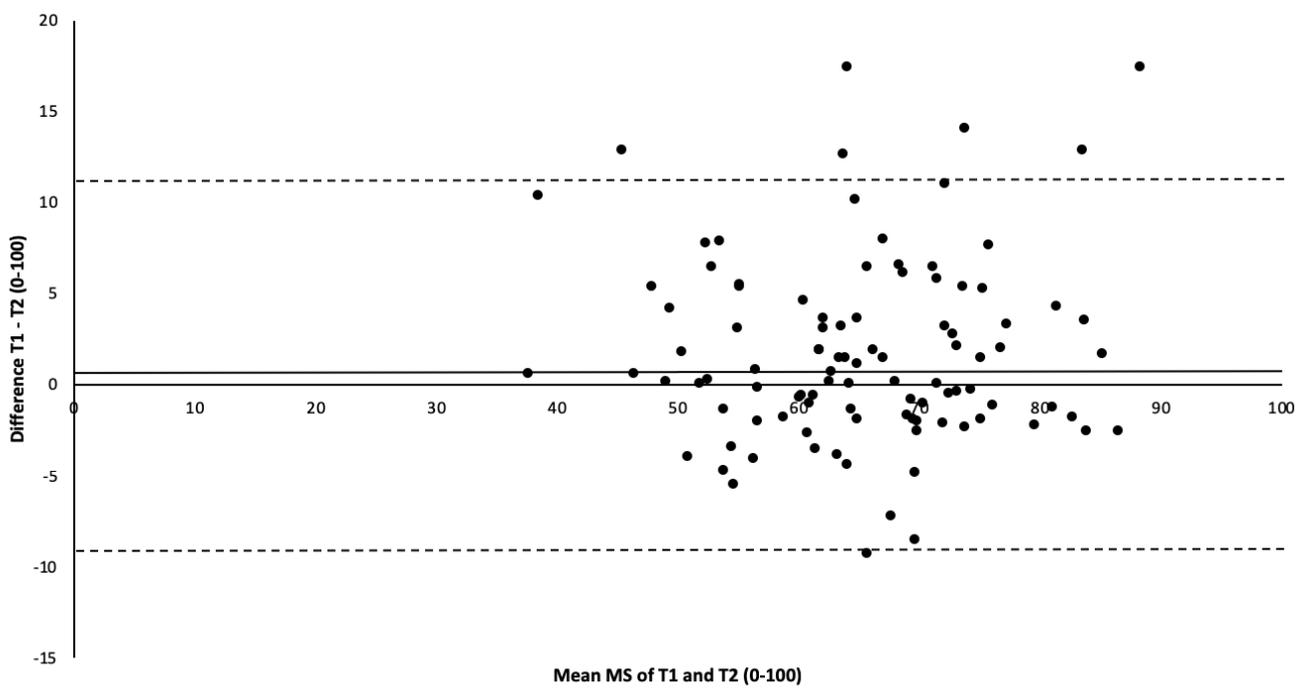
1.1 Bland Altman Plot at 50% VL.
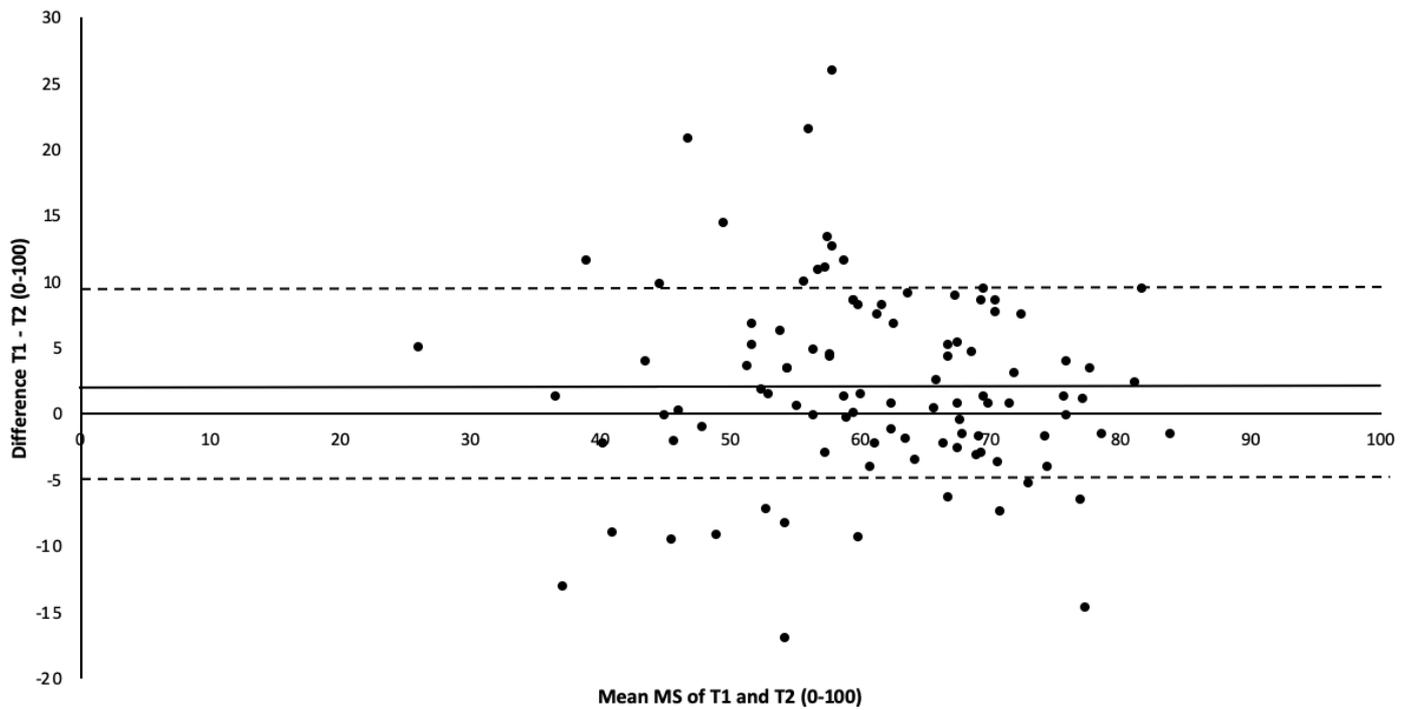
1.2 Bland Altman Plot at 50% RF

1.3 Bland Altman Plot at 50% VM

Food Diary

Personal Details	
Name	
Weight (kg)	
Height (cm)	
Age	
Playing Position	
Round & Opposition	

Portion Sizes & Macro Nutrient Content

Pasta Shells	Pasta Shells	Pasta Shells
Weight 88g	Weight 148g	Weight 205g
Servings 1	Servings 1	Servings 1

88g White Pasta	148g White Pasta	205g White Pasta
30g CHO	50g CHO	70g CHO
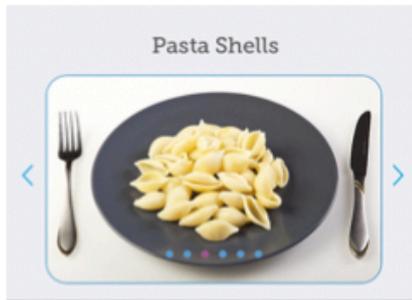
Pasta Shells	Basmati Rice	Basmati Rice
Weight 265g	Weight 32g	Weight 96g
Servings 1	Servings 1	Servings 1

205g White Pasta	32g White Rice	96g White Rice
90g CHO	10g CHO	30g CHO

Basmati Rice	Basmati Rice	Basmati Rice
Weight 163g	Weight 225g	Weight 290g
Servings 1	Servings 1	Servings 1

163g White Rice	225g White Rice	290g White Rice
51g CHO	71g CHO	91g CHO
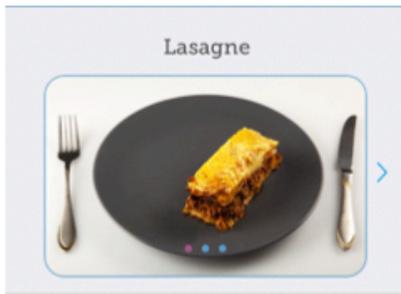
Lasagne

| Weight | 165g |
| Servings | 1 |

165g Lasagne

25g CHO

Lasagne

| Weight | 330g |
| Servings | 1 |

330g Lasagne

50g CHO

Lasagne

| Weight | 500g |
| Servings | 1 |

500g Lasagne

76g CHO

Wedges (baked)

| Weight | 110g |
| Servings | 1 |

110g Wedges

34g CHO
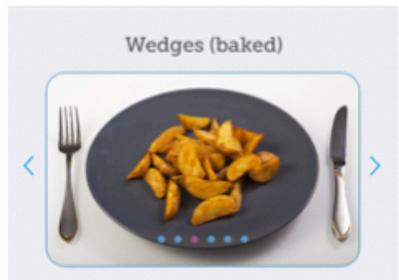
Wedges (baked)

| Weight | 165g |
| Servings | 1 |

165g Wedges

50g CHO

Wedges (baked)

| Weight | 220g |
| Servings | 1 |

220g Wedges

67g CHO

Gnocchi

| Weight | 160g |
| Servings | 1 |

160g Gnocchi

52g CHO

Gnocchi

| Weight | 240g |
| Servings | 1 |

240g Gnocchi

77g CHO
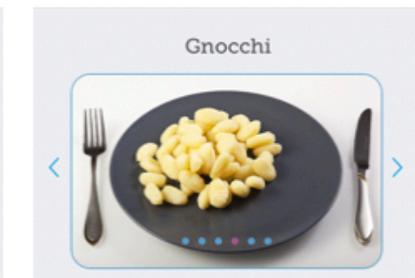
Gnocchi

| Weight | 322g |
| Servings | 1 |

322g Gnocchi

104g CHO

Date:

Weight (kg):

☐ Game Day - 1 ☐ Game Day ☐ Game Day + 1

(please tick)

Time	Food / Brand	Cooking Method (boiled, grilled, fried etc)		Portion Size (weight, volume, household measurement or estimation)
7.30am	Porridge Oats - Quaker			80g
	Semi-skimmed milk			200ml
	Wholemeal bread - Tesco	Toasted		1 slice
	Eggs	Poached		2 medium
	Apple Juice - Tesco			1 large glass
10.00am	Water	**EXAMPLE ONLY**		1 large glass
	Banana			1 large
12.30pm	Chicken Fillet	Grilled		1 medium
	Sweet Potato	Baked		1 large
	Mixed Veg	Boiled		1 handful
	Apple Juice - Tesco			1 large glass
3.30pm	Myprotein Whey Protein			1 scoop (30g)
	Sem-skimmed milk			200ml