Fuel for the damage induced: The metabolic requirements of recovery in elite rugby union match play

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy.

November 2022

Abstract

The physical movement demands of rugby union have been well researched. Although the position specific skills are diverse, there are commonalities in the desirable physical attributes. Athletes possessing high amounts of functional lean mass and relatively low levels of fat mass allow actions to be performed at speed with power and repeatability. Whilst nutrition interventions to promote preparedness to perform have been investigated there is relatively little known about the metabolic requirements of recovery from match play. Resting metabolism can now be determined via indirect calorimetry under strict outpatient conditions. The appreciation of the ability to capture all mechanistic biological pathways with the metabolome and the amplification of these processes by exercise makes it an exciting method to further our understanding of the metabolic demands of elite rugby union. Finally, the intense competitive season which can last for as long as nine months of the year places unprecedented pressure on athletes to recover and perform every week. The impact of these repeated events has not been studied with reference to the ability to maintain optimal physical profiles throughout a season. Therefore the overall aim of this thesis was to determine the metabolic requirements of recovery after elite rugby union match play which will ultimately contribute to the generation of improved recovery strategies.

The aim of the first study (Chapter 4) was to investigate whether exposure to elite rugby union training and match play alters resting metabolism on a day-to-day basis throughout a competitive match week. Indirect calorimetry was performed each morning of the competitive game week, in a fasted, rested state with 22 elite rugby union players. Internal and external training loads were monitored and recorded for all sessions and video analysis was used to record contacts throughout rugby match play. Mean (SD) resting metabolic rate (RMR) increased significantly ($p=0.005$) the morning after match play (231± 302 kcal). There were also significant changes to mean respiratory exchange ratio (RER) after match play at GD+2 ($p=0.030$) and GD+3 ($p=0.006$) due to a significant increase in carbohydrate oxidation at rest at GD+2 (0.22± 0.13g·min^{-1}) ($p=0.044$) and GD+3 (0.23± 0.13g·min^{-1}) ($p=0.003$) compared with GD-1 (0.16± 0.12g·min^{-1}) in the whole group. The monitoring of training and match loads revealed that comparable running and load metrics were experienced on training days and match days but that full collisions were not included in training. This led to the conclusion that the changes in metabolism at rest were due to the collisions experienced in match play rather than running based metabolic demands.

Having established the increased metabolic demands due to rugby union match play, the aim of the second study (Chapters 5 & 6) was to investigate the metabolic perturbations acutely and throughout recovery. Acute comparisons were made between samples collected on the day before match play and immediately post game, and the recovery comparisons were made between these same pre-match (GD-1) samples and the days

following match-play. Initially, a whole body, systems-based approach of untargeted metabolomics of serum was utilised (Chapter 5). Sample collection, processing, and statistical analyses were performed in accordance with best practice set out by the metabolomics standards initiative for studies employing 700 MHz NMR spectroscopy. The results demonstrated the acute energy needs of this high intensity sport are met primarily via glycolysis, the TCA cycle and gluconeogenesis, evidenced by significant increases in serum citrate (p= 0.032) and alanine (p= 0.017) immediately post-match with significantly ranked pathways of glucose-alanine cycle (p= 0.0019), glycolysis (p=0.005), and BCAA degradation (p= 0.030). The recovery period after cessation of match play and prior to training recommencing revealed a re-entry to gluconeogenesis denoted by a significant increase in serum alanine at GD+2 (p= 0.019) and the glucose-alanine cycle highly ranked (p=0.0005) coupled with pathways of oxidative stress such as glutathione metabolism (p= 0.031), glycine-serine metabolism (p=0.042), and alterations to fatty acid metabolism. The serum metabolome implicated the need for the increased metabolic demand to be met with an increase in carbohydrate intake to appease gluconeogenesis. Further investigation was required though to further understand what the impact of the increased amino acid degradation may be and to support the findings of the serum metabolome.

The second metabolomics study (Chapter 6) employed the same untargeted methodology but in less invasive biofluids; urine and saliva. Pathways most highly ranked in saliva were aligned with findings from the serum metabolome. The glucose-alanine cycle (p= 0.008) and gluconeogenesis (p= 0.028), together with pathways of amino acid degradation (arginine & proline metabolism p= 0.042, methionine metabolism p= 0.015) were identified throughout the recovery days. The metabolism of tryptophan via the kynurenine pathway was identified in urine samples as being significantly active, both acutely (p= 0.005) and in recovery (p= 0.035), providing further evidence for the acute high energy demands of the sport and in recovery. Markers of muscle protein and connective tissue breakdown were identified as increased in the days following competitive match play. We proposed these congruent findings support the concept of upregulated gluconeogenesis in the recovery period, due to the increased requirement of carbohydrate. If not provided, via dietary intake, then the degradation of amino acids is upregulated and these may be derived from muscle protein and connective tissue due to the collision-based activities the athletes are exposed to. These multi-biofluid data therefore suggest that the increased metabolic demand reported in Chapter 4 in the days post elite rugby union match play need to be met via an increase in dietary carbohydrate intake.

Having evidenced an increased metabolic demand in recovery from match play and gained novel insight as to the metabolic pathways responsible in meeting these increased demands, we sought to answer what the impact of players potentially not meeting increased carbohydrate requirements repeatedly over the course of a whole season and how this might impact body composition (Chapter 7). The aim being to use this data to estimate longitudinal energy balance and relate this to any changes in composition with other factors of training and match exposure. Forty-six premiership rugby union players underwent DXA scanning at the start, mid-point, and end of the competitive season. Over the season from start-end there were significant increases in body mass 1.38± 2.28kg

(p=0.0004)) and fat mass 1.26± 1.56kg (p=0.0001), but no significant reduction in lean mass 0.16± 1.77kg (p=0.57) in the whole group. There was, however, a loss of lean mass 0.72± 1.55kg (p=0.0055) start-mid, followed by an increase in lean mass 0.88± 1.40kg (p=0.0003) from mid-end. The forwards positional group experienced this pattern of significant variance in lean mass across the season (p=0.0042) whilst the backs saw no significant changes (p=0.2427). The loss of lean mass in the first part of the season which was typified by continuous intense weekly match play, occurred despite an estimated mean energy surplus. The gains in fat mass experienced over the season were significantly correlated with higher match exposure r= 0.6245 (p= 0.0014). This surprising finding that lean mass loss occurred despite a mean energy surplus whilst resistance training was undertaken throughout, may be because of the increased metabolic requirements in recovery. If, as we have proposed the increased demands for carbohydrate in recovery at GD+1 is not met, and the degradation of amino acids results in muscle protein breakdown acutely then a loss of lean mass can be explained. However, the concurrent mean energy surplus and gains in fat mass may be due to a desensitisation of the muscle to anabolism (termed anabolic resistance) in recovery explained by the inflammation and immunoendocrine response due to muscle damage from the collisions in match play. Further work is needed to investigate whether anabolic resistance is present in recovery and if so are there any nutritional strategies that can help overcome this.

In summary, the data presented in this thesis provide novel insights as to the increased metabolic requirements of recovery from elite rugby union match play. We propose the collisions inherent with the sport of rugby union are responsible for the significant increases in resting metabolic rate in recovery together with shifts in substrate oxidation. When investigated utilising a whole body, systems-based approach the upregulation of pathways pertaining to gluconeogenesis, amino acid degradation, and oxidative stress were revealed. The ramifications of which may compromise the integrity of muscle protein and connective tissues. The accumulation of these bouts of match play and recovery, repeated throughout a season, challenge the maintenance of optimal body compositions even when energy needs appear to be met. Nutrition interventions to shift the status quo and increase carbohydrate intakes in the day after competition may provide the necessary substrate for these metabolic requirements to be met. Indeed, elite rugby players should now ensure they are not only “fuelling for the work required” but also remember to “fuel for the damage induced”!

Declaration

I declare that the work in this thesis, which I submit for assessment on the programme of study leading to the award of Doctor of Philosophy was caried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and guidance acknowledged, the work within is entirely my own. In addition, all attempts have been made to ensure the work is original and does not, to the best of my knowledge, breach any copyright laws and has not been taken form the work of others, apart from the works that have been fully acknowledged within the text.

Publications & Presentations

Publications of the work within this thesis are as follows:

1. Hudson, J. F., M. Cole, J. P. Morton, C. E. Stewart and G. L. Close (2020). "Daily Changes of Resting Metabolic Rate in Elite Rugby Union Players." Med Sci Sports Exerc **52**(3): 637-644.
2. Hudson, J. F., M. M. Phelan, D. J. Owens, J. P. Morton, G. L. Close and C. E. Stewart (2021). ""Fuel for the Damage Induced": Untargeted Metabolomics in Elite Rugby Union Match Play." Metabolites **11**(8).

Presentation of the work within this thesis are as follows:

1. Hudson, JF. (2020) “Morning Metabolic Rate” Measuring RMR in the applied world. Invited oral presentation at the Trilogy sports nutrition conference, online presentation, August 4th, 2020.
2. Hudson, J. F., M. M. Phelan, D. J. Owens, J. P. Morton, G. L. Close and C. E. Stewart (2021). ""Fuel for the Damage Induced": Untargeted Metabolomics in Elite Rugby Union Match Play. Oral presentation recorded and available online as part of the European College of Sport Sciences (ECSS) 26th annual congress, Online, September 2021.

Acknowledgements

Getting to play professional sport will always feel like a happy coincidence. It was an incredible privilege to play the sport I love for 15 years. When my career as an athlete came to an end, I knew what I wanted to "be", but I needed a new goal to go after. Part of being able to play sport for so long I felt, was the balance that continuing my education gave me and this felt like the logical pursuit. It has been anything but easy and I am indebted to those who have made it possible.

I didn't know Professor Graeme Close personally, only his ever-growing body of work in Rugby before hobbling over at the ISENC conference in 2015 to have a chat. Starring down the barrel of retirement, when he asked the question, "What are you going to do…" was met with "I would love to do my PhD, is that something we could speak about please". He has probably regretted the swift positive response he gave me, but that demonstrates his generosity and kindness with his time and energy. I have learnt so much in his approach and application of research in sport. I am so grateful for his advice and positivity throughout this process.

As my director of studies Professor Claire Stewart has been incredible. A constant provider of support and knowledge. Her attention to detail and consistent high standards will continue to shape how I approach research questions evermore. Her calm attitude and resilience have certainly set an example to help navigate the last five years.

Finally on a supervisory front I would like to thank Professor James Morton. James has challenged the way I think, and most crucially asked the hard questions when needed the most. The measured way he formulates a reply has taught me many lessons.

I am incredibly grateful to all of you and hope to carry on answering some of the many questions left, over the years to come.

Although they are not officially supervisors there are two more people I must thank. Firstly Dr. Matt Cole at Birmingham City University who somehow got us permission to house the DXA scanner and metabolic cart at the training ground to pursue the first study of this thesis and making the access to the DXA possible for the final study. You have always been there to discuss ideas and give me an opinion I hugely respect. Also, to Dr. Marie Phelan at the University of Liverpool Metabolomics research facility. Thank you for your patience trying to teach a 'mature' student to write code and analyse reams of data in 'R' and for being a sounding board over many lockdown zoom catchups to discuss my findings.

I would like to acknowledge the financial support Gloucester Rugby Club provided but also the access to allow this research to be carried out. To all the staff in the performance and medical department, you make every day a joy. Challenging each other to deliver the best support for the players is a great environment to be part of. I must also say a huge thanks to our Head of performance at Gloucester Rugby, Dan Tobin. I am indebted to you for granting me the chance to be part of your team when I first retired. I have always and will continue to try and repay that. You set a phenomenal example of how to be a scientist but also a brilliant, applied practitioner.

The key members of all this research are of course the players, without whom this would not have been possible. To all who participated, I cannot thank you enough for taking an interest but also being generous with your time, and bodies! I just hope you can see and feel the impact of how the research has helped me try and deliver the best support for you as a group.

To my best mate and wife, Siobhan, thank you for your support, laughter, and the needed energy to keep going and get this project finished. It is undoubtedly a selfish pursuit, but I don't think either of us would be happy without taking on career challenges. Together the "day-to-day" and "special projects" will keep getting completed and continue to have a lot of fun as a family.

We often both reflect on how lucky we are with the parents we were blessed with. To Mum and Dad, thank you for the love, energy, and belief that I can succeed. The drive to not be "Mr. Average" is still there and the example you both set me in work ethic will stay with me forever. To Dougie P and P Niddy thank you for your support and kindness. Your willingness to step in and take on lockdown home schooling saved the day and all our sanities whilst making it an enjoyable few months.

Lastly to our boys, Barnaby, and Wilbur you are brilliant, passionate, beautiful souls. Through broken limbs, home schooling, curries, and cornettoes you never cease to make me smile. I hope through all the time Dad has been working on the 'thesis-aurus' I have never not played football, cricket, swam, read with you each day. We are so very proud of you both.

Ultimately, I hope this body of work will positively influence applied practice and the support practitioners can offer these athletes and allow them to recover from this exhausting, exhilarating, skilful and physical sport.

Abbreviations

$\dot{V}CO_2$ – carbon dioxide production

$\dot{V}O_2$ – oxygen consumption

^1H – protium, the lightest and most common isotope of hydrogen

3-HK – 3-hydroxykynurenine

AEE – activity energy expenditure

AFL – Australian football league

ANOVA – analysis of variance

AU – arbitrary unit

BCAA – branched chain amino acids

BM – body mass

BMR – basal metabolic rate

CK – creatine kinase

CO_2 – carbon dioxide

CPMG - Carr-Purcell-Meiboom-Gill

CRP – c-reactive protein

CV – coefficient of variation

Da – Dalton (unit of mass)

DLW – doubly labelled water

DOMS – delayed onset muscle soreness

DXA - dual-energy X-ray absorptiometry

EE – energy expenditure

EIMD – exercise induced muscle damage

EPOC – post exercise oxygen consumption

FDR – false discovery rate

FFM – fat free mass

FID – free induction decay

GC-MS - gas chromatography coupled with mass spectrometry

GD – gameday

GPS – global positioning systems

HIT – high intensity training

HIIT – high intensity interval training

hr – hour

Hz – hertz (a unit of frequency)

IDO – indolamine 2,3-dioxygenase

IIMD – impact induced muscle damage

IL – interleukin

KA – kynurenate

KATs – kynurenine aminotransferase

kcal – kilocalorie

kg – kilogram

km – kilometre

KMO – kynurenine 3-monooxygenase

KYN – kynurenine

LC-MS - liquid chromatography coupled with single stage mass spectrometry

m – metres

Mb – myoglobin

min – minute

MOD – moderate intensity training

MS – mass spectrometry

MSI - Metabolites Standard Initiative

NAD+ - oxidized form of nicotinamide adenine aminotransferase

NEAT -non-exercise activity thermogenesis

NEFA – non-esterified fatty acids

NFL – national football league

NMR - nuclear magnetic resonance

NOE - Nuclear Overhauser effect

O_2 – oxygen

PCA - principal component analysis

PGC-1α - proliferator-activated receptor-gamma coactivator-1alpha

PLS-DA - partial least squares discriminatory analysis

PQN – probabilistic quotient normalisation

QA – quinolate

RBE – repeated bout effect

RER – respiratory exchange ratio

RF – random forests

RFPM - remote food photographic method

RHIE – repeated high intensity efforts

RL – rugby league

RMR – resting metabolic rate

ROC – reporter operating characteristics

RPE – rating of perceived exertion

RQ – respiratory quotient

RU – rugby union

TCA – tricarboxylic acid cycle

TCI – Bruker brand triple resonance probe for NMR spectroscopy

TDO – tryptophan 2,3-dioxygenase

TEE – total energy expenditure

TEF – thermic effect of food

TEI – total energy intake

TMA – time motion analysis

TRP – tryptophan

TSP - trimethylsilyl propanoic acid

VIP – variable importance projection

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Forwards – ▲ (filled black triangle), ○ Backs- (empty circle).

*Denotes significant difference ($p<0.05$) for the whole group when compared to GD-1.

+Denotes significant difference ($p<0.05$) for the forwards group when compared to GD-1.

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Figure 6.12 Displays the heatmap for the saliva metabolites identified via PLSDA modelling that were attributed to dietary intake or environmental influence causing them to appear in samples. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

Figure 6.13 Displays the heatmap for the urine metabolites identified via PLSDA modelling that were attributed to dietary intake or environmental influence causing them to appear in samples. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

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Chapter 1

General Introduction

The aim of this General Introduction is to provide a brief overview and introduction to the area to provide the rationale for the aims and objectives of this thesis.

1.1 Background

Interest in collision sports has always been great, demonstrated by American football, which is the highest grossing sport worldwide with an estimated $13 billion generated each season. Whilst this is generated by the NFL league alone and played predominantly in North America, Rugby union is a truly global sport played by 9.6 million people across 159 registered unions worldwide(Rugby, 2020). This growing international movement which now comprises over 500 million fans, strengthens the need for further research in this arena (Rugby, 2022).

Collision sports are underpinned by the physical contact between competitors. In combat sports adversaries may score points with successful strikes or contacts, whilst sports such as American football and both rugby codes, also include contacts in which dominance contributes towards success (Gabbett and Ryan, 2009). Team ascendancy in rugby union depends heavily upon the ability to win the gain line of the tackle contest (Tierney et al., 2018) with winning teams completing more tackle events than the opposition (Ortega, Villarejo and Palao, 2009).

Rugby union is a dynamic collision team sport played between two teams of 15 players who contest a match for 80 minutes (Duthie, Pyne and Hooper, 2003). Each team is broadly categorised into forwards (numbers 1-8) and backs (numbers 9-15) (Duthie, Pyne and Hooper, 2003). The sport is comprised of intermittent, high-intensity activities incorporating high-speed running, sprinting, accelerations, and decelerations, as well as collision-based activities at the tackle area (tackle and breakdown contest) and the set piece (scrum and maul) (Roberts et al., 2008; Cunniffe et al., 2009; Austin, Gabbett and Jenkins, 2011a).

The “Fuel the work required” paradigm is well established in endurance sports (Impey et al., 2018) and there is evidence this framework is applied in team sport settings also (Bradley et al., 2015a; Anderson et al., 2017). The fluctuations in training load each day and desired adaptations specific to training sessions is combined with carbohydrate and energy availability to reduce risk of illness, injury, and to promote performance in competition (Burke, Loucks and Broad, 2006; Chamari et al., 2012; Bradley et al., 2016). There is evidence to suggest that applying this knowledge to contact sports requires understanding of the energy requirements arising due to the collisions inherent within the sports (Costello et al., 2018). This may also suggest position specific nutritional requirements, as forwards are exposed to more high level impacts during contact situations than backs (Cunniffe et al., 2009). We cannot capture these daily variations in energy expenditure using doubly labelled water (DLW) and the inability to utilise wearable devices during competition (Bradley et al., 2015a) leaves gaps in our knowledge. Therefore, investigating the major component of total energy expenditure (TEE), resting metabolic rate (RMR), each day may provide information as to how the metabolism of these athletes may change in response to training and competitive match play. This insight has never been provided in any sport, neither laboratory based or in the applied field.

Recent work in rugby has highlighted the importance of carbohydrate feeding in the day before gameday (GD-1) and reaching an intake of 6g/kg body mass appearing beneficial compared with 3g/kg body mass (Bradley et al., 2016). The replenishment of muscle glycogen also appears more effective when carbohydrate is fed immediately post-match play, rather than a delayed intake (Bradley et al., 2017). Muscle damage appears to impair glycogen resynthesis after forced eccentric exercise in the laboratory setting (O'Reilly et al., 1987; Costill et al., 1990). This may explain the importance of immediate carbohydrate

feeding post-match play. However, very little is known about the metabolic requirements in the extended recovery period after contact sports. Differences in training schedules and the load undertaken by the athletes in the days between competitive events will exist, but more information is needed as to how this recovery period is best fuelled as part of a periodised nutrition plan.

Investigating the alterations in metabolite profiles after exercise bouts has revealed alterations in pathways associated with energy production, amino acid metabolism and indicators of oxidative stress (Peake et al., 2014; Berton et al., 2017). Systematically reviewing data from blood, sweat, urine, and salivary metabolomes has identified 196 metabolites as significantly changed within 24hr of bouts of endurance or resistance training (Schranner et al., 2020). The complex and integrated nature of the whole-body exercise response means the use of metabolomics as an unbiased systems approach may be appropriate to fill these critical gaps in our understanding (Hoffman, 2017). If we can understand which metabolic pathways are active during the recovery processes, we may be able to devise dietary interventions to best recover from collision-based team sports and integrate these into the periodised model.

Ensuring energy needs are met throughout the in-season period may be necessary to maintain recovery, health, and optimal body composition, facilitating continual performance throughout a gruelling 9-month long competitive season in the top tier of European domestic rugby (Bradley et al., 2015a). Success in World Cup competitions has been associated with teams possessing heavier forwards and taller backs (Sedeaud et al., 2012; Barr, Newton and Sheppard, 2014). Not only do the basic anthropometry of the athletes appear crucial, but their body composition is important for the desired physical and fitness

characteristics (Posthumus et al., 2020) and the ability to repeatedly perform tasks in competition (Smart et al., 2014). Furthermore, as the level of competition moves from amateur to top level professional, so the physiology changes, with players possessing greater fat-free mass and lower body fat, resulting in faster, stronger and more powerful athletes (Smart, Hopkins and Gill, 2013).

Studies examining TEE using DLW reveal high energy requirements in both codes of rugby athletes (Morehen et al., 2016; Costello et al., 2018; Smith et al., 2018) but concerningly, a large disparity between TEE and total energy intake (TEI) has been observed (Morehen et al., 2016), indicating these athletes do not consistently meet their energy needs. The relationship between longitudinal energy balance has been investigated in military personnel (Fortes et al., 2011; Murphy et al., 2018), and athletic populations (Silva et al., 2017; Bartlett et al., 2019) providing insight as to how training and competitive periods affect energy balance derived from changes in body composition. There is currently no information available in rugby union as to how competitive periods of match play relate to changes in body composition at the elite level. There are also no data available as to the energy balance during extended periods of competition in these collision sport athletes.

1.2 Aims and objectives

The aim of this thesis is to determine the metabolic requirements of recovery in elite rugby union match play. This will be achieved by studying whole body metabolism, the metabolome in multiple biofluids, and body composition changes during the competitive in-season period by completion of the following objectives:

1) Investigate whether exposure to elite rugby union training and match play alters resting metabolism on a day-to-day basis throughout a competitive match week (Chapter 4).
2) Utilising a progressive, whole body, systems-based approach, investigate the metabolic perturbations associated with elite rugby union match play acutely and throughout recovery in serum (Chapter 5), urine, and saliva (Chapter 6).
3) Measure body composition employing dual-energy X-ray absorptiometry (DXA) scanning technology at multiple occasions throughout the in-season period in a population of elite rugby union players (Chapter 7).
4) Utilise this body composition data to estimate longitudinal energy balance in this population and relationships between energy balance and the training and match play demands these athletes are exposed to (Chapter 7).

Chapter 2

Literature Review

The aim of this Literature Review is to introduce the key theoretical concepts and provide a summary and critical appraisal of the relevant available literature.

2.1 Classifications

It is important to recognise the population we are studying within this thesis, the context of the participants within their sport and clarify specific terms.

2.1.1 The population

The demands of the sport of rugby union (RU), as outlined in this chapter, are positionally diverse (Smart et al., 2014; Posthumus et al., 2020) and the population is not as physically homogenous as the alternative code rugby league (RL) (Harley, Hind and O'Hara J, 2011; Morehen et al., 2015). The focus of this thesis is upon men's RU and all participating players are from an English Premiership squad. We will primarily review research carried out in this population. There are large gaps in the knowledge and research specifically for this population, and it is necessary to account for research carried out in other collision-based team sports such as RL, but also laboratory-based studies attempting to investigate the mechanisms of collisions and their effect upon muscle function and damage. Prior research outside of rugby union shall be reviewed to provide a full picture of the current understanding and the context of any other evidence will be provided as both the collisions and specific skills within these other sports are diverse.

2.1.2 Elite Rugby Union

It is crucial we also discuss the relative classification of the participants in this PhD programme of work. The English premiership is one of the four top competitions worldwide, with super rugby, the united rugby championship and the top 14 competition in France. Classification cannot be merely generated via time spent in skill acquisition, where the

proposed 10,000 hours of deliberate practice required to achieve mastery, cannot be applied to all activities (Baker and Young, 2014). Neither can the term “professional” be used without further clarity (McAuley, Baker and Kelly, 2021). These four leagues include international players from across the world and cover all the players involved in tier one nations who participated in the world cup in 2019. The cumulative teams within these four leagues are 54 with approximately 44 players per squad (Premiership, United Championship and Top 14 tend to be higher at 45-50 players whilst super rugby 33-36). Therefore, the top 2,376 players worldwide compete across these competitions equating to 0.00003% of the global population. A recent updated attempt to classify research participants utilised a number of 0.0025% of the global population of 7.9 billion as a threshold for “Elite” athletes (McKay et al., 2022). They did however set the parameters for team sport athletes with some room for debate, collating athletes in the national football league (NFL) (1344 athletes solely in North America), and the Australian football league (AFL) athletes of in the Elite bracket whilst detailing the Super rugby players of Australia as ‘Tier 3 or Highly Trained’. Others who have recently discussed the topic make the point that the country of origin of a sport and the context of popularity must be regarded when making this classification (McAuley, Baker and Kelly, 2021), which likely explains the status given to AFL (Australian rules football) and the NFL (American football) in their supplementary material (McKay et al., 2022). Finally, when accounting for the global participation of the sport, which for rugby union is 9.6 million (Rugby, 2020), those representing Premiership rugby teams are 0.0055% globally, also meeting the criteria set for Elite level (McKay et al., 2022). Considering this framework, we suggest that overall, a Premiership rugby squad should be classified as Elite (Tier 4) with developmental players yet to graduate from the academy system, as Highly trained (Tier 3).

2.1.3 Recovery

Recovery during the in-season or competitive period for team sports involves maintenance of the athletes' physical and mental readiness to perform in the next competition (Heaton et al., 2017). This term of "recovery" can be defined succinctly as the return to homeostasis of physiological systems following metabolic challenges and inflammation associated with muscle damage induced by the exercise bout (Hausswirth and Le Meur, 2011). In collision sport there is evidence rugby athletes are in pain and discomfort throughout the in-season period (Fletcher et al., 2016). It could be suggested therefore that recovery in this population is the readiness of the athlete to meet their previous level of performance in competition. This may not mean a full "recovery" and some residual fatigue, muscle damage, muscle soreness, and inflammation may persist (Dupuy et al., 2018).

To understand what these athletes are recovering from, the demands of the sport and the optimal physical profiles required to compete at the elite level will be reviewed.

2.2 Physical demands of rugby union

2.2.1 Movement demands in match play

The desire to quantify the physical demands of the sport at the elite level evolved quickly after the event of professionalism (Duthie, Pyne and Hooper, 2003). Initially the non-invasive method of time motion analysis (TMA), utilised video analysis by trained researchers to generate information on players movement patterns and time spent at different intensities (Deutsch, Kearney and Rehrer, 2007; Roberts et al., 2008; Austin, Gabbett and Jenkins, 2011a). This information formed a congruent picture of a sport dominated by low intensity activities but interspersed with intermittent, high intensity movements (Austin, Gabbett and Jenkins, 2011b). This time-consuming method was not without its constraints though, as the researchers needed to be able to distinguish between movement patterns and have a detailed understanding of the sport, whilst also making what are essentially subjective decisions on categorising the player's actions (Cunniffe et al., 2009).

The adoption of global positioning systems (GPS) units in elite rugby union is now the data collection tool of choice in the more recent studies of these match demands (Cunniffe et al., 2009; Coughlan et al., 2011; Cahill et al., 2013; Jones et al., 2015; Cousins et al., 2022). The earliest two of these only included two players (one forward and one back) in each study (Cunniffe et al., 2009; Coughlan et al., 2011), but later work has included more participants to allow the stratification of playing positional groups in more depth (Cahill et al., 2013; Jones et al., 2015; Cousins et al., 2022).

The evolution of GPS monitoring should also be considered. Early units were 1 and 5 Hertz (Hz) which have been suggested to lack sensitivity and accuracy in detection of changing

movement patterns in team sport athletes (Coutts and Duffield, 2010; Jennings et al., 2010). More recent research has utilised 10 Hz units (Jones et al., 2015; Cousins et al., 2022) which appear to be more accurate in detecting tasks completed at a range of velocities and at all phases of accelerations and deceleration activities (Varley, Fairweather and Aughey, 2012). Please see table 2.1 for an overview of the information gleaned from these match demand studies.

No matter the methodology or technology utilised in data collection, there are consistent findings across the research with significantly greater total distances reported in the backs positions compared with the forwards (Roberts et al., 2008), and at high intensities (Cunniffe et al., 2009; Jones et al., 2015). The biggest sample group of 120 English Premiership players revealed the backs covered greater total and relative distance (≈6500m and 71.1 $m \cdot min^{-1}$) compared with the forwards (≈5900m and 64.6 $m \cdot min^{-1}$) (Cahill et al., 2013). This is supported with the relative distances reported of 67.3 $m \cdot min^{-1}$ and 60.7 $m \cdot min^{-1}$ (Jones et al., 2015), and 74.3 $m \cdot min^{-1}$ and 66.3 $m \cdot min^{-1}$ (Cousins et al., 2022) in backs and forwards respectively in the most recent work. When it comes to evaluating these data though, it is imperative we remember that most of the early work incorporated specific banding of speed thresholds to determine what is “high speed”. For example, anything above 18$km \cdot hr^{-1}$ was described as high speed with above 20$km \cdot hr^{-1}$ determined to be “sprinting” (Cunniffe et al., 2009; Coughlan et al., 2011). However, recommendations to utilise individual relative speeds have been made (Dwyer and Gabbett, 2012). In the latest work in an English Premiership squad, 70% of the top recorded speed for an individual was used to define ‘high speed’ which may serve as to explain the relative high speed running intensities of 1.9 $m \cdot min^{-1}$ and 0.8 $m \cdot min^{-1}$ for the backs and forwards respectively, being much lower than previous research (Cousins et al., 2022). Due to the variety of sprint

abilities within a rugby union team, a predefined threshold close to an individual's maximum velocity could accentuate differences in relative distances covered at high velocity (Gabbett, 2013). Whilst there is no agreed consensus for specific percentages of an individual's top recorded velocity or of a positional threshold for rugby union at the time of research within this thesis, the descriptors herein are specified and consistent throughout.

The specificity of this literature review is kept to rugby union as there is evidence that although absolute distances covered by rugby union players are greater than rugby league (McLellan, Lovell and Gass, 2011b; Waldron et al., 2011), as much as 9.8 minutes can be spent in static exertions alone which are non-existent in rugby league (Roberts et al., 2008). This also then translates into relative running intensities being much higher in rugby league match play with forwards 95m·min^{-1} and backs 89m·min^{-1} (Waldron et al., 2011).

Whilst the overall distances covered are useful to compare positional demands of the sport, when constructing performance models to cope with the worst-case scenarios players are likely to face and ultimately be successful, it could be considered a crude measure. The actions rather than just overall ambulation, specifically the high-speed running, repeated high intensity efforts, and static exertions with collisions may provide more insight for when the ball is in play (Reardon et al., 2017b; Cunningham et al., 2018; Pollard et al., 2018).

Table 2.1 Rugby Union match play physical demands split into positional groups of backs (Back) and forwards (Fwd).

Study	Total Distance (m)		Relative Total Distance ($m \cdot min^{-1}$)		High Intensity Distance (m)		Relative High Intensity Distance ($m \cdot min^{-1}$)	
	Back	Fwd	Back	Fwd	Back	Fwd	Back	Fwd
(Roberts et al., 2008)	6127	5581			448	298		
(Cunniffe et al., 2009)	7227	6680	71.9	66.7	816	655	8.1	6.5
(Coughlan et al., 2011)	7002	6427	73.7	67.7	637	379	6.7	4.0
(Austin, Gabbett and Jenkins, 2011a)	5434	4962	67.1	56.4	738	524	9.1	6.0
(Cahill et al., 2013)	6545	5850	71.1	64.6				
(Jones et al., 2015)	6029	4995	67.3	60.7	528	225	6.1	2.7
(Cousins et al., 2022)			74.3	66.3			1.9	0.8

2.2.2 Repeated high intensity efforts and static exertions in match play

Repeated high intensity efforts (RHIE) were first measured in a TMA study and was defined as three or more sprints, scrums/rucks/mauls, and/or tackle efforts with less than 21 s recovery between high-intensity efforts (Austin, Gabbett and Jenkins, 2011b). The incorporation of GPS technology evolved this to three of more high acceleration (>2.79 $m \cdot s^{-2}$), high speed (5 $m \cdot s^{-1}$) or contact efforts with less than 21 s recovery between efforts (Gabbett, Jenkins and Abernethy, 2012) from rugby league research, but has transitioned across into later rugby union research (Jones et al., 2015). Investigations into the RHIE demands of RU show forwards perform significantly more bouts than backs (≈11-13 vs. ≈5-7) (Jones et al., 2015), and that these bouts are significantly longer in duration for forwards compared to backs (≈45-52 s vs. ≈26-28 s) (Austin, Gabbett and Jenkins, 2011b). The make-up of these periods is also highly diverse with forwards spending much of these intense periods rucking, mauling and in collision or static exertions and completing much less high intensity running and sprinting compared with backs (Cahill et al., 2013). The backs, spending less time involved in breakdowns and static collision episodes, perform much higher intensity running loads and also experience longer recovery periods during match play (Cahill et al., 2013). When examining the metabolic requirements of recovering from match play the facets of these high intensity periods may provide a greater insight. The composition of a player's high-speed running, sprinting, accelerations, and decelerations may provide more detail of the demands rather than simply the number and repeated nature of high intensity efforts. The challenge of quantifying the demands of collisions is more challenging though.

2.2.3 Collision demands in match play

As we have discussed, the use of both TMA and GPS to equate the physical demands of rugby union has evolved throughout professionalism with GPS now being accessible and readily used at the elite level to monitor training and match play (Cahill et al., 2013; Jones et al., 2015; Cousins et al., 2022). However, the use of GPS technology to measure the collision demands of the sport is fraught with challenges. The collision counts utilising GPS for forwards range from 1274 for a forward and 798 for a single back (Cunniffe et al., 2009) and forwards (≈32-38) compared to backs (≈16-21) in a larger (n=33) sample size (Jones et al., 2015). In the earlier TMA research an average of 89 collisions was recorded to forwards and 24 for backs per game (Roberts et al., 2008). The discrepancies between TMA and GPS methods but also between GPS micro-technology studies may be explained with the frequent non-collision events of running, acceleration, and deceleration involving g forces resulting in recorded collisions by the devices.

Research as to whether a manipulation of these g force thresholds based upon playing positions could improve collision detection using automated micro-technology was unsuccessful (Reardon et al., 2017a). The conclusion being that video analysis remains the more accurate technology for detecting collisions in rugby union (Reardon et al., 2017a).

The commonalities across collision research in rugby union is that forwards undergo a greater number of collisions per match than backs (Roberts et al., 2008; Cunniffe et al., 2009; Cahill et al., 2013; Jones et al., 2015) but it should also be regarded that the less frequent collisions experienced by backs may be at a higher force due to the positional capacity for higher running velocity (Reardon et al., 2017a). We should also understand that

the cumulative force of impact for these collisions cannot be quantified accurately in the same way internal running loads can be.

Any research investigating collisions should record frequency not an estimated load and utilise video analysis rather than micro-technology.

2.2.4 Training demands

As we have discussed, numerous studies have examined the demands of rugby union match play, with several at the elite level (Duthie, Pyne and Hooper, 2003; Duthie, Pyne and Hooper, 2005; Cunniffe et al., 2009; Cahill et al., 2013). The aim of these being to understand the physical demands of match play, but also to aid in the design of training practices (Duthie, Pyne and Hooper, 2005) and rehabilitation processes to prepare players to perform competitively (Coughlan et al., 2011). Despite this knowledge there is relatively little research into the training demands at the elite level.

Hartwig and colleagues observed adolescent rugby union athletes utilising TMA and highlighted disparities between training and match play (Hartwig, Naughton and Searl, 2011). Specifically, that no differences were observed between match play and training for the lower velocity movement zones, but that sprint intensity and distances as described per hour of play or training was significantly lower in training (Hartwig, Naughton and Searl, 2011). Despite a large volume of training (84 sessions) and matches (20) being observed, the group studied likely bore little resemblance to the physical capacities of elite players, nor would the sport science support guide training content to the same degree.

Most recently the best view of training practices were observed across two seasons in an English premiership squad (Cousins et al., 2022). Here backs were witnessed covering greater running distances by an average of 704m per session than forwards but without any

difference between positional groups in terms of perceived exertion per session (Cousins et al., 2022). There were no significant differences in high-speed running between forwards and backs, recorded at 50 ± 110m and 67 ± 88m per session respectively, despite the backs match demands of running and high speed running being 7.6m·min^{-1} greater and 1.22m·min^{-1} higher compared with forwards (Cousins et al., 2022).

A point to note is that when we compare training demands and match play metrics, the use of total distances or actions can be used for comparison (Cousins et al., 2022). However, the use of intensity measured as metres per minute cannot be used for training in its entirety as there may be structured blocks to exceed match metrics and prepare athletes for those worst case scenarios (Reardon et al., 2017b) but also lower movement, skill based periods of training. This may be to prepare for those episodes of static exertions in the scrum or maul, and also explain why the perception of effort in training was as high in forwards in training despite the significantly lower movement demands at high speeds observed compared with backs (Cousins et al., 2022).

Unfortunately, Cousins and colleagues did not investigate any daily variance in these metrics. A conclusion which could be taken, was that training demands are not as high as match demands in this population as average perceived exertion per session and running intensities were lower than match play. There are likely specific sessions of the week containing high speed effort for injury protection (Gabbett, 2016) as well as the development of physical qualities. Had there been a view of the periodisation of demands rather than averaged session content across a week used, there may have been further valuable information available. The authors may have also been presumptuous to declare

there was no prior data available in the literature. The primary focus of the studies may not have been training demands per se, but the information is there to review.

There exist two studies primarily examining nutritional intake of elite rugby union athletes, but they do also provide insight as to the training demands of the observational periods and these are displayed in table 2.2. the training week at the elite level is periodised in intensity and volume of work with the most demanding rugby sessions through the middle of the week with the lowest training load early in the week at GD+2 and the day prior to match play at GD-1 (Bradley et al., 2015a; Posthumus et al., 2021). There is a great difference between the loads and may be due to the studies having been based in the northern (Bradley et al., 2015a) and southern hemispheres (Posthumus et al., 2021). The duration of the competitive seasons does differ greatly with the southern hemisphere Super rugby competition being shorter with fewer total matches compared with the northern hemisphere domestic and European competitions which may go some way to explain the more than double running distance covered of ≈17km vs. ≈8km in the training weeks. The specific running intensities are not described sufficiently to make comparisons, but the perceived load also differed greatly at ≈3670 AU compared to 1776 AU (Bradley et al., 2015a; Posthumus et al., 2021). The perceived load of three training days was greater than the load for the match in the southern hemisphere group with one day containing comparable running distance (Posthumus et al., 2021). When recovery between fixtures and preparation across the training week are considered, any novel research needs to investigate the specific running volume and intensity of training as well as match play to understand and consider accurately the total demands on these athletes.

Table 2.2 Periodised training week content and session metrics for elite rugby union athletes available from the literature. Days are displayed relative to gameday (GD) using the +/- symbol

Study	Group	Content	GD+2	GD+3	GD+4	GD-2	GD-1	GD	GD+1	Total
(Posthumus et al., 2021)	All Players	Intensity	Low	High	Rest & recovery	High	Low	High	Rest & recovery	
		Field Sessions	1	1		2	1	Match		5
		Gym Sessions	1	1		1	0	0		3
		sRPE (AU)	784 ± 123	1277 ± 108		1367 ± 90	242 ± 42	716 ± 94		4386 ± 457
		Distance (km)	2.4 ± 1.0	6.7 ± 0.8		5.1 ± 0.7	2.7 ± 0.5	6.1 ± 0.7		23.0 ± 3.7
	Forwards	sRPE (AU)	781 ± 118	1266 ± 114		1377 ± 97	238 ± 41	695 ± 99		4357 ± 469
		Distance (km)	2.1 ± 0.8	5.7 ± 0.7		4.4 ± 0.7	2.5 ± 0.4	5.5 ± 0.8		20.2 ± 3.4
	Backs	sRPE (AU)	788 ± 132	1289 ± 104		1356 ± 84	247 ± 44	737 ± 86		4416 ± 449
		Distance (km)	2.8 ± 1.2	7.6 ± 0.9		5.7 ± 0.7	2.9 ± 0.5	6.7 ± 0.6		25.7 ± 3.9
(Bradley et al., 2015a)	All Players	Field Sessions	0	2	0	1		Match		3
		Gym Sessions	1	1	1	1				4
	Forwards	sRPE (AU)	Mobility & Strength (1hr)	Gym extra content, meetings. Rugby (45mins). Rugby (1 hr)	Mobility & Strength (1hr)	Gym extras & meetings Rugby (1hr)	Rest & recovery	Match data not included in the totals for the week	Rest & recovery	1776 ± 355
		Distance (km)								7.83 ± 0.95
		Running >4.4ms-1								≈863
		RHIE								19.2 ± 7.9
		Accelerations								15.3 ± 9.6
	Backs	sRPE (AU)								1523 ± 434
		Distance (km)								9.57 ± 1.23
		Running >4.4ms-1								≈1650
		RHIE								15.4 ± 10.3
		Accelerations								46 ± 14.6

2.3 Physical profile of rugby union athletes

2.3.1 Optimal body composition for performance

The profiles of rugby union players are more diverse than the partner code rugby league where a more homogenous group of athletes can be found (Morehen et al., 2015). This may be largely due to the specific set piece demands of rugby union requiring height and body mass to complement the specific skills of lineouts, scrummaging, mauling and rucking (Duthie, 2006). At international level, teams with the heavier and taller forwards in particular, seemed to progress to the latter stages of world cup competitions (Barr, Newton and Sheppard, 2014). As the level of play moves from amateur to the elite level the profile of rugby union athletes is augmented by increases in lean body mass and reductions in bodyfat translating into players possessing greater strength, power, and speed (Smart, Hopkins and Gill, 2013). The evolution of physical preparation with professionalism has resulted in similar observations, with increased overall body mass derived from significant increases in lean muscle mass and concomitant decreases in body fat mass (Bevan et al., 2022). When comparing elite club players with their international team mates over the last 20 years, the gap between these groups in all positions has been closed (Bevan et al., 2022). This highlights how the demands of the domestic competitions at the elite level have developed and what these athletes are exposed to every competition week.

The relationship between these evolving physical profiles and the desired application of these in match play demands and fitness qualities has been investigated (Smart et al., 2014; Posthumus et al., 2020). Broadly, forwards possess greater absolute power and possess greater overall mass and strength whilst backs are leaner, faster, have greater relative power and are aerobically fitter (Posthumus et al., 2020).

Speed is correlated with line breaks and the ability to break tackles, resulting in scoring tries more frequently (Smart et al., 2014). Again, this highlights the importance of relative power to generate speed. Excess body fat may constrict force production leading to decreased power, but also is linked to increased energy expenditure and the potential to exacerbate fatigue (Zemski, Slater and Broad, 2015). This evolution of body composition quality throughout professionalism is most apparent in the ability of front five players to significantly increase speed and running distance throughout a match (Bevan et al., 2022). The activity rate of all playing positions has been negatively related to body fat especially impacting the ability to sprint repeatedly (Smart et al., 2014) and aerobic fitness as tested by the Yo-Yo test distance achieved (Posthumus et al., 2020).

The observation that the vector force, momentum, is linked to overall body mass (Quarrie et al., 1995) has also evolved to align with improvements in body composition witnessed throughout professionalism (Bevan et al., 2022). This concept of momentum which relies upon a combination of player mass and acceleration to reach maximal velocity, has become a target of physical capacity to win the collision events within match play and can be developed even after maximal velocities have been reached in players (Barr et al., 2014). This again highlights how the development of large amounts of functional mass, especially in forwards will aid performance. There are, however, concerns that despite leaner forwards possessing more favourable fitness characteristics, too much overall mass may reduce power and slow sprint times (Posthumus et al., 2020). When reviewing body composition data in an elite squad there does appear to be a positive linear relationship between lean mass and total mass until a body mass of 116.04 kg total mass is reached, at which point lean mass accumulation reduced and relative amounts of bodyfat increased (McHugh et al.,

2021). This consideration is most apparent for front five forwards, especially props and locks (Zemski, Slater and Broad, 2015).

2.3.2 Changes in body composition during competition

The comprehension that optimising body composition is favourable to meet the physical demands of the sport is established. Furthermore, an understanding of whether the profile can be maintained during the competitive period needs to be assessed. There are some available research studies including highly trained or professional rugby union athletes (Lees et al., 2017; Walker et al., 2022), elite rugby league players (Harley, Hind and O'Hara J, 2011) and a squad containing both elite and highly trained rugby union players. The changes to measures of body composition derived from DXA scanning are summarised in Table 2.3.

The investigation of Harley and colleagues in elite rugby league players was the first to demonstrate decrements in lean mass of -1.54% in the second half of the competitive period with concurrent increases of 4.09% in fat mass (Harley, Hind and O'Hara J, 2011). The authors proposed that an increase in the relative match exposure of the squad throughout the second half of the season caused this unfavourable shift, especially as multiple matches with shorter recovery opportunities (e.g., 4 games in 16 days) were a feature of this period. The reasoning being reduced time for recovery between match events compared with the first period of the season (Harley, Hind and O'Hara J, 2011). The same study design applied to highly trained professional rugby union players yielded the same observation that despite no significant changes across the whole competitive season in body mass, there were unfavourable shifts in lean and fat mass compartments (Lees et al., 2017). Both forward (≈0.89kg) and back (≈1.26kg) positional groups saw a significant reduction in lean mass through the second period of the season with accompanying increases in fat mass for the

backs throughout the season, and the forwards in the latter half (Lees et al., 2017). There was however no analysis or comment on the match exposure of this group in relation to the observed body composition changes. Evidence from a professional group of Australian rugby union players again showed no significant changes in body mass across the season in the whole group, but with the forwards the only positional group to see a reduction in lean mass of ≈1.7kg and a significant increase in body fat percentage (Walker et al., 2022). These data were however derived from two measures at the start and end of the competitive period and lack any investigation into the training or match exposures.

Despite the major focus of the work of McHugh and colleagues being into visceral adipose tissue (VAT) changes during the in-season period in rugby union athletes, measures of some compartments are reported although not all statistical analysis accompany the data (McHugh et al., 2021). The reporting of very large reductions in body mass changes in the backs sub-group with a much great standard deviation at T3 of ±18.84kg compared to 7.52 and 7.80kg respectively for T1 and T2 has caused questions of whether there have been errors in reporting at this timepoint. The population included in this study appears to be the main first team squad together with developing players in the wider provincial program, a wide range from “elite” to “trained” has been included, but more detail is not provided in the research. Similarly to the prior research there are reductions in lean mass of ≈1.72kg in the forwards, and ≈1.42kg in backs groups from the mid-point to end of season measures with individual change analysis revealing forwards to have a greater tendency to lose lean mass throughout the season compared to backs (McHugh et al., 2021).

As previously discussed, the comparison of DXA compartment absolute values between studies is futile due to the large discrepancies between machines, both manufacturer and

between machine variability (Shepherd et al., 2012). However, what we can derive from the above studies is that significant changes to body composition have been witnessed throughout the in-season period in collision sport athletes. These changes may be more pronounced during periods of greater match exposure (Harley, Hind and O'Hara J, 2011) and may be more accentuated in the forwards positional group than backs (McHugh et al., 2021; Walker et al., 2022).

There are no data available in an elite rugby union population across the in-season period focussing on these changes in body composition compartments. There is also a lack of detailed information as to how individual player exposures to training and match play during the in-season period relate to changes in these body composition compartments. The desired physical profile of an elite rugby union athlete needs to be maintained throughout the competitive season, especially as competitions are decided in the final phase. Whilst the metabolic requirements of acute recovery to a match play event need to be determined, the cumulative effect of recovering each week across the season needs to be understood.

Table 2.3. DXA derived body composition changes during the competitive season. T1 – start of the competitive season, T2 – midpoint of the competitive season, and T3 – end of the competitive season. § - significant change from T1, ¶ - significant change from T2.

Research	Participants	Positional group	Timepoint	Body mass (kg)	Fat mass(kg)	Lean mass(kg)	Bodyfat (%)
(Harley, Hind and O'Hara J, 2011)	Elite rugby league	Whole squad	T1	95.28 ± 11.33	13.59 ± 3.66	77.38 ± 9.36	15.21 ± 3.55
			T2	95.55 ± 11.99	13.92 ± 4.06	77.40 ± 9.56	15.47 ± 3.66
			T3	95.05 ± 11.81	14.49 ± 4.05§¶	76.21 ± 9.44§¶	16.24 ± 3.79§¶
(Lees et al., 2017)	Highly trained professional rugby union	Forwards	T1	110.6 ± 7.6	20.64 ± 4.94	85.39 ± 5.38	19.3 ± 3.8
			T2	111.7 ± 7.8	21.52 ± 5.10	85.55 ± 5.50	20.0 ± 3.9
			T3	111.5 ± 7.5	22.20 ± 5.44§	84.66 ± 5.36¶	20.6 ± 4.3§
		Backs	T1	92.5 ± 6.3	13.47 ± 3.28	74.77 ± 5.76	15.3 ± 3.5
			T2	93.3 ± 7.4	14.05 ± 3.92	74.99 ± 5.68	15.7 ± 3.9
			T3	92.9 ± 7.5	14.96 ± 4.09§¶	73.74 ± 5.48¶	16.7 ± 3.9§¶
(Walker et al., 2022)	Highly trained professional rugby union	Forwards	T1	110.7 ± 8.6	19.3 ± 8.0	87.6 ± 5.6	17.0 ± 5.9
			T3	110.6 ± 9.9	20.2 ± 8.1	85.9 ± 6.3§	17.8 ± 5.9§
		Backs	T1	96.1 ± 9.6	14.0 ± 4.4	78.6 ± 7.1	14.3 ± 3.6
			T3	96.4 ± 9.8	14.4 ± 5.2	78.4 ± 6.4	14.6 ± 4.1
(McHugh et al., 2021)	Elite & highly trained rugby union	Forwards	T1	110.06 ± 9.32	Information and full statistics not available	86.38 ± 6.05	17.18 ± 3.95
			T2	109.89 ± 8.58		87.36 ± 5.48	16.26 ± 3.89
			T3	108.59 ± 8.80		85.64 ± 5.76	16.90 ± 4.20
		Backs	T1	91.57 ± 7.52		75.77 ± 6.46	12.68 ± 1.98
			T2	90.98 ± 7.80		76.15 ± 6.68	11.73 ± 2.08
			T3	86.81 ± 18.84		74.73 ± 6.96	12.28 ± 2.02

2.4 The metabolic requirements of elite rugby union

Understanding the metabolic requirements and energy needs of these athletes is important for maintaining health, whilst reducing illness and the risk of injury, enabling availability to train and perform throughout a season (Thompson, Manore and Skinner, 1993; Deutz et al., 2000; Burke et al., 2018; Elliott-Sale et al., 2018; Murphy et al., 2018). There is evidence that energy needs may not be met purposefully during the pre-season period to optimise body composition profiles and to therefore fulfil desired physical and fitness characteristics (Bradley et al., 2015b; Black et al., 2019). However, the competitive in-season period prioritises the maintenance of these optimal profiles and of physical performance (Bradley et al., 2015a) and ensuring these team sport athletes meet their energy needs is key in achieving this (Burke et al., 2018; Jenner et al., 2019). As has been discussed thus far, there are multiple facets to these metabolic requirements including movement demands, repeated high intensity bouts, together with positionally diverse collision demands including the set pieces of scrum and lineout with maul.

The knowledge of these demands across team sports has been reviewed extensively to generate key areas of consideration for practitioners (Mujika and Burke, 2010) together with position stands (Thomas, Erdman and Burke, 2016). However, when we compare the demands already discussed herein with the most studied team sport of soccer, there are glaring differences. Footballers cover far greater distances in running and high-speed running in match play and training (Anderson et al., 2017; Anderson et al., 2022). Whilst rugby union players have far greater overall body mass and lean mass profiles (Milsom et al., 2015). This is before we also consider the additional factors of static exertions and collisions in rugby (Cunniffe et al., 2009; Smart et al., 2014). Therefore, the transfer of

knowledge from other team sports into understanding the metabolic requirements of elite rugby and formulating any specific nutritional recommendations is lacking and requires targeted research.

To highlight the areas of specific knowledge gaps we will now review the current available information in this population considering energy intakes and expenditure data.

2.4.1 Energy intake

2.4.1.1 Dietary intake methodology

An accurate assessment of energy intake in any population is challenging due to both the intentional or unintentional omittance of foods, and the analysis of dietary choices by the assessor (Magkos and Yannakoulia, 2003). Team sport athletes can experience high levels of cognitive load as well as the fatigue and stress of their training content (Fuster, Caparrós and Capdevila, 2021) meaning the measurement of energy intake in an athletic population needs to acknowledge the limitations of methodology and what might fit best with the specific group (Magkos and Yannakoulia, 2003). Retrospective methods such as diet recall, food frequency questionnaires and diet history very much depend upon the memory and honesty of the athlete and can miss the diurnal variation in specific macronutrient intakes apparent in rugby union athletes (Bradley et al., 2015a; Posthumus et al., 2021). These methods also require time, often one-to-one with a researcher increasing the burden upon the participant (Capling et al., 2017). Prospective methods record the participants current dietary intake ongoing throughout the research period and can take the form of written diet records utilising scales to weigh foods or specific measures such as cup portions of foods. Again, the compliance required and commitment of athletes to collate this information has

revealed an underestimation by 19% when TEE is assessed concurrently using DLW (Capling et al., 2017).

The adaptation of prospective measures to reduce burden upon participants during 'free-living' conditions has utilised mobile phone technology to create the remote food photographic method (RFPM). The validity of this technique in adults has been proven with a mean underestimation of TEI by -6.6% significantly correlated with weighed TEI in free-living conditions (Martin et al., 2009). The method has also been examined in adolescent rugby players under the term coined 'Snap-n-Send' (Costello et al., 2017). It was observed to be an accurate method with only a small mean bias for underreporting throughout a 96hr free-living period of -0.75 MJ day^{-1} or 179 kcal day^{-1} (Costello et al., 2017). We should recognise though that the participants in this study did receive all food and drinks for consumption throughout the 'free-living' 4-day period. Elite team sport athletes with nutrition support will often be catered for during training periods at their club or institution but will be purchasing food for consumption away from there. This poses the question as to whether this methodology is a true reflection of real-life athlete habits. Recent work on this method in true free-living environments highlights the need for experienced practitioners when analysing photo records and that underreporting is greater on days containing more complex multi-ingredient meals (Stables et al., 2021). Nevertheless, the convenience and accessibility of mobile photographic devices removes much of the burden compared with other prospective and retrospective methods.

There is also the need to appreciate potential errors created in analysis of dietary records. Meals with the greatest errors in analysis were those where individual ingredients were difficult to identify and quantify such as lasagne, casseroles, or stir fries, but also 'niche'

market products such as sports foods (Braakhuis et al., 2003). Some of this can be resolved when the meals are catered 'in-house' by ensuring analysis is carried out by a trained, registered sports and exercise nutritionist prior to buffet selection, but also utilising modern dietary analysis software such as Nutritics (Nutritics Ltd, Ireland) to ensure databases are extensive as food composition values can be a major source of error, but also that the practitioner is able to manually enter and add foods to those databases (Larson-Meyer, Woolf and Burke, 2018). Nutritics software has an extensive database and has been used in team sport studies in rugby to design trial diets (Bradley et al., 2016) and analyse intakes (Bradley et al., 2015a; Bradley et al., 2015b; Morehen et al., 2016; Costello et al., 2017). The length of diet record may also reduce the variability in coding error with 7 days appearing to reduce this compared to 1- and 3- day records (Braakhuis et al., 2003).

Overall, we must choose methodology to balance the burden on participants and their total physical and cognitive load, with obtaining the truest reflection of their daily intakes. We must also appreciate underestimates may also be present in analysis even when experienced practitioners utilise modern database software.

2.4.1.2 Dietary intake in rugby union athletes

The macronutrient intakes of male rugby players throughout different phases of the season have been reviewed in adolescent players, amateur collegiate, and elite level players (Black, Black and Baker, 2018). The consensus being that whilst protein intakes are consistently greater than the 1.2-2.0g·kg^{-1} BM·day^{-1} guideline for athletes (Thomas, Erdman and Burke, 2016), and fat intakes are above the recommended proportional threshold of total energy intake at 20% (Thomas, Erdman and Burke, 2016), carbohydrate consumption is consistently meeting what would be considered a 'moderate' intake of between 300-400g·day or 3.3-4.8g·kg^{-1}·day^{-1} (Black, Black and Baker, 2018). As we have discussed, the pre-season period is

associated with the optimisation of body composition to complement the physical qualities desired to compete at the elite level of rugby union which may result in a purposeful energy deficit (Bradley et al., 2015b). This may be accompanied by a high protein intake greater than 2.5g·kg^{-1}·day^{-1} (Tipton and Wolfe, 2004) to support muscle hypertrophy and meet the desired physical profiles which may go some way to explaining why these high intakes persist into the in-season period (Black, Black and Baker, 2018). The two pieces of research available observing dietary intake across competitive match weeks are summarised in table 2.4 revealing that these high protein intakes also feature throughout the week.

Fat intake is different between the two observed groups where the southern hemisphere squad intake was highest, this may be reflective of the longer duration and lower intensity of training sessions (as discussed in section 2.2.4), with the mentality of 'eating (carbohydrate) to intensity' observed (Posthumus et al., 2021). Fat intakes may also be influenced by the high protein consumption derived from animal sources (Black, Black and Baker, 2018).

The daily intakes of protein and fat are relatively consistent across the days of the match weeks, but the periodisation of energy intake represents significant daily variation in carbohydrate intakes as is summarised in table 2.5.

Table 2.4 Summarises the available data on mean energy and macronutrient intakes throughout a competitive match week in elite rugby union athletes.

Study	Positional Group	Energy Intake		Carbohydrate	Protein	Fat
		kcal·day^{-1}	kcal·kg·day^{-1}	g·kg·day^{-1}	g·kg·day^{-1}	g·kg·day^{-1}
(Posthumus et al., 2021)	Forwards	4606 ± 719	40.5 ± 7.2	3.5 ± 0.8	2.5 ± 0.4	1.8 ± 0.4
	Backs	3761 ± 618	41.9 ± 7.2	3.7 ± 0.7	2.4 ± 0.5	1.8 ± 0.5
(Bradley et al., 2015a)	Forwards	≈3967	≈36.0	3.5 ± 0.8	2.7 ± 0.5	1.4 ± 0.2
	Backs	≈3394	≈36.3	3.4 ± 0.7	2.7 ± 0.3	1.4 ± 0.3

Table 2.5 The available data on macronutrient periodisation across a competitive match week in elite rugby union athletes.

Study	Macronutrient	Group	GD+2	GD+3	GD+4	GD-2	GD-1	GD	GD+1
(Posthumus et al., 2021)	Protein g·kg^{-1}	Forwards	2.5	2.7	2.1	2.7	2.6	2.5	2.1
		Backs	2.4	2.5	1.9	2.2	2.4	2.7	2.2
	Fat g·kg^{-1}	Forwards	1.7	1.7	1.5	1.8	2	1.9	1.7
		Backs	1.8	1.8	1.5	2	2	2.1	1.7
	Carbohydrate g·kg^{-1}	Forwards	3.5	3.2	3.1	3.8	3.3	4.4	2.7
		Backs	3.3	3.6	3.1	3.7	3.5	5.1	3.1
(Bradley et al., 2015a)	Protein g·kg^{-1}	Forwards	2.3	2.6	2.5	2.9	3.3	Not recorded	2.7
		Backs	2.0	2.4	2.4	3.0	3.8		2.6
	Fat g·kg^{-1}	Forwards	1.2	1.5	1.2	1.4	1.6		1.5
		Backs	1.1	1.4	1.3	1.4	1.9		1.5
	Carbohydrate g·kg^{-1}	Forwards	2.9	3.5	3.2	3.3	5.1		3.1
		Backs	2.9	2.9	2.6	4.4	4.2		3.1

2.4.1.3 Periodised energy intake

There is evidence that rugby union athletes periodise their nutrition across the match week but with quite different training structures and approaches (Bradley et al., 2015a; Posthumus et al., 2021). Despite small shifts in protein and fat intake over the 7-day period, carbohydrate has been consumed to 'eat to intensity' (Posthumus et al., 2021) or more akin to the 'fuel the work required' paradigm (Bradley et al., 2015a) with carbohydrate significantly increased in preparation for competition.

Research in rugby league match play, using muscle biopsies, demonstrated that a carbohydrate intake of 6g·kg^{-1} as opposed to 3g·kg^{-1} in the 36hr prior to kick off may be preferential in preventing glycogen levels becoming low enough to affect the athlete's ability to physically produce the repeated high intensity activities required (Bradley et al., 2016). This intake was not reached in northern hemisphere rugby players (Bradley et al., 2015a) and was even lower in southern hemisphere practices on GD-1 (Posthumus et al., 2021). Southern hemisphere players prior to kick-off on GD managed an intake of 339g (Forwards) and 271g (Backs) potentially making up some of the shortfall on GD-1 (Posthumus et al., 2021).

Prior investigations into glycogen replenishment after a rugby league simulation protocol recommended immediate high carbohydrate refeeding, to take advantage of the exercise induced glucose transport into the muscle compared to delayed feeding (Bradley et al., 2017). Despite Bradley and colleagues not recording dietary intake on GD (Bradley et al., 2015a), recent work in a southern hemisphere elite group revealed intakes of 167g (Forwards) and 190g (Backs) carbohydrate in the hours immediately post-match play and a total GD carbohydrate intake of 4.5 g·kg^{-1} (Forwards) and 5.1 g·kg^{-1} (Backs).

Both sets of observations do have a common theme at GD+1 though, with energy intake at the mean for the week (Forwards 16.6MJ or 3976kcal and Backs 14.2MJ or 3394kcal) in northern hemisphere players (Bradley et al., 2015a) and an energy intake below the mean for the week (Forwards 4606kcal and Backs 3761kcal) in southern hemisphere rugby union athletes (Posthumus et al., 2021). This corresponds to carbohydrate intakes below the mean for the week in each group, ≈2.7 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Posthumus et al., 2021) and ≈3.1 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Bradley et al., 2015a) for forwards & backs respectively.

This may be reflective of when the next competitive event is scheduled and the intensity of training sessions between fixtures. In soccer, the multiple fixtures often played within a 7-day period necessitate the restoration of muscle glycogen be made a priority and a high carbohydrate intake on GD+1 being part of a periodised nutrition plan (Anderson et al., 2022). From the available research, as reviewed above, this low intake of carbohydrate at GD+1 appears to be a common theme in rugby union. A greater understanding of the metabolic requirements of this recovery period may be able to guide whether the observed energy and macronutrient intakes are adequate in the elite setting.

2.4.2 Total energy expenditure

Energy expenditure is comprised of three components; 1. Basal metabolism, 2. Thermic effect of food also known as diet induced thermogenesis (TEF)), and 3. Activity energy expenditure (AEE).

2.4.2.1 Basal metabolism

Basal metabolism is the amount of energy needed to maintain normal homeostatic physiological functions at rest (Manore and Thompson, 2015). Measurements of basal metabolism are in a rested, fasted and thermoneutral state. The key difference between basal metabolic rate (BMR) and resting metabolic rate (RMR) being that BMR needs to be

measured at the bedside of a supine individual who has just woken, whereas RMR can be measured after the individual has risen but a period of rest has been undergone prior to measurement. RMR is therefore usually the more common measure in research and practice (Manore and Thompson, 2015). The two terms BMR and RMR are too often used interchangeably in the literature but provided stringent outpatient protocols are adhered to there is no difference between measures in an athletic population (Bone and Burke, 2018b). RMR tends to be the largest component (60-75%) of daily energy expenditure (EE) in humans (Speakman and Selman, 2003).

Fat free mass (FFM) is the most metabolically active compartment of the body. However, the tissues which make up this compartment are diverse, including different organs as well as skeletal muscle mass (Gallagher et al., 1998). The brain, heart, liver, and kidneys account for ≈60-70% of adult RMR but only accumulate to ≈6% of total body mass. Whereas skeletal muscle can account for ≈40-50% of adult body mass but only contribute ≈20-30% of RMR (Gallagher et al., 1998).

Calculating RMR from FFM is not linear though and a significant increase in lean mass of ≈2kg does not translate into a statistically significant or meaningful increase in RMR in rugby athletes (MacKenzie-Shalders et al., 2019).

Food and drink intake, specifically the energy and macronutrient content can influence measurement of RMR. Therefore, measurements need to be conducted under standardised, fasted conditions (Compher et al., 2006; Fullmer et al., 2015).

2.4.2.2 Thermic effect of food

The increase in metabolism above RMR following consumption of energy from food or drink is labelled as the thermic effect of food (TEF). It represents the amount of energy required

to digest, absorb, transport, metabolise and store nutrients following the consumption of food and, or drink (Manore and Thompson, 2015), and peaks ≈60-180 minutes after consumption (Compher et al., 2006). When measuring basal metabolism the fasting period of at least 8 hours should be adhered to as best practice (Fullmer et al., 2015) evidenced by TEF fully subsiding after a large ≈1300kcal meal in lean males after this time (D'Alessio et al., 1988). The main determinants of TEF are primarily the energy content of the food, followed by the protein fraction but all macronutrients do have an influence (Westerterp, 2004). For carbohydrates the TEF is 5-10% of the total energy content of the consumed carbohydrate, for fat 0-3% of the total energy content of the consumed fat, for protein 20-30% of the total energy content of the consumed protein and for alcohol 10-30% of the total energy content of the consumed alcohol (Westerterp, 2004). In healthy participants, consuming a mixed diet, TEF is approximately 10% of the total amount of energy ingested over 24 hours (Westerterp, 2004). Given the observed high protein intakes in rugby athletes (Black, Black and Baker, 2018) even with differing body composition goals (Black et al., 2019), and the evidence at the elite level of intakes at the highest end of the observed range (Bradley et al., 2015a; Posthumus et al., 2021), TEF may be even higher than this ≈10% figure. This also accentuates the importance of rigorous protocols when measuring resting metabolism and ensuring fasted periods prior to measures are at least 8 hours.

2.4.2.3 Activity energy expenditure

Activity energy expenditure (AEE) occurs as the result of physical activity and is the amount of energy expended above RMR and TEF to make up total energy expenditure (TEE). It is highly variable and is determined by both body movements and body size (Westerterp, 2013). AEE can be separated into non-exercise activity thermogenesis (NEAT) and planned exercise related energy expenditure (Levine, Schleusner and Jensen, 2000). NEAT is the

energy cost of activities of daily living including sitting, standing, walking, fidgeting and spontaneous muscle contractions such as shivering (Levine, Schleusner and Jensen, 2000). There is currently no research quantifying AEE in rugby union players but there have been attempts to quantify TEE in both academy and senior rugby union and league athletes during the in-season period (Bradley et al., 2015a; Morehen et al., 2016; Smith et al., 2018).

2.4.2.4 Methodological considerations in measuring total energy expenditure

To understand the metabolic requirements of recovery in a rugby player it is important to accurately quantify the energy requirements on a day-to-day basis. To achieve this a variety of methods to measure energy expenditure are available but their reliability and practicality in the applied setting must be considered. We can broadly classify them into either calorimetric (direct and indirect calorimetry) and non-calorimetric (estimation methods). Please refer to table 2.6 For an overview of the relevant methods.

Table 2.6 Overview of methods to assess energy expenditure with advantages and disadvantages, adapted from Pinheiro Volp et al. (Pinheiro Volp et al., 2011).

Method	Advantages	Disadvantages
Direct calorimetry	Highly sophisticated method, considered the gold standard for measuring TEE and allows for some activity during measurements	Highly complex methodology, high cost of equipment and requires the confinement of the subject for 24 hours or more
Indirect calorimetry	The gold standard for measuring both resting and basal EE. It is non-invasive, reasonably accurate and highly reproducible. Allows the quantification and identification of energy substrate oxidation. Allows short-term measurement of EE	High cost of equipment and relatively complex measurement. Requires trained personnel for its correct use
Doubly labelled water	Gold standard method for measurement of TEE in free-living conditions. It is a safe method employing deuterium (H2) and oxygen-18 (O18)	It is costly and requires sophisticated equipment and trained individuals. It does not provide the information of energy expended in physical activity neither does it provide information about substrate oxidation. Day-to-day variations in EE cannot be measured either
Wearable technology sensing heat and movement	Relatively inexpensive and reusable technology as an alternative to DLW. Day-to-day variations in EE can be estimated using these	Devices cannot be worn constantly due to need to remove when washing or bathing. Also, cannot safely be worn during specific exercise modalities and contact-based activities
Accelerometers (tri-axial) devices & heart rate monitors	Relatively inexpensive, non-invasive, portable, and reusable. Frequency, velocity, and duration of movements measured	Accuracy is dependent upon calibration of devices. Underestimates EE during team sport activities and intermittent exercise. Unable to detect and appreciate eccentric actions and collisions accurately. Factors other than exercise can significantly affect heart rate
Physical activity records	Low-cost method that estimates EE from an extremely detailed registry of all physical activity performed daily	Comparison across the literature is limited due to various existing codes for activities. Estimated EE does not consider inter-individual differences which may affect the energetic cost of a movement

Direct calorimetry

Direct calorimetry measures total heat loss from the body whilst indirect calorimetry measures total energy production by the body (Ainslie, Reilly and Westerterp, 2003). The highly sophisticated metabolic chamber required for direct calorimetry together with the limited activity participants can complete whilst in that environment means it is of little practical use whilst attempting to research team sport athletes (Ainslie, Reilly and Westerterp, 2003; Levine, 2005).

Indirect calorimetry

Indirect calorimetry measured utilising ventilated metabolic carts is the most used method to measure RMR in applied research and practice (Ainslie, Reilly and Westerterp, 2003; Levine, 2005). It has been used to determine RMR in young male (16-24 years old) rugby union and league players (Smith et al., 2018), and senior male rugby league athletes (Morehen et al., 2016).

The measurements of oxygen (O_2) and carbon dioxide (CO_2) together with consumption of oxygen ($\dot{V}O_2$) required for substrate oxidation, and carbon dioxide production ($\dot{V}CO_2$) are recorded from pulmonary ventilation by the metabolic cart, rather than the direct measurement of heat (Levine, 2005). The respiratory exchange ratio (RER) can be calculated ($\dot{V}CO_2/\dot{V}O_2$), the relative balance and absolute amounts of energy (kcal) and substrates ($g \cdot L^{-1}$ O_2) can be derived (Table 2.7) (Zuntz, 1901). When best practice guidelines are followed allowing for at least 20 minutes rest prior to measurements and a controlled fasted period of at least 8 hours is adhered to (Fullmer et al., 2015), the precision can be within 0.5-2.0% (Levine, 2005). The typical coefficient of variation (CV) is ≈2-3% and has a much lower variation than total daily EE measurements as the element of non-exercise activity is not included (Donahoo, Levine and Melanson, 2004).

Table 2.7 Derivation of energy (kcal) and substrate oxidation via indirect calorimetry.

Reproduced from Zuntz (1901).

Nonprotein RER	kcal. L^{-1} O_2	Percentage kcal derived from		g. L^{-1} O_2	
		Carbohydrate	Fat	Carbohydrate	Fat
0.707	4.686	0.0	100.0	0.000	0.496
0.71	4.69	1.1	98.9	0.012	0.491
0.72	4.702	4.8	95.2	0.051	0.476
0.73	4.714	8.4	91.6	0.090	0.460
0.74	4.727	12.0	88.0	0.130	0.444
0.75	4.739	15.6	84.4	0.170	0.428
0.76	4.75	19.2	80.8	0.211	0.412
0.77	4.764	22.8	77.2	0.250	0.396
0.78	4.776	26.3	73.7	0.290	0.380
0.79	4.788	29.9	70.1	0.330	0.363
0.80	4.801	33.4	66.6	0.371	0.347
0.81	4.813	36.9	63.1	0.413	0.330
0.82	4.825	40.3	59.7	0.454	0.313
0.83	4.838	43.8	56.2	0.496	0.297
0.84	4.85	47.2	52.8	0.537	0.280
0.85	4.862	50.7	49.3	0.579	0.263
0.86	4.875	54.1	45.9	0.621	0.247
0.87	4.887	57.5	42.5	0.663	0.230
0.88	4.899	60.8	39.2	0.705	0.213
0.89	4.911	64.2	35.8	0.749	0.195
0.90	4.924	67.5	32.5	0.791	0.178
0.91	4.936	70.8	29.2	0.834	0.160
0.92	4.948	74.1	25.9	0.877	0.143
0.93	4.961	77.4	22.6	0.921	0.125
0.94	4.973	80.7	19.3	0.964	0.108
0.95	4.985	84.0	16.0	1.008	0.090
0.96	4.998	87.2	12.8	1.052	0.072
0.97	5.01	90.4	9.6	1.097	0.054
0.98	5.022	93.6	6.4	1.142	0.036
0.99	5.035	96.8	3.2	1.186	0.018
1.00	5.047	100.0	0.0	1.231	0.000

Doubly labelled water

The use of DLW for the assessment of TEE in humans can be described as free-living indirect calorimetry (Ainslie, Reilly and Westerterp, 2003). The method involves the enrichment of the body water pool with stable, non-radioactive isotopes and the elimination of the ^{18}O and ^{2}H reflects the rate at which carbon dioxide is produced (Ainslie, Reilly and Westerterp, 2003). The amount of DLW consumed by participants is calculated based upon body mass. The cost of DLW is high and when you consider the size and body masses of rugby union players, the cost can be prohibitive at ≈£1000 per dose explaining the small participant numbers often reported in the literature (Morehen et al., 2016; Costello et al., 2018). There are also methodological short comings appropriate to discuss in line with our aims. DLW is an indirect method and assumes a respiratory quotient (RQ) of 0.85 based upon a typical Western dietary macronutrient distribution (Westerterp, 2018) , which as we have discussed is not reflective of rugby athlete's dietary intake (Black, Black and Baker, 2018). The DLW technique is the gold standard to TEE over a period of time, but the end calculations derive a mean daily TEE and the method cannot provide practical information on the TEE for a specific day or any day-to-day variation (Ainslie, Reilly and Westerterp, 2003).

Non-calorimetric estimation methods

Practitioners in the applied field have several estimation equations available to them when either the availability of specialist equipment, technical expertise, or budget means a practical alternative to calorimetric measures is required. The error in applying these formulae to rugby players is amplified as they are often derived from non-athletic populations with lower body masses (Harris and Benedict, 1918; Cunningham, 1991; Henry, 2005). Even when athletic populations have been used to derive equations (De Lorenzo et

al., 1999) underestimations are still reported when a range of athletes are measured via indirect calorimetry using ventilated hood equipment (Jagim et al., 2018). Underestimations of RMR using the equations developed in non-athletic populations may be explained by the absence of FFM consideration in the equation (Schofield, Thorpe and Sims, 2019). Even when FFM is considered (Cunningham, 1991), significant discrepancies are reported with underestimations of RMR in male heavyweight rowers (Carlsohn et al., 2011) and overestimations in senior rugby league players (Morehen et al., 2016).

The ease of use, lower financial costs, and portability of wearable technology to estimate EE makes them attractive in the applied research setting. However, the need for calibrations, integration of physiological data with accelerometers (Strath, Brage and Ekelund, 2005), and challenges in heart rate measurements being greatly influenced by thermoregulation during exercise (Brage et al., 2005). Attempts to utilise the accelerometers housed within the routinely worn GPS units to calculate a metabolic power of intermittent activities including collisions have failed, with underestimates of EE the result in rugby league players (Highton et al., 2017). The use of SenseWear armbands in rugby union athletes has been employed but the plastic units worn on the arm and containing battery compartments are not safely worn during contact training, match play, and any water-based activities. Whilst insightful as it was in an elite rugby union population, this data set was therefore incomplete as match play measurements and some training could not be included (Bradley et al., 2015a).

2.4.2.5 Total energy expenditure of rugby athletes

The examination of in-season EE in rugby union athletes utilising SenseWear armbands did demonstrate how players periodised their EI across the match week in relation to EE and in preparation for game day (Bradley et al., 2015a). The mean daily EE was ≈3800kcal and ≈3346kcal for forwards and backs respectively, but this doesn't include game day and the wearable technology used cannot be worn in contact training, and is likely an underestimate of TEE (Bradley et al., 2015a). The EE of developing rugby league and rugby union players has also been reported with great interindividual variability with a range of 3452-6617 Kcal·day^{-1} (Smith et al., 2018). The eldest cohort in this study was U24 and their TEE was ≈900 Kcal·day^{-1} less than that reported in senior RL players (Morehen et al., 2016). There were no differences reported between positional groups but across the two week observational period there was a large increase in TEE between week 1 & 2 respectively, in both forwards (4565 Kcal·day^{-1} & 5736 Kcal·day^{-1}) and backs (3967 Kcal·day^{-1} & 5808 Kcal·day^{-1}). These large TEE requirements were attributed in part by the authors to NEAT as the monitoring of training between weeks and the perceived exertions not changing, although there was no statistical analysis to corroborate this (Morehen et al., 2016). The unintended exertions of the players away from prescribed training does need consideration, especially when we are seeking to understand the metabolic requirements of the recovery period, but the differences in TEE between weeks in this research may also be due to the metabolic demands of collisions. The research was conducted over the first two weeks of the European Super League season. Therefore, the first week may not have included the energy expenditure in recovery from match play, whereas the second week would have included any increased energy needs in recovery from match 1, and the expenditure for the performance of the second match with an acute recovery period.

2.4.3 The energy cost of damage

These high TEE demands documented in senior elite rugby league players (Morehen et al., 2016) are exceptional when compared to the mean daily TEEs reported in soccer (3566 ± 585kcals), even when soccer players engaged in multiple competitive fixtures within the research weeks (Anderson et al., 2017). Collision activities induce substantial muscle damage referred to as impact induced muscle damage (IIMD) and these may increase the energy cost of recovery periods (Naughton, Miller and Slater, 2018a).

A landmark piece of research investigated the relationship between TEE as measured via DLW and the inclusion of competitive collisions in a 5-day training microcycle (Costello et al., 2018). The young (age 16-18) male rugby league athletes participated in a training week without contacts, and then a matched training week including 20 competitive collisions (10 tackles and 10 ball carrying collisions) which resulted in a very likely higher TEE of 4.96± 0.97 MJ when collisions were included which represented an approximate 5% increase in TEE (Costello et al., 2018). This research was well controlled but the participants were younger and likely significantly below the strength and power capacities of senior players (Geeson-Brown et al., 2020), also indicated by the mean body mass of 87.3± 14.9 kg (Costello et al., 2018). The study design also incorporated crash mats either side of the competitive zones to ensure that direct collision could not be avoided, but as damage can be caused by player-player contact and player-playing surface contact, this may have further reduced the full extent of damage as it would be in full match play (Costello et al., 2018). We could expect therefore that as the overall body mass, strength, and power increases with development into senior elite players, so would the damage induced and increased energy requirements in recovery. There is no research available though as to whether collisions cause significant changes day-to-day in energy requirements. As we have reviewed, RMR is the only

compartment repeatably measurable each day (Compher et al., 2006; Fullmer et al., 2015) and under outpatient conditions can accurately utilise indirect calorimetry to achieve this (Bone and Burke, 2018b).

There has been research into how differing exercise modalities may change RMR in the days after exercise exposure. Early work revealed no difference in RMR each morning, when measured for seven days with the first three days including either a jogging or cycling energy matched protocols (Kolkhorst, Londeree and Thomas, 1994). RMR was also measured after both resistance and aerobic exercise interventions, where resistance training did significantly increase RMR 15hours later, on the morning of the following day (Gillette, Bullough and Melby, 1994). The authors postulated the eccentric muscle damaging actions of the resistance training compared with energy matched stationary cycling may have caused this difference (Gillette, Bullough and Melby, 1994). Resistance training has been studied for extended periods after exercise with significant elevations in RMR lasting 48hr (Dolezal et al., 2000) and 72hr (Hackney, Engels and Gretebeck, 2008) when the volume of training is high and the lifting focus is upon the eccentric portion of the exercise. Unfortunately, despite the early work from Kolkhorst measuring both respiratory exchange ratio (RER) and oxygen consumption with RMR (Kolkhorst, Londeree and Thomas, 1994), none of the rest of the research reviewed here, included these measures. Indeed, the later work of Hackney et al demonstrated RMR to be higher (3.61%) for the seven-days where four resistance training sessions were completed, when participants were fed an amino acid-carbohydrate drink acutely around training rather than isoenergetic carbohydrate only beverage (Hackney, Kelleher and Ploutz-Snyder, 2013). This is an interesting finding and may relate to the early work of Welle & Nair on RMR and protein turnover (Welle and Nair, 1990). RER was provided in the results and this lowered significantly during the week of

resistance training in both conditions from baseline, indicating lipid utilisation was not altered by training but neither oxygen nor carbon dioxide uptake data were provided (Hackney, Kelleher and Ploutz-Snyder, 2013). This information could be crucial in determining the metabolic changes after the exercise exposure as an understanding of carbon dioxide and oxygen consumption can allow investigation of substrate oxidation in recovery rather than just reporting an extended period of post exercise oxygen consumption (EPOC) manifesting itself as increased RMR (Fullmer et al., 2015).

Novel research investigating changes in resting energy requirements needs to include observations of substrate oxidation calculated from the consumption of oxygen and carbon dioxide derived from indirect calorimetry.

The work of Welle & Nair (Welle and Nair, 1990) is cited to explain the energy cost of 'protein resynthesis' accounting for as much as 20% of RMR (Burt et al., 2014). This figure of 20% of total RMR is protein turnover for all tissues with active cell mass, not just muscle (Welle and Nair, 1990). There is a crucial difference though as the authors stated in their manuscript; the statistical correlations witnessed between leucine flux and metabolic rate alone cannot prove that protein synthesis is entirely responsible for the relationship between protein turnover and resting metabolic rate (Welle and Nair, 1990). The relationship reported between metabolic rate and both protein breakdown and protein synthesis were nearly identical (Welle and Nair, 1990) so the later research reviewing changes to RMR after exercise cannot claim any changes to be due to protein synthesis exclusively.

It is not purely protein turnover that may alter the metabolic response to exercise. There are also considerations as to the inflammatory response associated with muscle damage

(Peake et al., 2017) and the activation of the immune system during recovery (Owens et al., 2019). This is important when examining the aetiology of exercise induced muscle damage (EIMD) and impact induced muscle damage (IIMD) experienced in collision sports.

2.5 Muscle damage

As discussed in section 2.1, the demands of elite rugby union require an athlete to perform repeated high-intensity actions while also being exposed to collisions during ball carrying, tackling and set-piece events. The resultant muscle damage due to these actions can be separated into EIMD due to the repeated high levels of mechanical stress (Hyldahl and Hubal, 2014) but also IIMD due to the blunt force trauma in contacts with other players on the field and also the playing surface itself (Naughton, Miller and Slater, 2018a). It is important to consider how both forms may contribute to the challenge of recovery from match play.

2.5.1 Exercise induced muscle damage

Throughout match play the accelerations, decelerations, violent changes of direction or plyometric movements are characterised by rapid and repeated muscle lengthening, resulting in EIMD (Hyldahl and Hubal, 2014; Peake et al., 2017). The resultant disruption to myofibrillar ultrastructure causes a loss of power and strength, delayed onset muscle soreness (DOMS), swelling, and a systemic efflux of myocellular enzymes and proteins such as myoglobin, creatine kinase and interleukins (Peake et al., 2017).

Following this primary mechanism of damage, a secondary phase commences allowing the removal, repair, and remodelling of damaged tissue whereby satellite cells (muscle stem cells), inflammatory cells (neutrophils, macrophages, T lymphocytes and mast cells), vascular, and stromal cells orchestrate the process. Effective recovery from muscle damage is determined by the dynamics of these cellular interactions (Peake et al., 2017).

Some of these indirect markers of muscle damage have been studied after rugby union match play (Takarada, 2003; Cunniffe et al., 2010; Cunniffe et al., 2011) and rugby league (Oxendale et al., 2016; Morehen et al., 2020) but they are not solely due to EIMD. There are complex interactions between EIMD and IIMD resulting in the apparent profiles of these markers in the available literature.

2.5.2 Impact induced muscle damage

Typically, muscle damage due to direct trauma results in myofibril damage, tissue necrosis, and the invasion of pro-inflammatory factors encompassing a similar primary and secondary phase of regeneration and remodelling as in EIMD (Naughton, Miller and Slater, 2018a). However, IIMD is thought to result in an enhanced inflammatory infiltrate and amplified secondary damage response (Merrick, 2002).

To isolate IIMD, a simulation experiment has been designed to capture and characterise the magnitude and duration of recovery from IIMD (Naughton, Miller and Slater, 2018b). Whilst the results demonstrated a reduction in the markers of physical performance correlated with subjective soreness 48-72hr post exposure, there were no significant differences between baseline circulatory measures in inflammatory markers (c-reactive protein (CRP)), and marker of muscle damage (myoglobin (Mb)) throughout the recovery period (Naughton, Miller and Slater, 2018b). Peak systemic concentrations of Mb immediately following an IIMD simulation (≈47 $mg \cdot ml^{-1}$) were far below those previously reported in rugby union (≈500 $mg \cdot ml^{-1}$) (Takarada, 2003) and American football (≈250 $mg \cdot ml^{-1}$) (Hoffman et al., 2002). The proposed explanation by the authors being that the likely amplification of muscle damage when EIMD and IIMD are suffered in the same physical exposure, as in collision sports (Naughton, Miller and Slater, 2018b). It could be argued therefore, that to truly investigate recovery from collision sports, participants will be exposed to both forms of

muscle damage, and the relationship between phases of recovery are intertwined with any resulting metabolic changes being an accumulation of, rather than attributable to either aetiology.

2.5.3 Markers of damage after rugby match play

Takarada tracked changes around match play in amateur rugby union, for creatine kinase (CK) and Mb (Takarada, 2003). Both markers demonstrated a significant transient increase compared to baseline measures at rest. Mb levels peaked at 45min post-match but remained higher until 48hr post. CK meanwhile peaked 24hr post-match (Takarada, 2003). It was interesting that both metabolites correlated significantly with the number of tackles made, but that these were assessed via video analysis and most importantly were only those tackles involving front on collisions. All others were discounted which was conspicuous in the absence of reasoning for this chosen method and may explain how such positive near perfect correlations were reported (Takarada, 2003). Either that, or the authors in reporting this way have implicated the front on collisions being the most damaging causing the significant relationship.

Further CK measures have been performed in elite RU research (Cunniffe et al., 2010; Cunniffe et al., 2011) and observed to be associated with match demands (McLellan, Lovell and Gass, 2011a; Oxendale et al., 2016) and perceptual fatigue (McLean et al., 2010) in RL. However, the differing times to peak concentrations in the blood across these studies may be explained by great variance in individual resting values and the rate at which individuals clear CK from the blood being diverse, highlighting the multiple limitations of using CK as a marker of muscle damage (Baird et al., 2012).

The leakage of Mb into the bloodstream following trauma and the loss of structural integrity is also associated with oxidative stress markers in urine. Specifically, the oxidation by Mb and iron of dihydroneopterin has been observed after rugby match play and correlated with collision events (Lindsay et al., 2016a). Neopterin is an oxidation product of dihydroneopterin produced by macrophages and is a marker of cellular immune system activation with a pro-inflammatory immune status (Lindsay et al., 2015). The acute peaks post-match in these pterins align with myoglobin increases but not with changes in immunoglobulins (Lindsay et al., 2015). These studies provide further evidence of oxidative and inflammatory stress after match play whilst utilising non-invasive sampling of urine and saliva (Lindsay et al., 2015; Lindsay et al., 2016a).

Research into immune cell profiles have been conducted, demonstrating total leukocytes, neutrophils, and monocytes peak immediately and then remain elevated above pre match levels at 14hr post international match play (Cunniffe et al., 2010) and within a series of three matches (Cunniffe et al., 2011). In the same research, the cytokine interleukin (IL), IL-6 peaked immediately post game (Cunniffe et al., 2010; Cunniffe et al., 2011) similarly to after rugby league match play (Morehen et al., 2020). The rises in CRP at 14hr and peaking at 38hr post game may be indicative of the secondary damage response requiring the recruitment of leukocytes for repair processes (Cunniffe et al., 2010). The only research to observe more cytokine activity in IL-8 and the anti-inflammatory IL-10 did not measure changes between immediately and 48hr post, which may have missed fluctuations in the cytokine response (Morehen et al., 2020). The timeline of changes to this entourage of inflammatory, immunoendocrine, and oxidative stress markers highlights the need to observe changes in metabolism at regular intervals throughout recovery.

2.5.4 Adaptation to muscle damage

The repeated bout effect (RBE) is a phenomenon identified as a protective adaptation of the muscle to eccentric exercise, such that if consecutive bouts of similar intensity and load are performed, the sensitivity of the muscle to damage is reduced (McHugh et al., 1999). These adaptations persist for over 6 months following the initial bout of damaging exercise (Naughton, Miller and Slater, 2018a) with reductions in the severity of several of the descriptors of fatigue such as force loss, and perceptual soreness after the second bout (Naughton et al., 2021). There has been a proposed 'contact adaptation' presented in American football research utilising comparative CK concentrations showing lower levels through the in-season period compared to the pre-season measures (Kraemer et al., 2013). However, between the limitations in CK measures we have already discussed and no information on relative training or contact loads through those phases of the season, further research outside of the scope of this thesis is needed to verify this theory.

The mechanisms for RBE are still to be clarified but adaptation of the extracellular matrix remodelling with changes to connective tissues has been evidenced (Hoffman et al., 2016). The alternate theory of adaptation to the inflammatory cellular response seems less likely as the number of neutrophils and macrophages was still significantly greater after the second bout of unilateral eccentric exercise compared with pre-exercise values (Stupka et al., 2001) and neutrophil levels remained unchanged in well trained runners after multiple downhill running bouts (Peake et al., 2005). This is interesting as even if structural changes do occur to better cope with a familiar load or intensity of exercise, the cellular response may still be unchanged, meaning the markers of fatigue may not be as severe, but that metabolic changes in recovery may still be prominent.

There is also the consideration that players are exposed to a combination of EIMD and IIMD which may be unique after each bout with collisions happening across the body in a non-uniform profile and the unpredictable nature of movement patterns consistently creating unfamiliar eccentric loading. This notion is supported in the perception of soreness being consistent throughout an entire season of rugby league, only reducing to a lower level prior to another match play event throughout the season and players never being entirely free from soreness (Fletcher et al., 2016).

2.5.5 Current recovery strategies from muscle damage

The understanding that fatigue manifests itself in rugby populations as soreness and reductions in neuromuscular performance underpinned by muscle damage and the ensuing inflammation (Naughton et al., 2021) has caused recovery strategies to target these perceptual and inflammatory roots. The most researched methods being compression garments, cryotherapy, cold water immersion, contrast bathing, active low intensity exercise and passive forms such as massage and electrostimulation (Tavares, Smith and Driller, 2017; Dupuy et al., 2018). Evidence that cold water immersion and contrast bathing are effective in reducing loss of jump height, and allaying muscle soreness ready for training sessions to re-commence (Webb et al., 2013), and that cold exposures and massage are the most effective at assuaging inflammatory markers (Dupuy et al., 2018) has led to those modalities being readily utilised in contact sports.

Crucially though, these modalities still don't consider any potential metabolic changes, especially when cold water immersion may in fact reduce the T-cell and monocyte activation in response to high intensity exercise, thereby potentially reducing the effectiveness of the immune cascade as part of the secondary response to muscle damage (Lindsay et al., 2016b).

Nutrition interventions have focussed their attempts to attenuate inflammation and oxidative stress with omega-3 polyunsaturated fatty acids, vitamins C, D, and E (Owens et al., 2019). Despite any disruption to the adaptive response not necessarily being a priority after match play, further work is needed on the dose and frequency of these to assess their validity (Howatson and van Someren, 2008). Employing functional foods or concentrated versions has also received much attention as an in-season strategy, such as antioxidant rich tart cherry, blackcurrant, and pomegranate (Heaton et al., 2017). The issue with many of the controlled functional food studies is that the prescribed diets are devoid of fruit and vegetables in the days leading up to the exercise bout (Bell et al., 2016) , thereby not reflecting the real world of athlete dietary intakes (Close, Kasper and Morton, 2019). In fact, when athletes are allowed to consume their normal diet, the addition of polyphenols via tart cherry juice confer no benefit on markers of inflammation, muscle soreness and function following rugby league match play (Morehen et al., 2020). These findings are echoed in other recent work in soccer (Abbott et al., 2020) and rugby union (Kupusarevic, McShane and Clifford, 2019).

2.5.6 Potential alterations to metabolism after muscle damage

As we have discussed, the primary and secondary responses to damaged tissue after EIMD and IIMD include cellular interactions causing oxidative damage together with an inflammatory response and accompanying efflux of immune cells to repair damage and remodel tissue. There is evidence these cellular activities have an energy cost. The metabolism of fatty acids (Wolowczuk et al., 2008) with the availability of energy and glucose (Von Ah Morano et al., 2020) may have a profound impact on whole body metabolism (Pearce and Pearce, 2013) facilitating an effective immune response. Whilst there is a body of evidence supporting the incidence of this immunoendocrine response to

match play, there is no deeper knowledge of the metabolic requirements of the recovery period. The cytokine markers of inflammation previously investigated also have multiple purposes across body tissues (Pedersen et al., 2003) and effect substrate metabolism (Wolsk et al., 2010; Knudsen et al., 2017). We therefore propose investigating the metabolic changes these recovery processes invoke on the body as a whole system is essential.

2.6 Metabolomics

Metabolomics is a rapidly expanding field allowing large quantities of metabolic data to be generated opening up previously inaccessible areas of systems biology (Kell, 2004). The term metabolomics is the comprehensive characterisation of metabolites and other chemical species in biological specimens in response to a variety of perturbations or interventions (Khoramipour et al., 2022). These metabolites are classified as small molecules <1500 Da (Fiehn, 2002). The metabolome is the complete set of these low molecular weight metabolites that can be found in a cell, tissue, biofluid, or an organism (Khoramipour et al., 2022). The metabolites which make up the metabolome can be primary or secondary metabolites, endogenous and exogenous compounds. The study of these metabolites is the product of complex interactions occurring within the genome, transcriptome, and proteome of the cellular compartment, combined with environmental influences outside the cell (Macel, Van Dam and Keurentjes, 2010; Patti, Yanes and Siuzdak, 2012).

Prior to the development of this technique, much of our detailed knowledge of exercise physiology was generated utilising highly invasive muscle biopsies, but with the ability to utilise blood, urine, sweat, and saliva in a less invasive manner, the challenge to whole body homeostasis which exercise provokes may be investigated (Hawley et al., 2014).

2.6.1 Metabolomics biofluids

Unless access to tissue samples is easier using animal or cellular models, the collection of relevant biological fluids is generally preferred due to this simpler and less invasive collection of samples (González-Domínguez et al., 2020). These biofluids do also provide more information about the dynamic organ-organ interactions, and the complexity of the metabolome at the systemic level (Zhang et al., 2012). The recent review from Schranner

and colleagues highlights those biofluids most used in exercise studies as blood (serum or plasma), and urine (Schranner et al., 2020). The potential of saliva has been realised and has been utilised more readily since 2015 (Gardner, Carpenter and So, 2020). Table 2.8 Includes an overview of the most used biological samples in exercise and sport metabolomics studies and has been adapted from Khoramipour and colleagues (Khoramipour et al., 2022).

Table 2.8 Advantages and disadvantages of the most used biological samples in exercise and sport metabolomics studies. Adapted from the review of (Khoramipour et al., 2022).

Biological sample	Advantages	Disadvantages
Blood (serum or plasma)	Contains metabolites secreted or excreted by multiple tissues Includes endogenous metabolites primarily Indicates physiological changes well and tracks change well over time Can be analysed using all methods	Still an invasive collection method Difficult to detect small metabolites by NMR Samples must be processed consistently and efficiently as blood analytes can react with enzymes in the sample and degrade sample quality
Urine	Non-invasive collection method Contains endogenous and exogenous metabolites Contains higher concentrations of waste products of metabolism Generally free of larger proteins and macromolecules Processing and storage simple	High salt and urea concentration which makes MS analysis problematic Can be contaminated with bacteria and effected by environmental and dietary exposures Samples can be very complex metabolically
Saliva	Contains a wide range of low molecular weight metabolites Non-invasive collection method Processing and storage simple Provides a good reflection of the whole-body physiological conditions	Can be contaminated with bacteria and high molecular weight proteins Sample volume can be limited in individuals Pathological conditions of the mouth can affect sample quality, as can oral intake Concentration of endogenous metabolites is lower than in blood
Tissue (biopsy collection)	High concentrations of measurable metabolites Most accurate representation of conditions of the immediate location Specific sample sites can alter composition greatly May exclusively contain endogenous metabolites	Very invasive sampling Sampling volume or amounts are very limited Often contaminated with high molecular weight proteins

2.6.2 Analysis methods

The three most used techniques in metabolomics research are; liquid chromatography coupled with single stage mass spectrometry (LC-MS), gas chromatography coupled with mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy (Emwas et al., 2019). The mass spectrometry techniques are considerably cheaper and proved a greater degree of sensitivity than NMR which explains in part why so many studies have employed these LC-MS and GC-MS techniques so far in exercise metabolomics (Schranner et al., 2020; Khoramipour et al., 2022). NMR is non-destructive and so samples can be stored and re-examined later to validate findings as well as being highly reproducible in comparison to MS methodology (Emwas et al., 2019). Whilst NMR will return 50-200 identified metabolites, these will be unambiguous compared with the potentially 1000+ metabolites generated via MS techniques. This is due to the ability of NMR to attenuate the signals of higher molecular weight metabolites which can cause problems in spectral analysis without extraction techniques being required (Emwas, 2015). MS and NMR techniques can be used in targeted or untargeted metabolomics investigations. Each method has strengths and weaknesses but, the greater structural details provided by NMR allowing for more certainty in metabolite identification and the ability to examine the more abundant compounds from biological fluids means NMR may be the preferred form of analysis used in untargeted metabolomics (Markley et al., 2017). Please see table 2.9 For a summary of the major differences and considerations when choosing analysis methods.

Table 2.9 Major differences between mass spectrometry and NMR spectroscopy considering application in metabolomics research.

Consideration	Mass Spectrometry	NMR Spectroscopy
Sample processing	More time and labour intensive due to the ionisation steps which cause sample destruction	Less time required, non-destructive
Detection	Targeted, predefined metabolites	Untargeted and unbiased
Sensitivity	High sensitivity, but ionisation step can potentially cause metabolite loss	Only metabolites with higher abundance
Advantages	Identifies specific metabolites	Metabolic pathways can be analysed with confidence, cost-effective, and high throughput for large sample numbers

2.6.2.1 NMR Spectroscopy

Proton (^{1}H) NMR spectroscopy (^{1}H NMR) is employed in the vast majority of NMR-based metabolomic studies because ^{1}H atoms are found in almost every known metabolite (Emwas et al., 2019). Utilising this technique, a spectrum of distinct signals arising from compounds which contain one or more ^{1}H atoms in their structures can be generated and as ^{1}H provides the greatest signal intensity they create spectra with incredibly high resolution (Emwas, 2015).

NMR spectroscopy informs on molecular structure utilising magnetic moments of atomic nuclei. Angular momentum nuclear spin is dependent upon interactions between protons and neutrons within the nucleus (Bharti and Roy, 2012). ^{1}H nuclei have an odd number of protons and neutrons and therefore exhibit a spin of ½ and can adopt two different

orientations of equal energy. When a magnetic field is applied externally the energy levels split into higher and lower planes denoted by a magnetic quantum number (m). The change in energy state (ΔE) can then be calculated (Emwas, 2015). The resonance of the nucleus is measured after exposure to a pulse of radiofrequency radiation. The pulse and relaxation periods are calibrated for each sample and the decay of the oscillation is termed the free induction decay (FID). A Fourier transform is applied to the FID signal and this produces an NMR spectrum with the signal intensities proportional to the number of nuclei (Bharti and Roy, 2012).

The final NMR spectrum has separate peaks generated for each ^{1}H chemical environment within a sample. These are expressed by the dimensionless parameter, parts per million (ppm) from a reference standard. This allows spectra to be compared irrespective of the field strength in which the spectra were acquired (Emwas, 2015).

The next steps involve spectral cleaning, baseline corrections, and what is termed 'peak binning' to allow metabolite identification. These can also be complemented with specific biofluid software and reference libraries which can be online or generated 'in-house' utilising historical spectral data (Khoramipour et al., 2022).

2.6.3 Data analysis methods

Due to metabolomics being in its relative infancy, and especially when it comes to examining exercise exposures, there is no standardised statistical approach utilised. Employing a more classical univariate approach to initially reduce the large number of metabolites to only those showing the strongest changes across treatments or between sample groupings and correcting for the multiple comparisons ensures best practice (Bartel, Krumsiek and Theis, 2013). It is interesting that when compiling their recent review on

exercise metabolomics only ‘raw p-values’ were used to determine inclusion of metabolite changes (Schranner et al., 2020) as the use of corrections based on the number of spectral bins is not always employed, leading to potential confusion in conclusions from the literature. Whilst feature selection of biological samples using p-values is of value, predictive models based on the many metabolites detected necessitates employing multivariate methods (Gowda et al., 2008).

Multivariate analysis can be broadly divided into supervised and unsupervised methods. Principal Component Analysis (PCA) is the most used unsupervised method in metabolomic analysis. PCA explains the variance between biological samples by generating orthogonal and ranked principal components, creating a two- or three- dimensional plot clustering datapoints which share similarities (Gowda et al., 2008).

Supervised methods are used to classify differences between samples rather than cluster commonalities and build a predictive model. They use data to train the model before employing it to differentiate certain groups (Khoramipour et al., 2022). Partial least squares discriminatory analysis (PLS-DA) is a supervised statistical method used to optimise separation between predefined and identified sample groupings (Gowda et al., 2008). PLS-DA can handle noisy data, rank variables, and is resistant to multi-collinearity when the number of variables is larger than observations (Gromski et al., 2015). PLS-DA can be accompanied by neural networks and machine learning methods if the data set and experimental hypothesis requires. Random Forests (RF) is a supervised machine learning method utilised in metabolomics but due to a tendency of RF to over-fit models under some data distributions (Gromski et al., 2015), and concerns RF may not always be adapted to

spectral data due to classification methodology (Menze et al., 2009) it may not be optimal for NMR spectroscopy.

2.6.4 Biological interpretation of data

If unsupervised methods are used to generate principal components, further work of network plots is needed to understand relationships between identified metabolites. The interpretation of data may be negatively affected when focussing upon maximum variation between samples (Rosato et al., 2018). When supervised methods, such as PLS-DA are employed to identify metabolites discriminating between sample groups, these can then be entered into databases mapping them on human metabolic networks (Rosato et al., 2018). MetaboAnalyst provides a selection of tools online for pathway enrichment analysis, identification, multi-omics integration and biomarker analysis (Chong et al., 2018).

2.6.5 Metabolomics exercise studies

As we have reviewed, the exercise bouts rugby union players are exposed to at the elite level are complex with resistance training, high intensity running, and static exertions included together with rugby specific skills. We will now review those exercise metabolomics studies including modalities included in the sport and aligned to the physical qualities and demands of the sport as best we can from the literature.

A recent investigation into different phenotypes of athlete; bodybuilders, sprinters, and endurance athletes revealed very different responses of the metabolome to graded ergometry (Schranner et al., 2021). Blood levels of branched chain amino acids (BCAAs) were depleted to a greater extent in bodybuilders compared to other phenotypes after exhaustive exercise and may be caused by higher rates of protein synthesis and a greater habitual dietary intake (Schranner et al., 2021). Protein intake was observed in this research group to be ≈2.4g·kg body mass (Schranner et al., 2021) and not dissimilar to observations in

rugby union athletes (Bradley et al., 2015a; Black, Black and Baker, 2018; Posthumus et al., 2021).

The metabolic perturbations after resistance training have been investigated utilising NMR spectroscopy, and in healthy young male participant s the reductions in blood BCAA levels were also observed at 60 min post exercise (Berton et al., 2017). The participants were fed prior to the exercise bout and metabolites of leucine breakdown were observed immediately post resistance exercise with Krebs cycle intermediates implicating energy provision pathways in meeting training demands (Berton et al., 2017). 2-hydroxybutyrate was also identified as significant post exercise, indicating a cumulative metabolic stress after resistance training (Deminice et al., 2011), together with hypoxanthine (Berton et al., 2017).

Studies exploring the response of the metabolome to endurance exercise has also consistently identified changes in BCAA metabolism, but also products of glycolysis and TCA cycle intermediates, together with acylcarnitines associated with fatty acid metabolism (Khoramipour et al., 2022). The effect of running a marathon upon the metabolome has demonstrated the immense strain on energy systems indicating carbohydrate metabolism, amino acid degradation, and fatty acid metabolism all contribute to meeting fuel needs (Stander et al., 2018; Schader et al., 2020; Bester et al., 2021). The difference between these studies being that fatty acid metabolism is indicated in all three to meet energy needs during the event but, NMR highlights the increased concentrations of ketone bodies (Bester et al., 2021) whilst MS techniques include the appearance of specific fatty acid groups as significant in analysis (Stander et al., 2018; Schader et al., 2020). Acylcarnitines and fatty acids are best detected using the mass spectrometry methods rather than NMR spectroscopy which we should regard when reviewing these exercise studies as it may not

be the absence of evidence, rather the absence of the appropriate detection method having been utilised (Schranner et al., 2020). This is reinforced when the endurance phenotype athletes showed increased blood acylcarnitines compared with sprinters or bodybuilders when measured using MS techniques. All phenotypes increased metabolites associated with glycolysis and gluconeogenesis (Schranner et al., 2021). Exhaustive endurance exercise utilising NMR spectroscopy has identified significant rises in blood ketone bodies; 3-hydroxybutyrate, acetoacetate, and acetone in line with significant rises in lactate and alanine, implicating liver derived ketogenesis and gluconeogenesis for energy production (Kirwan et al., 2009).

As discussed, rugby union is not an endurance sport but requires the ability to repeat high intensity bouts of exertion. The best research into differing demands of intensity from Peake and colleagues matched the overall work of the participants whilst comparing high intensity interval (HIIT) training with a moderate intensity (MOD) bout (Peake et al., 2014). In this research, carbohydrate oxidation and blood lactate were significantly higher in HIIT compared with MOD, with fat oxidation and blood fatty acids not significantly different between exercise exposures (Peake et al., 2014). Alanine and the TCA cycle intermediates citric acid, aconitic acid and, succinic acid were higher immediately post HIIT compared to MOD, but then declined in the first two hours post exercise (Peake et al., 2014). Finally, one metabolite which did not fall within the study's classical metabolite groupings was 2-hydroxybutyrate indicating the HIIT exercise caused extensive metabolic stress resulting in its formation (Peake et al., 2014).

In all these studies the acute sampling did not extend beyond 3 hours post exercise bout. The review of metabolomics exercise studies by Schranner and colleagues highlighted that

research has not ventured past 24 hours post bout when researching acute exercise changes to the metabolome (Schranner et al., 2020). There are however 'long-term' studies whereby the prescription of exercise over a series of weeks has been researched to investigate chronic responses to training (Khoramipour et al., 2022). Any investigation into the effects of rugby union match play would be examined as acute changes to the metabolome, but the timeline for recovery may extend past the 24hour mark when investigating any effects of muscle damage as discussed in section 2.5.

2.6.6 The evolution of 'Sportomics'

There has not been any research specifically in rugby or collision-based team sports utilising metabolomics. However, there is relevant research which we can review and use to guide our investigations together with how the application of metabolomics in sport has evolved.

The term Sportomics was first described in 2011 via Resende and colleagues when assessing and then programming the metabolic and physical preparation of a world-class windsurfer (Resende et al., 2011). It was an exciting case study demonstrating the potential to incorporate metabolomics into the programming of athletes and improve performance (Resende et al., 2011). Bassini and Cameron then published a conceptual paper in 2014 which appeared to be a claim to the 'Sportomics' protocols and techniques (Bassini and Cameron, 2014). There was however little in the way of specific directions given in this paper and it was more of a review attempting to validate a number of the authors previous observations and appears to be them staking their claim to the 'Sportomics' brand promoting its use primarily with elite athletes (Bassini and Cameron, 2014). They did make some valid proposals around the value in small subject numbers and ensuring they are representative of a larger population whilst also providing caution as to the number of variables in this type of research and how hypotheses should be treated (Resende et al.,

2011). It wasn't until the 2019 review by Bongiovanni et al where the landscape was given clarity in the statement that 'Sportomics is non-hypothesis-driven research on an individual's metabolite changes during sports and exercise' (Bongiovanni et al., 2019)(page 11012).

The early Sportomics publication suggested that unsupervised methods such as PCA were appropriate for data analysis due to their ability to detect small associations (Bassini and Cameron, 2014). The argument was poorly constructed and based upon the differences between 1st and 10th place in major games in the 100m sprint to be 2% or less, it is ironic that they also placed this under 'statistical methods' which are not based upon statistical methodology for data analysis. The later review does appreciate the inclusion of supervised and unsupervised methods (Bongiovanni et al., 2019) but for reference and comparison, the wider clinical metabolomics community already included the reasoning for including supervised and unsupervised methods as part of the workflow in best practice of data analysis (Alonso, Marsal and Julià, 2015). As we have already discussed under section 2.6, the most recent review aligns the data analysis and workflows of the wider metabolomics research applications with sport and exercise research pushing forward the ability to appraise the value of research in 'Sportomics' (Khoramipour et al., 2022).

2.6.7 The application of 'Sportomics' in highly trained and elite athletes

In elite athletes there is one large scale observational study of 191 participants from a variety of sports which successfully distinguished between 'power' and 'endurance' athletes utilising MS methods and a combination of supervised and unsupervised multivariate techniques for data analysis (Al-Khelaifi et al., 2018). The authors recognised the serious limitations of this study due to the samples being generated via doping control samples, meaning they were both 'IN' and 'OUT' of competition samples so there was no control over

whether the participant had just completed exercise or competition, but also no control over the fed or fasted state of the athlete. For these reasons the separation or identification of an athlete 'phenotype' is interesting but specific metabolites should be viewed with caution from this research (Al-Khelaifi et al., 2018).

As the quality of participants moves towards the highly trained and elite level, the use of less invasive techniques, the reasons for which we have discussed herein, becomes more apparent. The saliva metabolome was able to separate the best and worst performing professional footballers when tested physically using the Yo-Yo test but there was no information as to which metabolites differentiated the participants (Santone et al., 2014). Football players have been investigated in-season utilising both urine (Prado et al., 2017; Quintas et al., 2020), saliva samples (Ra et al., 2014; Pitti et al., 2019), and multiple biofluids (Alzharani et al., 2020). Commonalities across these studies are the presences of purines such as hypoxanthine after match play with a reduced level in saliva (Pitti et al., 2019), increases in urine (Prado et al., 2017) and increases in both saliva and urine after moderate intensity football training (Alzharani et al., 2020). Hypoxanthine increases were also associated with external training loads when the urine metabolome was sampled at five timepoints during the football season (Quintas et al., 2020) making it a metabolite of interest potentially associated with fatigue. When 122 male football players were exposed to three matches on consecutive days the salivary metabolome was investigated with traditional indices of fatigue such as heart rate, body mass and mood scores (Ra et al., 2014). The authors reported metabolites associated with changes in energy provision indicating increased gluconeogenesis with fatigue, but also increases in potential skeletal muscle degradation signified by the marker 3-methylhistidine (Ra et al., 2014). The saliva metabolome yielded the same metabolite indicative of muscle breakdown in the latter

stages of competitive basketball match play (Khoramipour et al., 2020). Metabolites evidencing the high intensity nature of basketball match play identified at the beginning of each half were lactate, pyruvate, and TCA cycle intermediates together with hypoxanthine (Khoramipour et al., 2020). As each match entered the second and fourth quarters, the appearance of alanine indicative of gluconeogenesis and fatty acid metabolism signified by acetoacetate and glycerol (Khoramipour et al., 2020)

The only research involving combat and potential damage to muscle after competition utilised elite Brazilian Ju-Jitsu practitioners to investigate ammonemia and immune responses (Gonçalves et al., 2012). The authors supplemented with arginine and revealed a novel attenuation of ammonia production and lymphocyte response to competition. However, the athletes were predisposed to ammonemia via a low carbohydrate diet in the days leading up to competition, very different to the likely preparation of these athletes normally entering competition, or collision team sport athletes (Gonçalves et al., 2012). There were no details of metabolic pathway enrichment analysis in the study publication (Gonçalves et al., 2012).

Overall, the application of metabolomics in sport is an exciting and rapidly growing field. The research we have discussed demonstrates the ability to identify metabolic pathways and specific metabolites affected by exposure to sport and exercise providing an unbiased perspective of the whole biological system. However, as we have also tried to demonstrate there are many considerations when designing metabolomics research to ensure the results have been processed and analysed in the most suitable way to generate worthwhile hypotheses.

2.6.8 Considerations for the application of metabolomics

As discussed, research in rugby union has previously investigated specific markers of fatigue, muscle damage and inflammation but the metabolic perturbations around competition and training have never been studied.

An initial piece of research should be untargeted in nature and as per the prior discussion, NMR spectroscopy would appear the preferred technique to acquire spectra due to the unambiguous identification of metabolites and non-destructive steps in sample preparation.

The most common biofluids used in exercise and sport specific metabolomics research are derived from blood (Schranner et al., 2020), with serum being utilised more frequently since 2018 (Khoramipour et al., 2022). Urine has been utilised more readily since it has been comprehensively screened (Bouatra et al., 2013). Saliva is the least utilised but may also hold the most potential (Santone et al., 2014; Dame et al., 2015). There is also evidence utilising more than one biofluid to build a complementary picture of the active metabolic pathways, may be the best proposal (Do et al., 2015).

However, the concentration of biomarkers in saliva and blood cannot be used interchangeably in healthy adults (Williamson et al., 2012). Whilst the comparison of lactate levels for example, in both biofluids is better in trained athletes, they do exhibit a differing response to maximal exercise (Tekus et al., 2012). The review of exercise studies carried out by Schranner and colleagues illustrated this point well in that metabolites from blood and urine associated with carbohydrate metabolism, TCA cycle, and amino acid metabolism, could differ greatly in response to exercise (Schranner et al., 2020). Metabolite changes in lactate and pyruvate for example, were a similar positive fold change whilst citrate and succinate could be seen to have opposite changes with increases in blood mirrored with

decreases in urine. The converse responses were also true of some amino acids (Schranner et al., 2020). We would propose the gathering of multiple biofluids at the same sample timepoints serves to investigate these differences further and to establish whether these differing responses occur due to athlete phenotype as well as exercise stimulus (Schranner et al., 2021)

Finally, in data reduction and statistical analyses, a combination of univariate analysis and supervised multivariate modelling to identify significant metabolites between samples should provide the information necessary to investigate changes to biological pathways throughout the sample collection period. It is crucial though that the quality of any models generated and the thresholds for metabolites to be taken forward to pathway analysis comply with best practices as too few studies have reported aspects such as reporter operating characteristics (ROC) to provide assurance models are not over-fitting the data and true separation between samples is achieved (Alonso, Marsal and Julià, 2015; Khoramipour et al., 2022). Another standard which has not yet translated from clinical studies into sport and exercise metabolomics, is the deposition of data for public access (Khoramipour et al., 2022). To achieve much needed transparency in this area, depositories such as MetaboLights should be used to make data publicly accessible (Haug et al., 2013).

2.7 Summary and directions for research

In Summary, the sport of rugby union at the elite level has evolved since professionalism with the demands and physical profiles of domestic players similar to that of international competition. Whilst total distances covered have remained consistent, the intensity of movement at higher speed thresholds and increased demands placed upon the ability to accelerate and generate force into collisions has never been greater. The physical profile of

players requires high levels of lean muscle mass with accompanying pragmatic levels of bodyfat to complement these physically intense demands. With the relentless nature of a season of rugby union, the aim of 'recovering' between fixtures, or more accurately the ability to reproduce a level of physical performance week-on-week has never been more important despite the realisation residual soreness will be present throughout.

Despite an understanding that competition results in muscle damage both derived from the movement and collision demands of the sport, and that these may cause the exceptionally high energy expenditures reported, there is no knowledge of whether the metabolic requirements of the athletes change during recovery. The insight into markers of inflammation, immunoendocrine, and oxidative stress responses to match play has resulted in attempts to improve perceptual fatigue and decrease loss of function rather than trying to understand how we might meet any metabolic requirements.

For those reason we propose the examination of the only facet of energy expenditure we can measure in a controlled fashion regularly to ascertain how resting metabolism may change on a day-to-day basis throughout a competitive match week. The metabolic perturbations due to these stressful responses to match play are also unknown. With methodology now available to investigate this viewing the effect of a competitive match week on the whole system may provide valuable insight as to changes to metabolic pathways throughout recovery. Finally, the impact of this repeated process upon the physical profiles of these players is also unknown. Understanding what factors contribute to elite rugby union players maintaining optimal physical profiles at the 'business end' of a long season where competitions are won, may support consistent performance levels throughout.

Chapter Three

GENERAL METHODS

This chapter details the methods employed across multiple studies within this thesis. Methods unique to a particular chapter are presented in the methods section of that specific study chapter.

3.1 Ethical approval and location of testing

The local ethics committee of Liverpool John Moores university (LJMU) approved studies 1 (Chapter 4) and 2 (Chapters 5 & 6) in this thesis. Studies 1 & 3 were also approved by the ethics committee of Birmingham City university (BCU) due to the use of DXA scan facilities loaned to the lead researcher for use on-site at Gloucester rugby club (Study 1) and further access to the scanner at (BCU) for body composition analysis (Study 3). All subjects were fully informed of the nature of any testing and the requirements of studies. This information was provided verbally and in writing. The participants provided fully informed consent and understood they were free to withdraw at any time during the studies.

DXA scanning for study 3 took place at the South City Sports Centre at BCU, Birmingham (Fig 3.3). The DXA scanning and metabolic gas exchange data for study 1 were both collected on-site at Gloucester Rugby training facilities located at Hartpury College, Gloucestershire (Fig 3.1). The training and match GPS, load, and session content data for all studies were collected at both the Gloucester Rugby training facility and Kingsholm Stadium, Gloucester where any match play events took place (Fig 3.2). The collection of biofluids for study 2 were carried out at both the Gloucester Rugby training facility and the Kingsholm stadium medical facilities. All biofluid processing was carried out at the training facility in the medical room together with temporary storage at -24^{o}C in a securely locked medical freezer before permanent storage at LJMU at -80^{o}C. Gatekeeper consent was obtained, and risk assessments thoroughly conducted for all venues. All corresponding documents for research were logged and stored on the LJMU SharePoint facility to ensure latest versions were accessible.

Figure 3.1 The Gloucester Rugby training centre. Where all data collection for Chapter 4 took place, together with training day samples and data for Chapters 5 & 6.

Figure 3.2 Kingsholm Stadium, the home of Gloucester Rugby Club in the heart of the city of Gloucester, UK. Where match data was collected for all chapters and biofluid samples were collected immediately post-match for Chapters 5 & 6.

Figure 3.3 Birmingham City University South Campus where body composition measurements were taken for Chapter 7.

3.2 Participants

All participants were full time professional rugby union players part of the Gloucester Rugby squad. A total of 75 participants were involved in the studies across three full playing seasons. Some players took part in more than one study, and the characteristics of each study group can be seen in Table 3.1.

Table 3.1 Summary of participant characteristics from all three studies including age, height, and body mass. Data are presented as mean ±SD.

	N	Age (yrs)	Height (cm)	Body mass (kg)
Study 1 (Chapter 4)	22	26 ±4	185.3 ±6.9	104.6 ±12.6
Study 2 (Chapters 5 & 6)	7	22 ±3	182.6 ±7.4	102.5 ±13.7
Study 3 (Chapter 7)	46	24 ±4	186.3 ±6.9	101.9 ±13.9

3.3 Anthropometric assessments

Chapters 4 and 7 included measurements of participant anthropometry. Prior to measurements all participants removed jewellery and wore only underwear ensuring no zips or metallic objects were in the garments.

3.3.1 Height and body mass assessment

Body mass and height were assessed to the nearest 0.01kg and 0.1cm respectively, using a digital measuring station (Seca, Hamburg, Germany). These measurements were taken when the players arrived at the testing site, either the Gloucester Rugby training ground (Chapter 4) or Birmingham City University South campus (Chapter 7) prior to eating or undertaking any exercise.

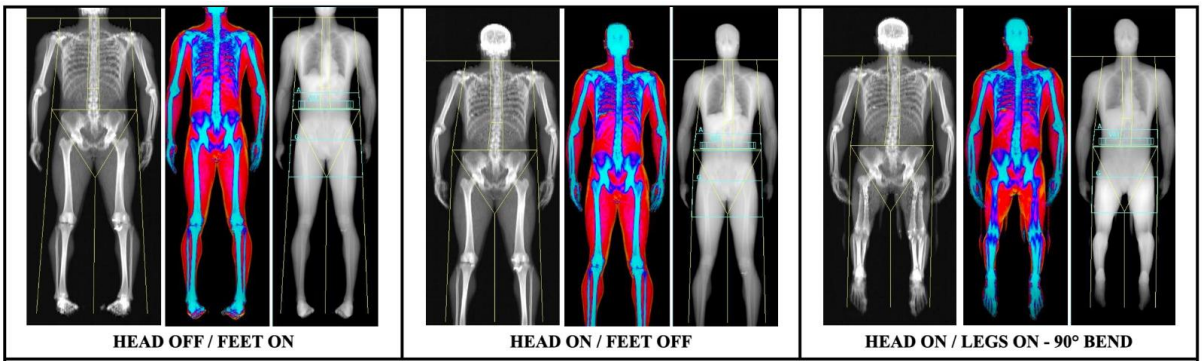
3.3.2 Dual energy X-ray absorptiometry

In studies 1 (Chapter 4) and 3 (Chapter 7), the body composition of participants was assessed via dual-energy X-ray absorptiometry (DXA). Each assessment meant the

participant underwent a whole-body fan beam scanner (Hologic Horizon W, Hologic, Bedford, MA) where the effective radiation dose was 8µSv or 0.008mSv. This is considered a safe and ethical radiation dose (IRMER, 2006). Every morning prior to player scans, calibration was carried out as per the manufacturer's guidelines using a spine and step phantom and then followed by a radiographic uniformity scan.

Players presented for each DXA scan under standardised conditions: rested, having not partaken in any exercise for at least 18 hours, and fasted, having not eaten for at least 8 hours (Nana et al., 2015). Urine osmolality was tested prior to each scan to ensure satisfactory hydration levels as this may affect measurement of lean tissue mass (Toomey, McCormack and Jakeman, 2017). Each scan lasted ≈420 seconds. Players lay supine on the DXA scanner bed with the palmer surface of the hand facing towards each leg alongside the body, and the feet in a plantar flexed position. These positions were comfortably maintained throughout the duration of the scan and kept in position utilising tape to ensure standardised gaps between limbs were consistent (Nana et al., 2015). The wide bed of the newer Horizon W machine meant all participants could fit without the need to sum two partial scans. The decision was made to remove the head from all scans for uniformity and to accommodate the taller players without multiple scans each time. Scanning techniques to accommodate taller individuals have highlighted substantial errors as the "head" section of the scan is not directly measured, rather, assumptions are made within the machine algorithm leading to overestimations in both lean and fat mass (Nana et al., 2012). Figure 3.4 includes a test utilising three body positions for a taller subject which was included in the narrative review of body composition testing methods (Kasper et al., 2021) and as part of the preparation for the research herein. It was decided that although lean mass was not

greatly affected, the variation in fat mass was consistent with the prior research and that the head would be removed from analysis (Nana et al., 2012).



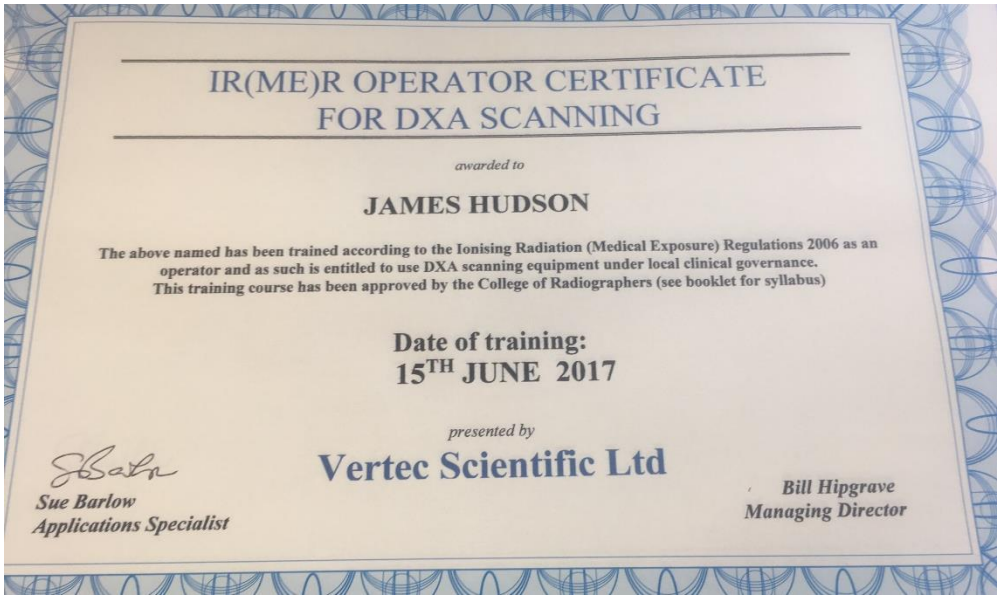
Characteristics - Caucasian, Male, Age: 35 years, Height: 201.0 cm, Weight: 103.5 kg
DXA - QDR Series Horizon, Hologic Inc., Bedford, MA

SCAN	Fat Mass (kg)	Lean Mass (kg)	BMC (kg)	Body Mass (kg)	Body Fat (%)
HEAD OFF / FEET ON	13.3	79.3	4.0	96.5	13.8
HEAD ON / FEET OFF	16.6	79.4	4.1	103.0	16.6
HEAD ON / LEGS ON	14.2	79.1	4.2	97.6	14.6

Figure 3.4 An example of how the constraints of the DXA bad may affect results depending upon technique of measurement chosen. From left to right; head off/feet on, head on/feet off, and head on/legs on – 90˚ bend. This was published as part of the narrative review (Kasper et al., 2021).

The Hologic Horizon has been precision tested and the coefficient of variation (CV) measured for total body fat mass (FM) 0.89% and lean mass (LM) 0.51% (Cheung, Roff and Grossmann, 2020). Two machines of the same model when tested in parallel were found to have CV of 0.78% and 0.77% for FM, with 0.52% and 0.40% for LM (Nowitz and Monahan, 2018). Our CV for the period of scanning within this study was 0.253% as recorded by the individual machine we used for all scans. This CV was calculated from the daily quality control (QC) scans of the spine phantom throughout the research period.

All scans were automatically analysed using the Apex software version 13.5.3.1(Hologic, Bedford, MA) by the same trained operator (Figure 3.5 displays operator qualifications of the PhD candidate). The values presented in Chapters 4 and 7 are sub totals (without the head region as discussed above) for lean mass or fat free soft tissue mass (kg), fat mass (kg), and per cent body fat (%).


IR(ME)R OPERATOR CERTIFICATE
FOR DXA SCANNING

awarded to

JAMES HUDSON

The above named has been trained according to the Ionising Radiation (Medical Exposure) Regulations 2006 as an operator and as such is entitled to use DXA scanning equipment under local clinical governance.
This training course has been approved by the College of Radiographers (see booklet for syllabus)

Date of training:
15TH JUNE 2017

presented by

Vertec Scientific Ltd

Sue Barlow
Applications Specialist

Bill Hipgrave
Managing Director

Figure 3.5 Evidence of the PhD candidate having undertaken training to operate and analyse DXA scans utilising the Hologic machine for this research in accordance with IRMER regulations 2016.

3.4 Quantification of training and match load

3.4.1 Global positioning systems

Throughout studies 1 and 2 the external demands of all rugby training sessions and match play were recorded using micro-technological units worn by players containing GPS (10Hz) and accelerometer (100Hz) (Catapult Innovations, Melbourne, Australia). All players consistently wore the same unit throughout each season for all sessions unless a unit had to be replaced due to hardware failures. The device was secured in a custom-built manufacturer provided vest (Catapult Innovations, Melbourne, Australia) which positioned the device between the scapulae. Acquisition of satellite signals and synchronisation of the GPS clock, with the satellite's atomic clock was performed 30 minutes prior to data collection(Maddison and Ni Mhurchu, 2009). Data were downloaded and analysed using Catapult Sprint software (Catapult Innovations, Melbourne, Australia). The total distance covered, number of high-speed efforts (>60% positional average) and the number of very high-speed efforts (>80% individual average) were recorded (Reardon, Tobin and Delahunt, 2015; Tierney et al., 2017). The GPS sampling frequency of 10Hz is the most reliable in team sports measuring high-speed running activities (Rampinini et al., 2015).

3.4.2 Session ratings of perceived exertion

Internal loads for each training day and the game day were assessed by the session rating of perceived exertion (sRPE) using a modified 10-point Borg scale (Foster et al., 2001). These included all gym based, skill focussed and rugby specific pitch sessions. This RPE of the training session was multiplied by the training duration to calculate a player load in arbitrary units (sRPE; AU) (Foster et al., 2001). The specific training weeks and session contents are detailed in each Chapter.

3.4.3 Subjective ratings of wellness, recovery, and sleep

Throughout the competitive match week studied in Chapters 5 and 6 the participants provided ratings of perceived muscle soreness, vigour, non-training stress, and sleep quality using a 1-7 Linkert scale. The players also reported sleep duration in hours slept each day. The participants were familiar with this process, and it was part of their normal requirements each day. They reported individually to prevent any potential influence from other players or staff. This process was formulated based upon the most effective forms of questions asked to gain subjective feedback form athletes (Saw, Main and Gastin, 2016) and previous work in rugby league has utilised similar panels of questions (McLean et al., 2010; Twist et al., 2012) when studying fatigue and recovery from match play.

3.4.4 Dietary Intake

Study 3 (Chapters 5 and 6) recorded dietary analysis of the whole competitive match week. Dietary intake was recorded using the participants mobile phone device incorporating the 'Snap'n'Send' method (Costello et al., 2017). The athletes were educated in their nutrition requirements. A wide range of meals and snacks designed by the team nutritionist were provided at the training facility. Their choices and portions in the club dining facility and whilst away from there were self-selected. The dietary analysis software Nutritics (Nutritics Ltd., Dublin, Ireland) was used by a registered sports and exercise nutritionist (SENr) to analyse food intake over the match week. Analysing dietary intake has also allowed us to account for metabolites associated with the ingestion of foods and any dietary supplements in our conclusions (O'Gorman, Gibbons and Brennan, 2013).

3.6 Biofluid collection and primary processing

3.6.1 Blood sample collection

Blood samples were collected every morning apart from the GD time point when the samples were taken post-match play. Post-waking, participants came straight into the training ground in a fasted state and provided a blood sample. Blood samples post-match were collected within 30 minutes of the final whistle, enabled by two researchers operating in the medical room of the stadium (Figure 3.6). Whole blood samples (10ml) were drawn from a superficial vein located in the antecubital fossa of the forearm using standard venepuncture techniques. Samples were collected using serum tubes (Vacutainer Systems, Becton Dickinson) which did not contain clotting gels or additives as these may interfere with metabolomics analysis (Phelan and Lian, 2016). Samples were allowed to clot at room temperature (18-22C̊) for 40 minutes prior to centrifugation at 1600g for 15 minutes.

3.6.2 Urine and saliva sample collection

Urine was collected and centrifuged at 1600× *g* for 15 min in 15 mL urine centrifuge tubes (Sarstedt, Leicester, UK) which contained no citrate or other stabilizers.

Saliva samples were collected using the previously validated Salivette swabs (Salivette Sarstedt, Nubrecht, Germany) without additives, centrifuged at 1500× *g* for 15 min (Santone et al., 2014).

All samples were aliquoted into 2 mL cryovials (Fisherbrand, Loughborough, UK) and immediately frozen at −24 °C. Upon completion of data collection all samples were transferred to -80 ^{o}C for longer term storage, prior to metabolomic processing and spectral analysis. The samples were used well within the nine month guide for storage best practice

(Teahan et al., 2006). Again, the time taken to process the samples each day was recorded and rigorously replicated to ensure reduced between-sample variability as a result of sampling and the highest possible sample quality (Figure 3.7) (Beckonert et al., 2007).

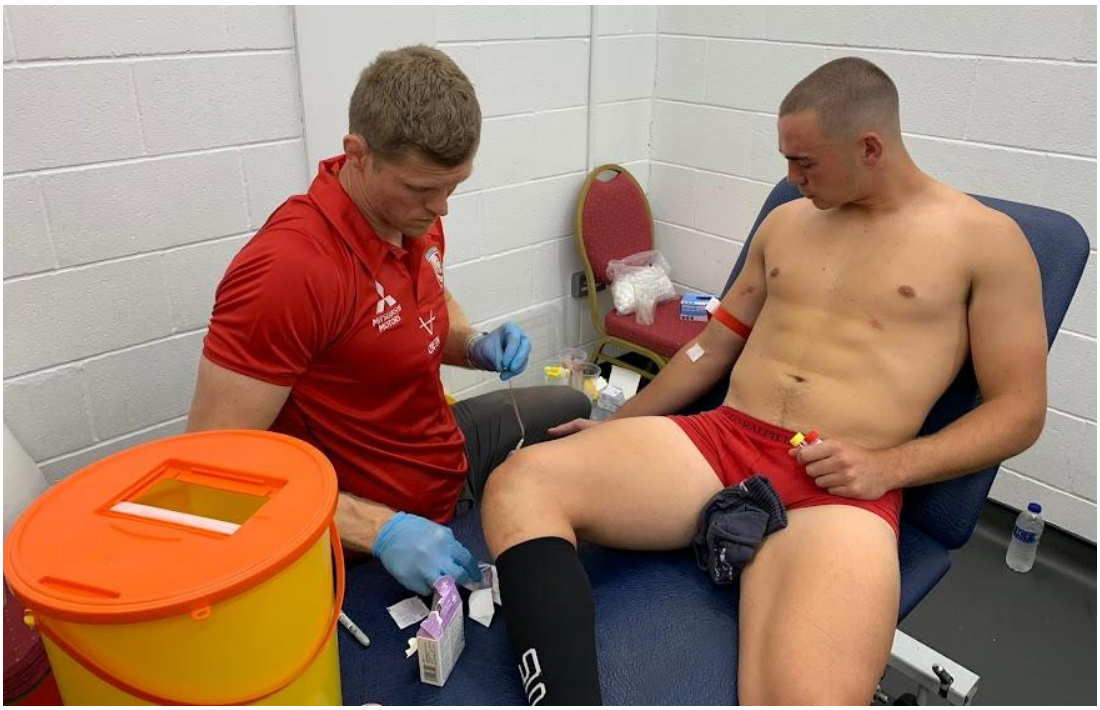


Figure 3.6 Blood samples being collected in the Kingsholm stadium medical room immediately post-match.

Figure 3.7 Blood, urine, and saliva samples collected from participants with timings recorded to allow accurate replication of processing times every morning and post-match.

3.7 NMR Spectroscopy

3.7.1 NMR sample preparation

Aliquots were thawed and 500 µL of serum was diluted to a final volume containing 50% (v/v) serum, 40% (v/v) dd $^{1}H_2O$ (18.2 MΩ), 10% (v/v) 1 M PO_4^{3-} pH 7.4 buffer (Na_2HPO_4, VWR International Ltd., Radnor, Pennsylvania, USA and NaH_2PO_4, Sigma-Aldrich, Gillingham, UK) in deuterium oxide ($^{2}H_2O$, Sigma-Aldrich) and 1.2 mM sodium azide (NaN_3, Sigma-Aldrich). Samples were vortexed for 1 min, centrifuged at 13,000 × *g* at 4 °C for 2 min and 600 µL transferred into 5 mm outer diameter NMR tubes (Bruker, Coventry, UK) using a glass pipette.

Urine and saliva samples (500 µL) were thawed at room temperature before addition of 500 µL 1 M phosphate buffer (Na_2HPO_4 and NaH_2PO_4) at pH7.4 with 20% $^{2}H_2O$, 200 µM TSP and 2.4 mM sodium azide. The samples were vortexed for 30 s prior to 5 min centrifugation at 21,500× *g* and 4 °C before transferring 600 µL of sample to Bruker SampleJet 5 mm (outer diameter) NMR tubes. The final concentration in the NMR tube was 50% urine or saliva, 10% $^{2}H_2O$, 1.2 mM sodium azide and 100 µM TSP.

3.7.2 NMR acquisition

Non-targeted 1D ^{1}H NMR spectra were acquired at 37 °C using a 700 MHz Bruker Advance IIIHD spectrometer equipped with a TCI cryoprobe and chilled Sample-Jet autosampler. 1D ^{1}H NMR standard experiment with the cpmgpr1d filters (Bruker, Coventry, UK) for selective observation of low molecular weight components with optimal water suppression was acquired, pulse sequence is vendor supplied using Carr-Purcell-Meiboom-Gill (CPMG) sequence. Spectra were acquired with 32 transients a 30-ppm spectral width, 64k points, 9.6 ms echo time and a 3.1 s acquisition time and a 4 s interscan delay.

Urine and saliva 1D ^{1}H-NMR spectra were acquired at 25 °C to facilitate analysis via Chenomx Standard library. Urine spectra were analysed via 1D ^{1}H-NMR standard pre-saturation experiment for optimal water suppression (vendor supplied noesypr1d). Nuclear Overhauser effect (NOE) spectra were acquired with 32 transients a 25-ppm spectral width, 96k points, 2.7 s acquisition time and a 4 s interscan delay.

Full spectrum parameter sets are available with the data deposited at MetaboLights public repository (Haug et al., 2013) ID number MTBLS2967.

3.7.3 Spectral processing and annotation

All spectra were analysed to ensure conformity with the recommended minimum reporting standards set out by the Metabolites Standard Initiative (MSI) (Sumner et al., 2007),(Considine et al., 2017). Serum spectra were aligned to glucose anomeric peak at 5.24 ppm. Urine and saliva spectra were aligned to the TSP peak at 0 ppm. Spectra underwent automated data processing, Fourier transformation and phasing carried out in Topspin v3.6 software using standard Bruker routines (apk0.noe).

Serum spectral peaks were annotated using a combination of Chenomx standard spectra and in house metabolite libraries with pattern files produced for both biofluids to enable spectral binning. Serum spectra were integrated into 160 bins with 104 (65%) annotated corresponding to 38 metabolites and 56 unknown metabolite bins. Spectra were binned or bucketed per peak into a matrix of metabolite peak intensities using tameNMR.

Saliva spectral peaks were annotated using a combination of Chenomx standard spectra and in house metabolite libraries with pattern files produced for both biofluids to enable spectral binning. Saliva spectra were integrated into 251 bins with 134 (53%) annotated

corresponding to 82 metabolites and 117 unknown metabolite bins. Spectra were binned or bucketed per peak into a matrix of metabolite peak intensities using tameNMR.

Chenomx v8.2 software was used to perform metabolite annotation on the individual urine spectra using automated fit all metabolites routine. Of the 251 metabolites annotated in Chenomx for the 117-sample set 46% (13,696 values) were missing. To provide a missing value estimation for this dataset metabolites with too many missing values <50% were removed—this reduced the number of annotated metabolites to 131. The remaining missing values were replaced by an estimate of the limit of detection corresponding to 0.2 of the minimum positive value of each variable. Manual confirmation of identities where possible to in-house standards for metabolite peaks found to be significantly variable.

Bin annotation and binned data for all spectra is available with the dataset in Metabolights (MTBLS2967).

3.7.4 Data analysis

Univariate and multivariate analyses was performed using R (Version 3.6.1, The R foundation for statistical computing). The scripts used were provided by the Computational Biology facility at the University of Liverpool (UK). Prior to univariate analysis, normalization was performed. The spectra can be seen in figure 3.8, with no visible difference between normalisation utilising total area (3.8.b) or post PQN (3.8.c). As PQN normalisation is reported to be the most robust method in the analysis of complex biofluids (Dieterle et al., 2006; Kohl et al., 2012) that technique was used.

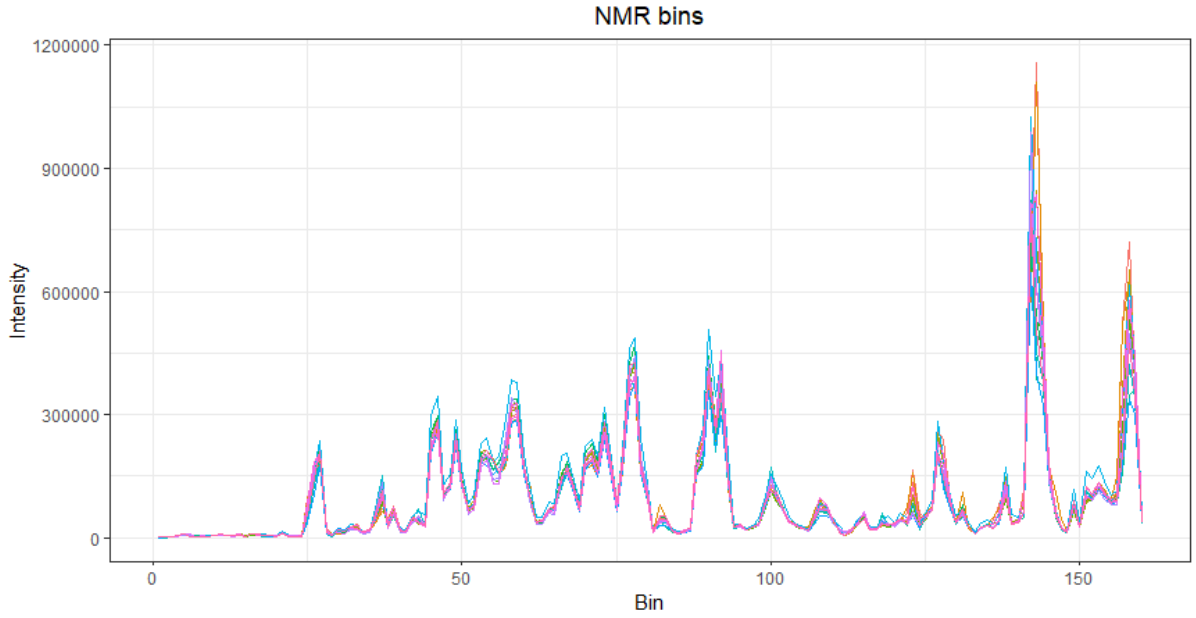
Further to this, data were scaled and mean-centred prior to multivariate analysis. Figure 3.9 demonstrates visually the difference between ‘Auto’ (3.9.a) and ‘Pareto’ scaling (3.9.b). Pareto scaling, where the square root of the standard deviation is used as a scaling factor

rather than standard deviation alone, with mean centring was performed as the preferred method to ensure the ability to identify small biologically significant variations in metabolites (Smolinska et al., 2012). This was expected as NMR spectra is less suited to auto-scaling (Euceda, Giskeodegard and Bathen, 2015).

Univariate analysis was performed on the PQN normalized spectra across the week using a one-way ANOVA using a Benjamini-Hochberg (FDR) method of multiple correction at a significance level of $p < 0.05$. A post-hoc Tukey analysis provided pairwise comparisons of specific time points. Partial least squares discriminant analysis (PLS-DA) was used for multivariate analysis, specifically identifying differences in metabolites between time points. Models generated via PLS-DA were evaluated using a random 30% of the data held back to test the model and produce receiver operator characteristic (ROC) scores. Specific metabolites within each model were only used for further analysis if the Variable Importance in Projection (VIP) scores were above 1.00 and ROC scores ≥ 0.75.

Pathway analysis was performed using MetaboAnalyst (Enrichment analysis, version 4.0, metaboanalyst.ca) (Chong et al., 2018). Ranked *p* values are reported here where $p < 0.05$ without adjustment. Only specific metabolites identified using PLS-DA between time-points were entered into the enrichment analysis. Heatmaps were generated incorporating metabolites identified from univariate and multivariate analysis. The fold change is reported relative to GD-1 and then natural log is displayed.

3.8a).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900000
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300000
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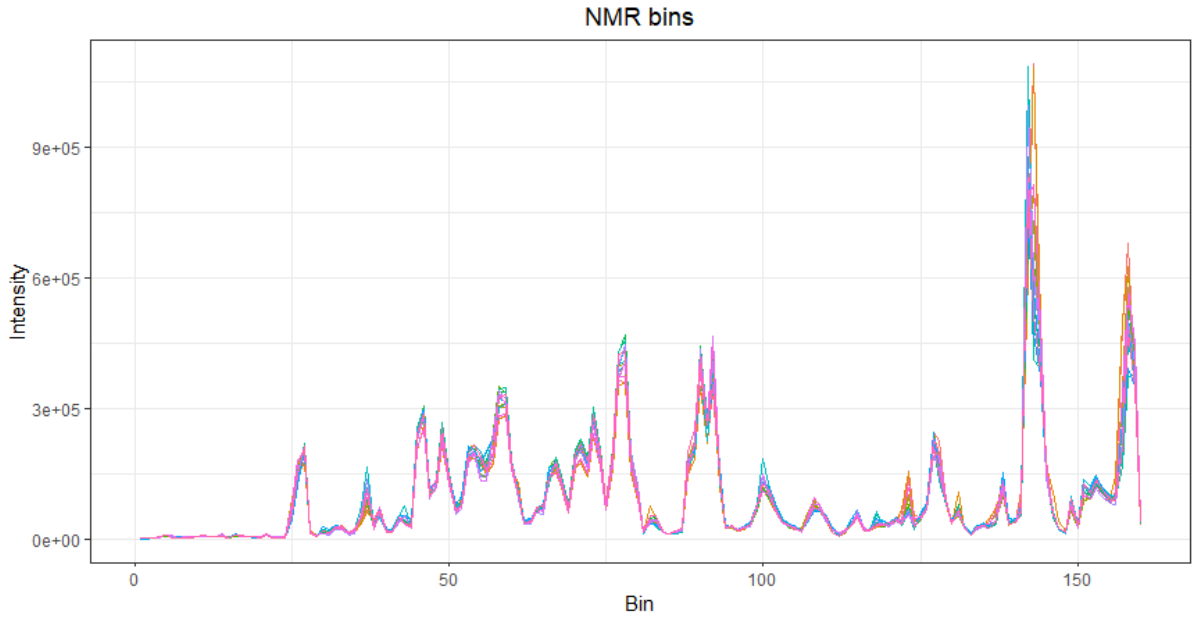
100
150
Bin

3.8b).


NMR bins
9e+05
6e+05
3e+05
0e+00
Intensity
0
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100
150
Bin

3.8c).

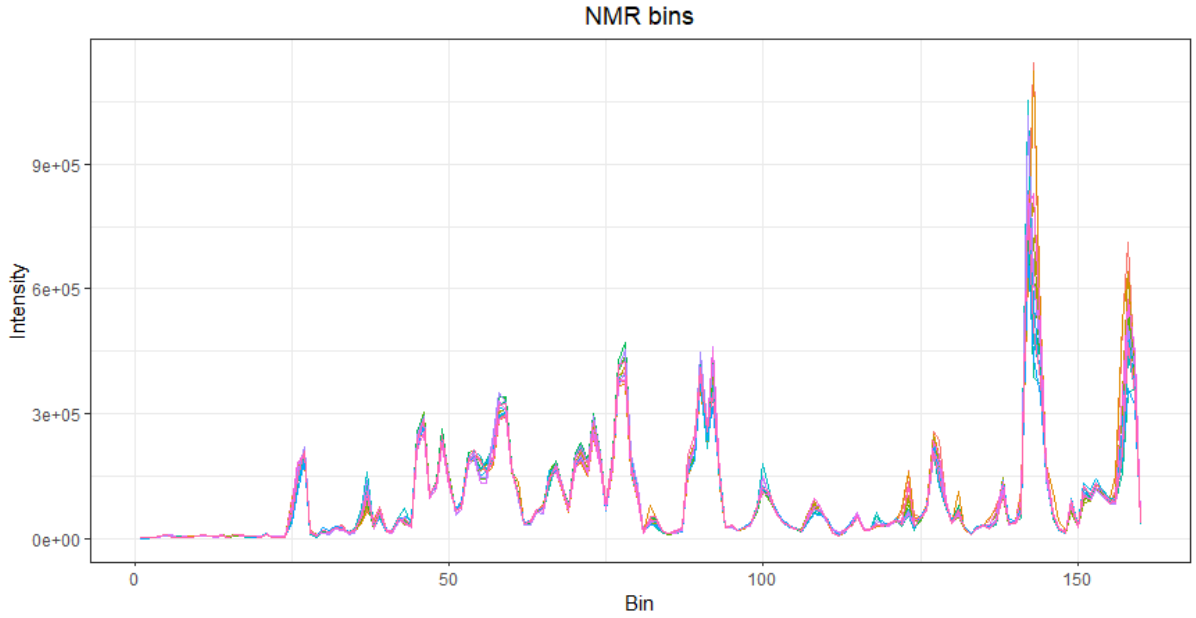
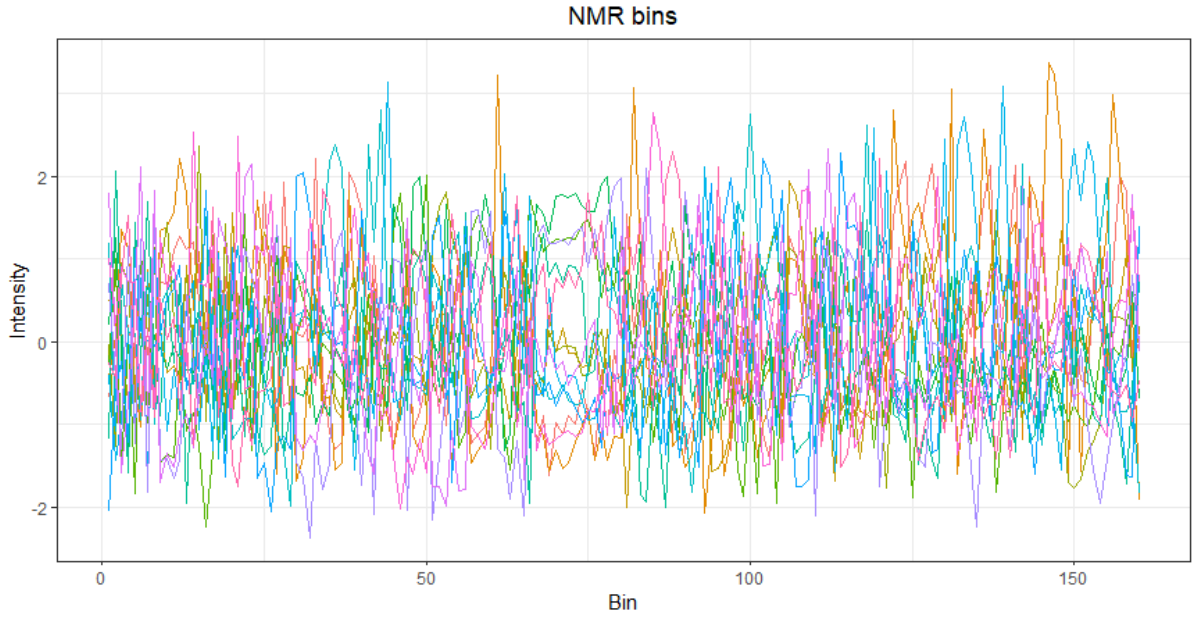


Figure 3.8 Representative NMR spectra a). prior to normalisation, b). post normalisation by 'Total area', and c). post probabilistic quotient normalisation (PQN).

3.9.a)



3.9.b)

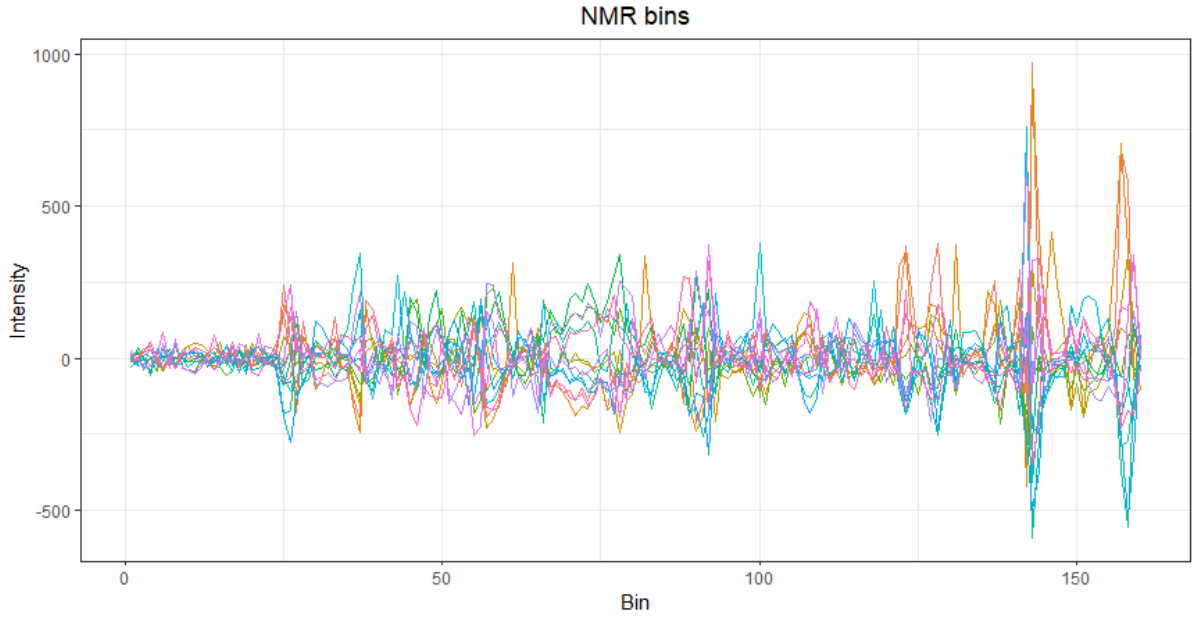


Figure 3.9 The results of scaling the normalised spectra via a). auto-scaling, and b) Pareto scaling.

3.8 Statistical analysis

Statistical analyses for Study 1 (Chapter 4) were completed using SPSS (Version 24 for Windows, SPSS Inc., Chicago, IL) and statistical analyses of Study 2 (Chapters 5 and 6), and Study 3 (Chapter 7) were completed using SPSS (Version 26 for Windows, SPSS Inc., Chicago, IL). The metabolomics specific analysis is detailed above in section 3.7.4. The analyses utilising SPSS for study 2 were the comparisons of daily training and match play physical metrics, subjective load, and scores of muscle soreness, sleep, and vigour. Throughout the thesis all data are presented as mean (± SD).

Data were initially assessed for normality of distribution using the Shapiro-Wilk's test. In all studies statistical comparisons between days of the competitive week (Studies 1 and 2), and timepoints (Study 3) employed a one-way repeated measures analysis of variance (ANOVA). The tests of within subjects' effects provided values for Mauchly's test for sphericity. If this was violated, then a Greenhouse-Geisser correction was used. The difference between means were tested at a significance level of $p<0.05$.

When testing for associations between data in studies 1 (Chapter 4) and 3 (Chapter 7) data was assessed for normality using the Shapiro-Wilk's test first. Parametric data associations were tested using a Pearson's correlation coefficient (r). If the data was non-parametric a Spearman's rank-order correlation coefficient value (rs) was generated (Spearman, 1987). The significance of any relationships found was tested at $p<0.05$. Ninety-five % confidence intervals (95% CI) are displayed, with r^2 for Pearson's correlation, and r_s value for Spearman's rank order as recommended (Spearman, 1987).

Chapter Four

Daily Changes of Resting Metabolic Rate in Elite Rugby Union Players

This study was published in the journal 'Medicine & Science in Sports & Exercise' (MSSE) in 2020.

Hudson, J. F., M. Cole, J. P. Morton, C. E. Stewart and G. L. Close (2020). "Daily Changes of Resting Metabolic Rate in Elite Rugby Union Players." Med Sci Sports Exerc 52(3): 637-644.

4.1 Abstract

INTRODUCTION: Preparation for competitive contact sport has been extensively researched. There are, however, limited data to guide players on how the demands of their sport affect the metabolic requirements of recovery. We aimed to provide novel data on changes in resting metabolic rate (RMR) in contact sport athletes and relate these to the physical demands of training and competition.

METHODS: 22 Elite professional Premiership Rugby Union players were recruited to the study. Indirect calorimetry (Vyntus CPX canopy, CareFusion) was used to measure RMR each morning of the competitive game week, in a fasted, rested state. External loads for training and game play were monitored and recorded using global positioning systems (Catapult Innovations, Australia), whilst internal loads were tracked using rate of perceived exertion scales. Collisions were reviewed and recorded by expert video analysts for contacts in general play (breakdown and tackle area) or the set piece (scrum or maul).

RESULTS: The players were exposed to internal and external loads during the training week comparable to that of a match day, however, despite the equivocal loads between training and game play, there were no significant increases in RMR following training. There were, however, significant ($p=0.005$) mean increases in RMR of 231± 302 kcal the morning after (GD+1) (2544± 409 kcal) and 3 days after the game (GD+3) (2424± 322 kcal), compared with the day before the game (GD-1) (2313± 292 kcal).

CONCLUSION: The collisions experienced in rugby match play, but absent from training, are likely to be responsible for the significant increases in RMR at GD+1 and GD+3. Consequently, the measurement of RMR via indirect calorimetry may provide a non-invasive measure of the

metabolic effects of collisions. This study provides a novel insight to the energy requirements of recovering from contact sport.

4.2 Introduction

As discussed in section 2.1, Rugby Union is a global, dynamic and collision team sport with diverse positional demands (Duthie, Pyne and Hooper, 2003). Backs cover greater distances and at greater relative intensity compared with forwards who are involved in greater static exertions at the set piece (scrum and maul), tackle area (tackle and breakdown contest), and in more collisions (Roberts et al., 2008; Cunniffe et al., 2009; Jones et al., 2015; Cousins et al., 2022). As much as these movement demands can be well measured and monitored, there are significant limitations of using GPS technology to determine contact occurrence and quantitative measurement of force, rendering it unreliable to determine the physical strain placed on the players (Reardon et al., 2017a).

Whilst the technology to accurately quantify physical collisions in rugby is currently lacking, the recognition of their impact upon the athlete is not (Tavares, Smith and Driller, 2017). The forces and mechanical stress in rugby can cause exercise induced muscle damage (EIMD) and impact induced muscle damage (IIMD) which may be distinct in their symptomology and recovery time course (Naughton, Miller and Slater, 2018a). These physical collisions have been shown to increase indirect markers of muscle damage (Takarada, 2003; Cunniffe et al., 2010; Cunniffe et al., 2011; McLellan, Lovell and Gass, 2011a; Oxendale et al., 2016), reduce neuromuscular function (McLean et al., 2010; McLellan and Lovell, 2012), and increase perception of muscle soreness (McLean et al., 2010). Sport scientists have examined a wide array of modalities to enhance recovery from the damaging collisions of rugby match play, some of which may mildly alleviate symptoms (Calleja-Gonzalez et al., 2018). However, despite multiple interventions being implemented, elite rugby players report painful soreness every day throughout a competitive rugby

season, and this pain remains significantly sorer than pre-match levels, four days after match play (Fletcher et al., 2016). It is therefore crucial that accurate and quantitative markers are developed to assess the extent of the IIMD to allow more targeted interventions to be developed. One potential candidate is assessing the energy expenditure of players given that the total energy expenditure (TEE) of young rugby league players was 5% higher when training weeks involved collisions (Costello et al., 2018).

Resting metabolic rate (RMR) is the primary component of TEE and is the energy expended to maintain homeostasis at rest. Indirect calorimetry (IC) requiring both oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) to be measured is the most accurate method of assessing RMR (Fullmer et al., 2015). Large variations in the estimation of RMR using prediction equations have been noted in a variety of sports (Jagim et al., 2018), especially in athletes with a high fat free mass (Carlsohn et al., 2011) such as elite and highly trained rugby players (Morehen et al., 2016; Smith et al., 2018; MacKenzie-Shalders et al., 2019). It is therefore imperative that RMR is accurately measured rather than simply predicted mathematically. Importantly, much of the existing understanding around effectively calculating an athlete's energy requirements are based upon studies which primarily utilise recreational or youth athletes and are thus limited by lower training ages and exposures to lower absolute intensities of work. To our knowledge there are no data on the daily variations in RMR across an entire competitive match week in any sport. It is therefore crucial that potential changes in RMR are explored in highly trained professional athletes with indirect calorimetry performed prior to and the days following a competitive fixture.

To facilitate recovery, it is essential that rugby players are provided with the correct nutrition in terms of both the total energy intake and the provision of recovery promoting foods. Nutrition research in rugby has focussed upon preparation for match play, ensuring muscle glycogen concentrations are optimal for performance (Bradley et al., 2016). It appears elite players now have a good understanding of this (Bradley et al., 2015a), however, the nutritional intakes in the days following a match are much more variable (Bradley et al., 2015a) with many players decreasing total energy intake the day after a game. If muscle damage arising from match play causes an increase in energy requirements in recovery, current guidelines could be underestimating player's needs post competition.

To this end, the aims of this study were two-fold. 1) To assess changes in RMR in an elite group of professional rugby union players measured using indirect calorimetry under strict outpatient procedures throughout a competitive week, including the days before and after a game; 2) to explore the relationship between game day physical exertions and any changes in metabolism observed, as some actions are only experienced during match play compared with weekly training exposures. The hypothesis being that metabolic rate will increase in the days after competition and that this will be associated with the collision events inherent with elite rugby union match play.

4.3 Methods

4.3.1 Participants

Twenty-two healthy elite rugby union players, all members of an English Premiership squad, were recruited for this study. The participants included six internationals, and many established Premiership or Super 15 players (mean ±SD, age; 25.7 ±4.1 years, body mass; 104.6 ±12.6 kg). Five participants were excluded from the analysis having sustained an injury during games which prevented them from completing all aspects of the study. All playing

positions were covered in the remaining 17 players who were eligible for the full study analysis. All participants gave written informed consent prior to commencing the study. Ethical approval (18/SPS/004) was granted by the university research ethics committee at Liverpool John Moores University, UK.

4.3.2 Research Design

The study was designed to allow RMR to be measured within the training schedules of elite rugby players during a complete microcycle. Timepoints throughout the study are described relative to game day (GD) using +/- symbols for days before (-) and days after (+) GD. Due to the timing of team selection defining when recruitment could occur, the first measurement was taken at GD-2. Measurements were then repeated every day, apart from the game day itself, as this was deemed too disruptive to the players' habitual routine. Table 4.1 details the training schedule for the match week. Seven micro cycles were used to attain the total data set, with all games played on the Saturday afternoon (Game Day). This ensured that the training schedules throughout the microcycle were the same and there were no conflicting kick-off times, which would alter the time relative to match play of the subsequent measures. Internal and external loads for training and match play were recorded throughout the week. The weeks chosen were throughout the middle of the season (weeks 13-30), so the players were accustomed to the training load and rigours of match play.

4.3.3 Resting Metabolic Rate

The RMR of participants was assessed 6 times in total. All measures were completed at the same time between 7-9am and players arrived after an overnight fast, with their last meal at least 8 hours prior to measurement. Players awoke and came straight to the training ground

as per reliable outpatient protocol (Bone and Burke, 2018a). To ensure best practice, a private room was established at the training facility away from the main building where temperature was maintained at 21-23 ℃, the room was dimly lit, and quiet (Fullmer et al., 2015). Players lay in a comfortable supine position and were reminded to stay awake. A twenty minute resting period was prescribed, as the minimum sufficient time to achieve rest (Schols et al., 1992). A ventilated hood was employed rather than mouth piece and nose clip to reduce day-to-day variance (Roffey, Byrne and Hills, 2006). The coefficient of variance for our protocol was measured at 1.13% for RMR and 1.62% for RER. This was generated in healthy male nonplaying squad participants who undertook measures on three consecutive days whilst not engaging in exercise for the two days prior to the first measurement and throughout the three days of measures.

The ventilated hood was placed over the head of the athlete and expired gas was analysed using the dilution canopy method (Vyntus CPX canopy, CareFusion, Hoechberg, Germany). The gas analyser was calibrated every day using the manufacturer's automated flow and digital volume transducer calibration (15.92% O_2 and 5.03% CO_2). The first 5 minutes of measurements were discarded following best practice guidelines (Fullmer et al., 2015). Measurements were subsequently recorded for 15 minutes continuously at 10 second intervals for $\dot{V}O_2$ and $\dot{V}CO_2$. Data were exported into Microsoft Excel (2018, Seattle, USA), and mean respiratory exchange ratio (RER) across the measurement period generated, with the calorific value, carbohydrate and fat oxidation rates determined according to the table of Zuntz (Zuntz, 1901).

4.3.4 Measurement of lean body mass

Lean body mass was measured using a dual-energy-X-ray absorptiometry (DXA) fan beam scanner (Hologic Horizon W, Hologic, Bedford, MA) as per the protocols detailed in section

3.3.2 of this thesis, with scanning and analysis performed by the same trained individual using Apex software version 13.5.3.1(Hologic, Bedford, MA). Players were scanned twice during the period of data collection for this study and the scan corresponding closest to their week of participation used, which was no longer than 4 weeks.

4.3.5 Training and Match loads

External demands of all rugby training sessions and match play were recorded using micro-technological units worn by players containing GPS (10Hz) and accelerometer (100Hz) as detailed in section 3.4.1. The corresponding internal loads were assessed by session rating of perceived exertion (sRPE) as explained I section 3.4.2 of this thesis.

4.3.6 Data analyses

All data are presented as mean (± SD). All statistical analyses were completed using SPSS (Version 24 for Windows, SPSS Inc., Chicago, IL). A one-way repeated measures ANOVA was used to compare all gas exchange measures and the work completed by players throughout training days and during the competitive game day. The tests of within subjects' effects provided values for Mauchly's test for sphericity. If this was violated, then a Greenhouse-Geisser correction was used. The difference between means were tested at a significance level of $p<0.05$. The least significant difference (LSD) was used post hoc to compare specific time points when the ANOVA revealed a significant difference between measures over the week. This was examined in the whole group (n=17), sub-groups forwards (n=11) and backs (n=6). A Spearman's correlation was used to assess any associations between changes in RMR throughout the microcycle, with the metrics of physical load and collision data gathered from the competitive match play (n=17). A Spearman's rank-order correlation

coefficient value (rs) was generated and this was tested at $p<0.05$ to test the significance of any relationships found (Spearman, 1987).

Table 4.1 The training sessions throughout the competitive micro cycle. Game Day (GD).

Time Point	GD-3	GD-2	GD-1	GD	GD+1	GD+2	GD+3	GD+4
Purpose	Rest & Recovery	Intensity	Team Run	Match Play	Rest & Recovery	Installation	Volume	Rest & Recovery
Resistance Training Content	None	Upper Limb Strength (30 min)	None	None	None	Lower Limb Strength (45 mins)	Upper Limb Strength (45min)	None
Rugby Content	None	Specific Game Prep (35 mins) Unit Split (15-25mins)	Agility warm-up, Execution of specific game prep at a low-moderate intensity (35min)	Individual & Team Warmups. Rugby Match Play (80 mins).	None	Low-moderate intensity attack shapes and defensive systems. Running top-ups for some players. (60 mins)	High Intensity throughout rugby specific drills. Units Split- Forwards - Scrum/Maul Backs - Strike plays. (75 min)	None
Targets	Recovery	Execution of tactical game specifics at a high intensity. Rehearsal of set pieces in a unit split. Forwards – Lineouts Backs – Strike and skill execution.	Execution of specifics at a lower intensity, low intensity unit rehearsal of set pieces. Forwards – Lineouts Backs – Strike plays.	Full competitive rugby match play. Target physical performance and win.	Recovery	Learning of specifics for the following fixture and recovery.	High running volume, aiming to overload running volume relative to time.	Recovery

4.4 Results

4.4.1 Training and match demands

The training schedule and structure of sessions can be seen in Table 4.1 with the internal and external demands of the week in Table 4.2. It should be noted that data are presented as n=14 for these analyses due to faults with GPS data collection, resulting in lost running metrics for some training sessions in three of the participants.

4.4.2 Player Load

There was no significant difference (p=0.842) in player load on GD+3 (631.07± 110.67) compared with GD (622.36± 98.70). This was also true for the sub-groups of forwards (p=0.308) and backs (p=0.407). The player load on all other days of the training week were significantly lower than the game day in the whole group and when subdivided into forwards, and backs.

4.4.3 High-Speed Running Distance

In the whole group, there was no significant difference (p=0.609) in high-speed running (HSR) distance covered on GD+3 (254.64± 214.15m) compared with GD (285.43± 113.09m). In the forwards sub-group, there was only significantly less HSR distance covered on GD-1 (9.25± 12.10m) (p=0.001) and GD+2 (72.00± 102.86m) (p=0.013) compared with GD (215.88± 96.82m). In the backs sub-group, there was significantly less HSR distance covered on GD-2 (214.83± 42.94m) (p=0.005), GD-1 (2.17± 5.31m) (p<0.0005), GD+2 (51.67±92.63m) (p<0.0005), and GD+3 (221.33± 127.33m) (p=0.019) compared with GD (378.17± 45.46m).

4.4.4 Number of High-Speed Running Efforts

In the whole group, there were significantly fewer HSR efforts on GD-2 (11.43± 3.34) (p=0.002), GD-1 (0.93± 1.69) (p<0.0005), GD+2 (4.93± 6.86) (p<0.0005), and GD+3 (14.79± 8.91) (0.031) compared with GD (20.29± 7.23). In the forwards sub-group, significantly fewer HSR efforts were completed on GD-1 (1.38± 2.07) (p=0.001) and GD+2 (5.63± 7.05) (p=0.014) compared with GD (16.63± 6.99). In the backs sub-group, significantly fewer HSR efforts were completed on GD-2 (12.67± 2.88) (p=0.003), GD-1 (0.33± 0.82) (p=<0.0005), GD+2 (4.00± 7.13) (p=0.001), and GD+3 (13.83± 6.18) (p=0.001) compared with GD (25.17± 4.17).

Table 4.2 Comparison of metrics recorded for training and match play throughout the competitive micro cycle.

*****Denotes values significantly different (p<0.05) when compared with **game day (GD)** shown in bold.

Time Point		GD -2	GD -1	GD	GD +1	GD +2	GD +3	GD +4
Player Load (sRPE x Time)	Whole group	404.07± 103.88*	27.00± 34.52*	**622.36± 98.70**	Rest & Recovery	238.14± 186.90*	631.07± 110.67	Rest & Recovery
	Forwards	391.25± 83.93*	31.25± 35.65*	**595.13± 106.45**		243.13± 212.10*	654.37± 100.19	
	Backs	421.17± 132.60*	21.33± 35.39*	**658.67± 81.85**		231.50± 166.58*	600.00± 125.57	
High Speed Running Distance (m)	Whole group	168.00± 60.12*	6.21± 10.15*	**285.43± 113.09**		63.29± 95.43*	254.64± 214.15	
	Forwards	132.88± 45.89	9.25± 12.10*	**215.88± 96.82**		72.00± 102.86*	279.63± 268.19	
	Backs	214.83± 42.94*	2.17± 5.31*	**378.17± 45.46**		51.67±92.63*	221.33± 127.33*	
High Speed Running Efforts (n)	Whole group	11.43± 3.34*	0.93± 1.69*	**20.29± 7.23**		4.93± 6.86*	14.79± 8.91*	
	Forwards	10.50± 3.55	1.38± 2.07*	**16.63± 6.99**		5.63± 7.05*	15.50± 10.90	
	Backs	12.67± 2.88*	0.33± 0.82*	**25.17± 4.17**		4.00± 7.13*	13.83± 6.18*	
Very High-Speed Running Distance (m)	Whole group	17.86± 16.28	0.00*	**16.50± 15.89**		0.36± 0.93*	23.29± 31.10	
	Forwards	9.88± 10.64	0.00	**10.75± 16.46**		0.38± 1.06	23.75± 38.99	
	Backs	28.50± 17.10	0.00*	**24.17± 12.42**		0.33± 0.82*	22.67± 19.66	
Very High-Speed Running Efforts (n)	Whole group	1.29± 0.91	0.00*	**1.14± 1.17**		0.14± 0.36*	1.29± 1.14	
	Forwards	1.13± 0.99	0.00	**1.00± 1.51**		0.13± 0.35	1.25± 1.28	
	Backs	1.50± 0.84	0.00*	**1.33± 0.52**		0.17± 0.41*	1.33± 1.03	

4.4.5 Very High-Speed Running Distance

In the whole group, very high speed running (VHSR) distance was only significantly lower on GD-1 (0.00± 0.00m) (p=0.002) and GD+2 (0.36± 0.93m) (p=0.002) compared with GD (16.50± 15.89m). Within the forwards sub-group, there was no significant difference (p=0.196) in VHSR distances covered on any day compared with GD (10.75± 16.46m). The backs covered significantly fewer VHSR metres on GD-1 (0.00± 0.00m) (p=0.005) and GD+2 (0.33± 0.82m) (p=0.006).

4.4.6 Very High-Speed Running Efforts

In the whole group, the number of VHSR efforts completed was only significantly lower on GD-1 (0.00± 0.00m) (p=0.003), and GD+2 (0.14± 0.36m) (p=0.013) compared with GD (1.14± 1.17m). In the forwards sub-group, there was no significant difference in VHSR efforts on all training days compared with GD (1.00± 1.51m). In the backs sub-group, there were only significantly less VHSR efforts on GD-1 (0.00± 0.00m) (p=0.001), and GD+2 (0.17± 0.41m) (p=0.013) compared with GD (1.33± .52m).

4.4.7 Changes in resting metabolic rate

Changes in RMR adjusted for lean body mass across the microcycle can be seen in Figure 4.1 with the absolute (kcal·day^{-1}) and relative (kcal·kg·day^{-1}) RMR measures displayed in Table 4.3. Lean body mass (measured by DXA) was 74.8±7.4kg for the whole group, 78.2±5.6kg for the forwards, and 68.6±6.0kg for the backs. The forwards possessed significantly greater lean body mass (p=0.0074). In the whole group, there was a significant increase in RMR from GD-1 (31.1± 4.7 kcal·kg·day^{-1}) to GD+1 (34.1± 5.3 kcal·kg·day^{-1}) (p=0.005) and GD-1 (31.1± 4.7

kcal·kg·day^{-1}) to GD+3 (32.5± 4.2 kcal·kg·day^{-1}) (p=0.04). In the forwards sub-group, there was a significant increase in RMR between GD-1 (30.81± 2.6 kcal·kg·day^{-1}) to GD+1 (33.98± 4.13

kcal·kg·day^{-1}) (p=0.017) and GD-1 (30.81± 2.6 kcal·kg·day^{-1}) to GD+3 (32.38± 2.81 kcal·kg·day^{-1}) (p=0.045). However, in the backs sub-group, there was no significant difference in RMR across the week.

Table 4.3 Absolute and adjusted measurements of RMR across the competitive microcycle for all players (n=17).

Time Point	GD-2	GD-1	GD+1	GD+2	GD+3	GD+4
Absolute RMR (kcal)	2318± 182.1	2313± 283.0	2544± 396.9	2391± 274.2	2424± 312.0	2327± 305.3
Adjusted RMR (kcal·kg^{-1}·day^{-1})	31.2± 3.0	31.1± 4.7	34.1± 5.3	32.1± 4.1	32.5± 4.2	31.3± 4.7

4.4.8 Changes in respiratory exchange ratio

Changes in RER across the microcycle can be seen in Figure 4.1 In the whole group, there were significant increases at GD+2 (0.88± 0.10) (p=0.030) and GD+3 (0.86± 0.08) (p=0.006) compared with GD-1 (0.82± 0.08). In the positional subgroups there were no significant differences across the microcycle p=0.065 and p=0.177 for forwards and backs respectively.

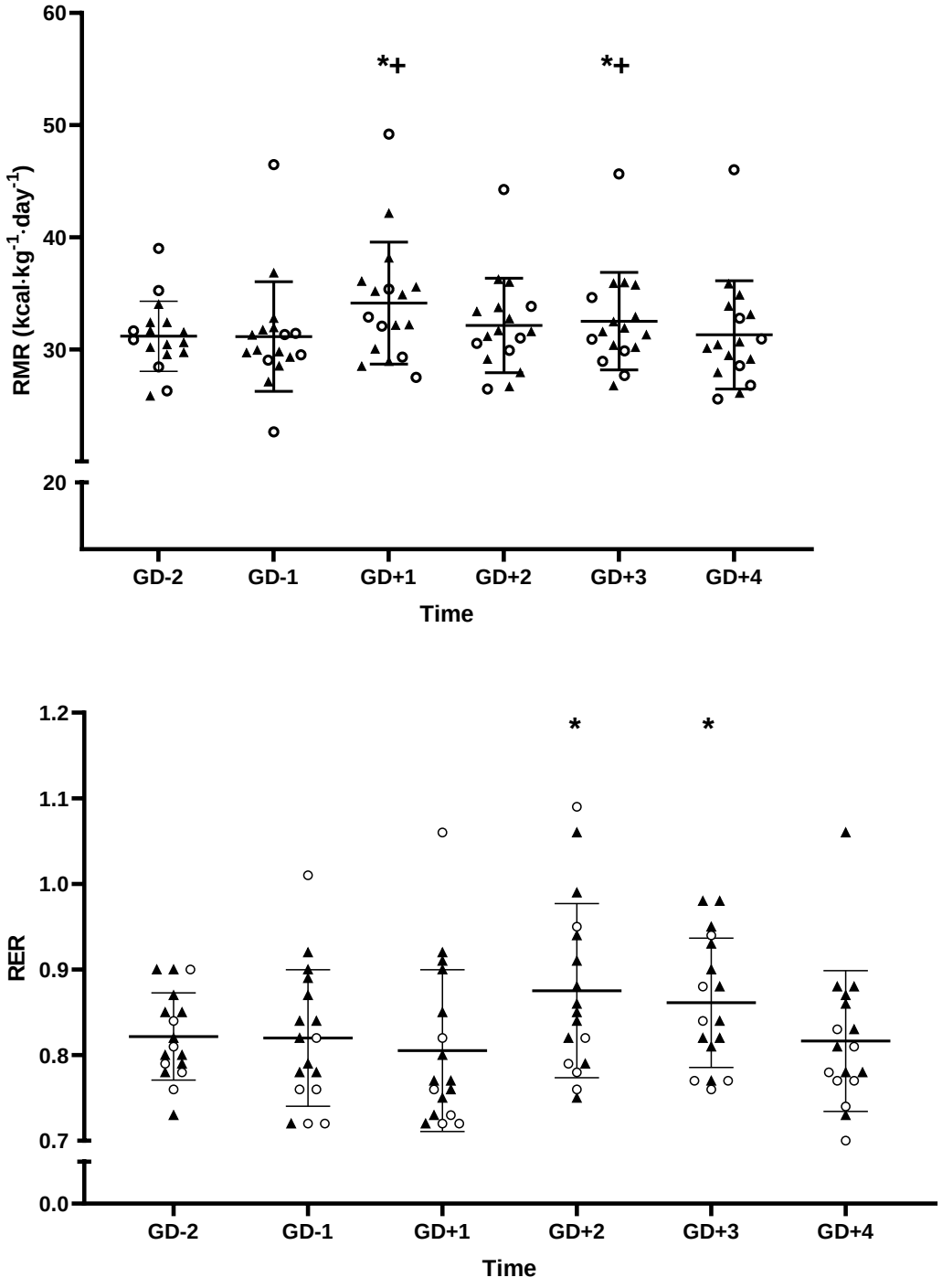


Figure 4.1. Gas exchange measurements across the microcycle. RMR (kcal·kg·day^{-1}) accounting for lean body mass and RER.

Measurements displayed as mean± S.D. with individual data points for all participants.

Forwards – ▲ (filled black triangle), Backs- ○ (empty circle).

*Denotes significant difference ($p<0.05$) for the whole group when compared to GD-1.

+Denotes significant difference ($p<0.05$) for the forwards group when compared to GD-1.

4.4.9 Changes in $\dot{V}O_2$ and $\dot{V}CO_2$

Figure 4.2 shows the measures of $\dot{V}O_2$ and $\dot{V}CO_2$. There were significant increases in $\dot{V}O_2$ in the whole group at GD+1 (0.37± 0.06L·min^{-1}) (p=0.008) and GD+3 (0.35± 0.04 L·min^{-1}) (p=0.041) compared with GD-1 (0.33± 0.04 L·min^{-1}). These significant increases were also observed in the forwards at GD+1 (0.38± 0.06 L·min^{-1}) (p=0.025) and GD+3 (0.36± 0.03 L·min^{-1}) (p=0.027) compared with GD-1 (0.35± 0.03 L·min^{-1}). There were no significant differences for $\dot{V}O_2$ in the backs' subgroup across the week.

There were significant increases in $\dot{V}CO_2$ in the whole group at GD+1 (0.30± 0.06 L·min^{-1}) (p=0.008), GD+2 (0.30± 0.05 L·min^{-1}) (p=0.01), and GD+3 (0.30± 0.05 L·min^{-1}) (p=0.001) compared to GD-1 (0.27± 0.05 L·min^{-1}). These significant increases were also observed in the forwards at GD+1 (0.31± 0.04 L·min^{-1}) (p=0.037) and GD+3 (0.32± 0.04 L·min^{-1}) (p<0.001) compared to GD-1 (0.29± 0.03 L·min^{-1}). There were no significant differences across the week in measures of $\dot{V}CO_2$ in the backs.

4.4.10 Changes in carbohydrate and fat oxidation

Measures of carbohydrate and fat oxidation are displayed in Figure 4.3. Carbohydrate oxidation significantly increased at GD+2 (0.22± 0.13g·min^{-1}) (p=0.044) and GD+3 (0.23± 0.13g·min^{-1}) (p=0.003) compared with GD-1 (0.16± 0.12g·min^{-1}) in the whole group. In the forwards a significant increase was measured at GD+3 (0.26± 0.12g·min^{-1}) (p=0.003) compared with GD-1 (0.18± 0.09g·min^{-1}), whilst there were no significant differences across the microcycle in the backs for carbohydrate oxidation.

Fat oxidation decreased significantly at GD+3 (0.08± 0.04g·min^{-1}) (p=0.029) in the whole group compared with GD-1 (0.10± 0.04g·min^{-1}) and at the same time point in the forwards,

GD+3 (0.08± 0.05g·min^{-1}) (p=0.028) compared with GD-1 (0.1± 0.03g·min^{-1}). There were no significant differences measured for fat oxidation across the microcycle in the backs.

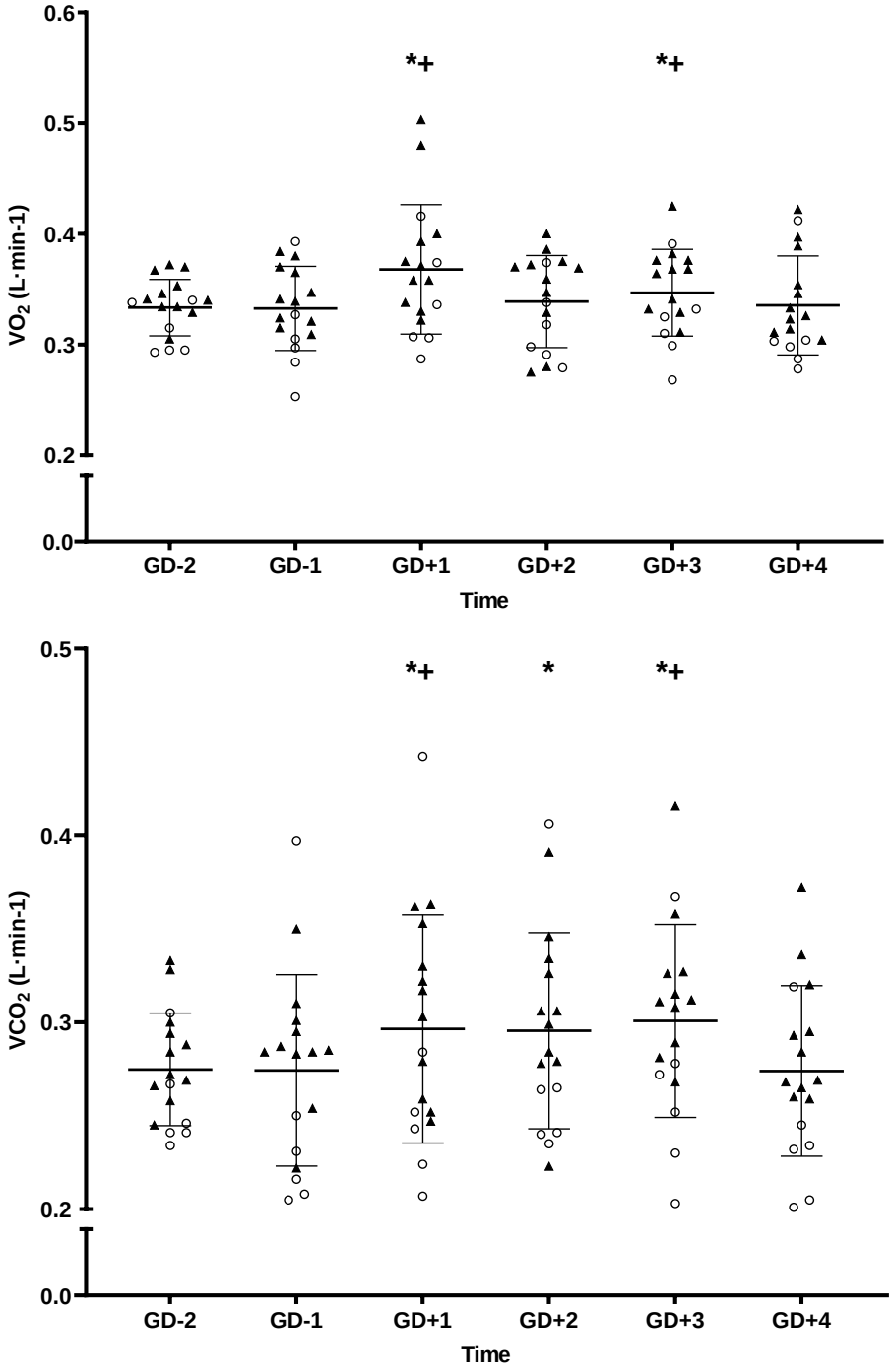


Figure 4.2. Gas exchange measurements across the microcycle. $\dot{V}O_2$ (L·min^{-1}) and $\dot{V}CO_2$ (L·min^{-1}).

Measurements displayed as mean± S.D. with individual data points for all participants.

Forwards – ▲ (filled black triangle), Backs- ○ (empty circle).

*Denotes significant difference ($p<0.05$) for the whole group when compared to GD-1.

+Denotes significant difference ($p<0.05$) for the forwards group when compared to GD-1.

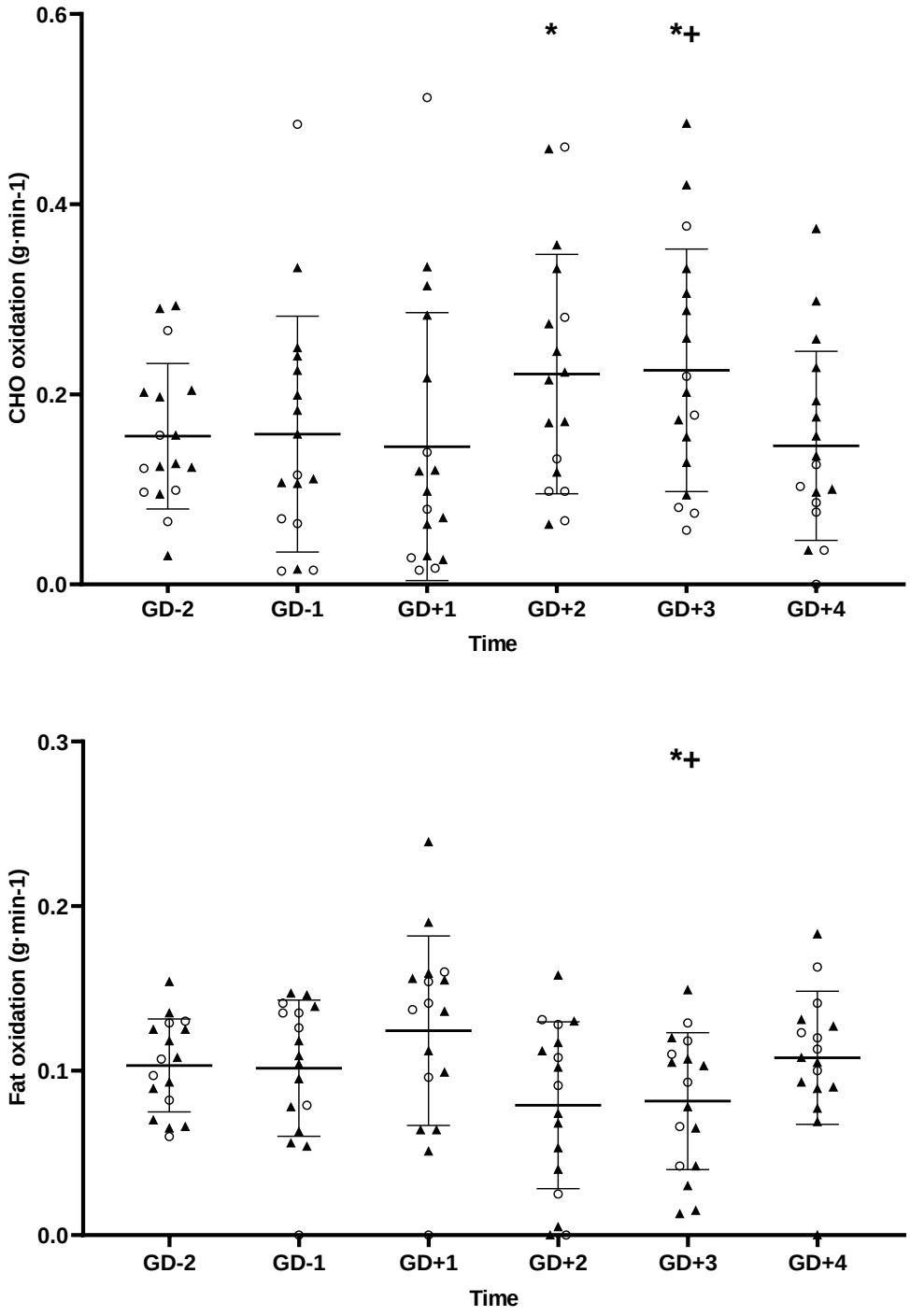


Figure 4.3. Gas exchange measurements across the microcycle. Carbohydrate (CHO) oxidation (g·min-1) and Fat oxidation (g·min-1). Measurements displayed as mean± S.D. with individual data points for all participants.

Forwards – ▲ (filled black triangle), Backs- ○ (empty circle).

*Denotes significant difference ($p<0.05$) for the whole group when compared to GD-1.

+Denotes significant difference ($p<0.05$) for the forwards group when compared to GD-1

4.4.11 Associations of match demands with changes in metabolic measurements

Table 4.4 displays the Spearman's coefficient associations between the physical match demands, and changes in RMR. In the whole group, there were no significant associations found between phase contacts, total contacts, player load, HSR meters, HSR efforts, VHSR meters, VHSR efforts and the change in RMR observed between GD-1 to GD+1. This was also true when the positional sub-groups of forwards and backs were analysed.

Table 4.4 Spearman's coefficient (r_s) associations derived from changes in RMR between GD-1 and GD+1.
*denotes significant p<0.05 association.

Timepoints for comparison	**Group**	**Phase Contacts**		**Total Contacts (Phase+set piece)**		**Player Load (sRPExTime)**		**HSR (m)**		**HSR (efforts)**		**VHSR (m)**		**VHSR (efforts)**	
		r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*
Change in RMR GD-1 to GD+1	Whole Group	0.05	0.84	0.23	0.38	-0.17	0.95	-0.13	0.62	-0.26	0.31	-0.11	0.97	0.19	0.48
	Forwards	-0.10	0.77	0.16	0.63	-0.19	0.58	-0.28	0.40	-0.24	0.47	0.19	0.57	0.32	0.34
	Backs	0.09	0.87	0.09	0.87	0.34	0.51	0.37	0.47	-0.44	0.39	-0.09	0.87	0.00	1.00

4.5 Discussion

The objective of the present study was to investigate whether exposure to elite rugby union training and match play alters metabolism on a day-to day basis throughout a competitive match week. To this end, we monitored RMR using indirect calorimetry alongside game day and training demands in 22 Premiership RU players throughout a game week. We report, for the first-time, that RMR increased significantly following elite rugby union match play, a change that was not observed following intense training with the same training loads. These data therefore illustrate those changes in RMR following match days exist, reflecting a yet unreported increased metabolic demand in the days after a game of elite rugby. This may allow the further development of positional strategies to meet the metabolic requirements of recovery. Furthermore, increased RMR may also represent the physical collisions of match play and indeed could suggest that RMR may be used as a non-invasive marker of muscle damage.

We have reported a mean increase in RMR following match play of 231± 302kcal per day at GD+1, a 10% increase from GD-1. This represents a significant increase given that it is greater than the suggested 6% required as meaningful change using the canopy method (Roffey, Byrne and Hills, 2006) and the 4.5% coefficient of variance between days measured using the Vyntus system (Iraki et al., 2021). The rigour in our protocol also resulted in a lower coefficient of variance than reported previously (Roffey, Byrne and Hills, 2006; Iraki et al., 2021). Importantly, these increases in RMR were due to significant increases in $\dot{V}O_2$ and $\dot{V}CO_2$ and are not merely EPOC being measured as increased $\dot{V}O_2$. The range of increased RMR was large, with individual responses between 240-1000kcal. The greatest increases in RMR were seen in the forwards, who undergo more physical collisions during a game and

greater periods of static exertion at the scrum, maul and tackle area vs. backs (Cunniffe et al., 2009). The whole group, and forwards positional group, also experienced increased RMR which remained elevated 3 days post-game. This sustained increase at GD+3 may be a result of the lower limb resistance training session on GD+2 given that resistance training, especially with an eccentric component, has been shown to increase RMR (Dolezal et al., 2000). It is possible that this sustained increase in RMR, because of the resistance training session, increased the metabolic demands of the recovery from match play, therefore extending the period during which RMR remained elevated, although this suggestion remains speculative and requires further investigation.

Along with changes in RMR in the days after the game we also reported significant changes in RER. The increased RER at GD+2 and GD+3 corresponds with significant increases in resting carbohydrate oxidation coupled with a significant reduction in fat oxidation at GD+3. These significant changes in substrate oxidation are occurring at a time where the secondary response to the combination of EIMD and IIMD are repairing and remodelling damaged tissue (Naughton, Miller and Slater, 2018a). The inflammatory cytokine activity, immunoendocrine changes, and associated oxidative stress resulting from both forms of muscle damaging exercise have been observed after rugby match play (Takarada, 2003; Cunniffe et al., 2010; Cunniffe et al., 2011; Lindsay et al., 2015; Lindsay et al., 2016a; Morehen et al., 2020). It is possible that the responses to damage, together with the presence of immune cells such as neutrophils and macrophages (Peake et al., 2017), may alter substrate oxidation in the recovery period (Wolowczuk et al., 2008; Pearce and Pearce, 2013; Von Ah Morano et al., 2020). Muscle damage induced reductions in glucose transport may also result in a decreased whole-body glucose tolerance which has been reported after a laboratory-based muscle damage protocol (Gonzalez et al., 2015). Further exploration into

the metabolic perturbations after match play is required to investigate potential pathways responsible for changes to substrate oxidation.

Taken together, we have demonstrated increased RMR and altered carbohydrate oxidation, following match play, which suggests that post-exercise nutrition should be specifically tailored to the unique metabolic demands of this period. Moreover, we have shown highly individual responses with some players increasing their RMR by 500-1000kcal. It is crucial to identify such players and tailor their energy intakes to facilitate recovery accordingly.

Given that the participants in the present study were full-time professional players, in the middle of a competitive playing season, it was not possible to either control or record dietary intake. Whilst there is evidence that the thermic effect of food and the total energy content of a meal may alter resting metabolic rate measures (Compher et al., 2006; Fullmer et al., 2015) we do not believe that the player to player variations in diet would have any meaningful effects on RMR or RER in the present study. Previous research has reported that a large meal containing 1300kcal had negligible effects upon measuring RMR and RER when measured 7 hours later, and in lean male participants both measures had returned to baseline at 8 hours following this meal (D'Alessio et al., 1988). Given that both the forwards and backs in the present study had undergone a minimum of an 8 hour fast prior to having their RMR and RER assessed, it is unlikely that differences in diet would be a primary contributor to the observed changes. Moreover, we believe that this group of players consumed adequate energy, as indicated by no major changes in body mass over the testing periods. However, future work should attempt to measure or control dietary intake to fully explore these changes to metabolism.

An additional challenge identified was the potential for alcohol consumption after match play to interfere with the data collection at GD+1. Alcohol consumption even at the elite level can still form part of the cultural behaviours and can negatively affect recovery after match play (Barnes, Mundel and Stannard, 2012). When RMR is measured immediately after consumption of alcohol there is evidence the thermic effect can be recorded acutely as an increase in oxygen consumption (Rosenberg and Durnin, 1978) and a rise in RMR of 4-6% similarly to fat or carbohydrate consumption (Weststrate et al., 1990). Therefore is the fasted period was adhered to, there would not be a significant concern if a small amount of alcohol was consumed. However, the participants were asked to abstain completely as the likelihood of excess consumption and the fasted period not being strictly adhered to may be increased leading to the thermic effect being witnessed in the GD+1 measure. To enable this to be understood by the players this was explained prior to giving their consent and they were reminded of this need to abstain. The testing weeks were also chosen carefully by the lead researcher to omit any potential team social events or gatherings which may make adherence more challenging. There was also an occasion during data collection where a player arrived late for testing and when the lead researcher feared the abstinence had not been adhered to, the player was sent home, their data omitted from the final analysis and they took no further part in the research.

We propose that the muscle damage because of elite rugby union match play could be a key factor in accounting for the changes in metabolism we have witnessed. By carefully monitoring the internal and external demands of the competitive week we have shown that when contact sport athletes are exposed to comparable player load (including HSR and VHSR metrics) to that of a match day but without the physical collisions, there is no change in RMR in the following days. We therefore speculate that the collisions encountered on a

game day could be responsible for the significant changes in RMR reported at GD+1. This may account for the increases in TEE previously observed in youth players when a training session contained collisions like that of match play (Costello et al., 2018).

When we investigated the positional groups of forwards and backs there were differences in how they reacted to match play. The backs' sub-group did not show any significant changes in RMR or RER post-match, albeit they did show a similar pattern across the week as seen in the forwards sub-group. The backs did not experience as many contact incidents as the forwards as has previously been shown (Cunniffe et al., 2009), and they were not involved in the static exertions of the scrum and maul which are potentially damaging. These positional differences may further substantiate our hypothesis that the contact-based activities are responsible for the metabolic changes reported here.

The total number of contacts were rigorously evaluated; however, the Spearman's correlations did not show any significant correlations of changes in RMR with the match demands or collisions experienced. There was one back who exhibited a large increase of ≈796kcal in RMR. Although the actual number of contacts performed by this player were not significantly different to the mean of the backs group, subjective analysis of these collisions (by experienced rugby staff) classified the magnitude and intensity of these as being much greater than typical. Examples like this, coupled with the current inability to accurately quantify collision activities, emphasises the need for a practical measure of the impact contact sports have upon these athletes to be developed.

4.5.1 Practical implications.

From an applied perspective the periodisation of nutrition to optimise adaptation and ultimately performance is well established under the 'Fuel for the work required' paradigm

(Impey et al., 2018) and apparent in team sport practice (Bradley et al., 2015a; Anderson et al., 2017).

The novel data presented here could enhance the application of this in team sports involving collision-based activities. Despite differences in periodisation tactics in rugby union athletes the commonality at GD+1 is an energy intake at or below the mean for the week with corresponding carbohydrate intakes below the mean for the week in each group, ≈2.7 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Posthumus et al., 2021) and ≈3.1 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Bradley et al., 2015a) for forwards & backs respectively. With the next fixture usually at least 6 days following, and glycogen restoration not necessarily the priority, and in a population who habitually appear to consume lower than the recommended carbohydrate intakes (Black, Black and Baker, 2018), this may require a conscious intervention based on these observed practices (Bradley et al., 2015a; Posthumus et al., 2021).

The immediate consumption of carbohydrates does appear advantageous for glycogen resynthesis, rather than delaying feeding post-match (Bradley et al., 2017). However, the work of Bradley and colleagues was carried out after a simulation exercise rather than real match play which may not reflect the true effect of live collisions on metabolism (Bradley et al., 2017). further work is needed to investigate changes to metabolic pathways during the recovery period to understand how this metabolic requirement in recovery may be fuelled. We speculate the timing of carbohydrate feeding may also require further investigation though, if indeed substrate oxidation is altered until the muscle damage due to match play is resolved (Costill et al., 1990; Gonzalez et al., 2015).

Given that the true definition of resting metabolic rate involves ‘strict and steady resting conditions’ it could be argued that the present study did not actually measure RMR at any

time point where in fact Morning Metabolic Rate (MMR) was measured. Indeed, it could be argued that rugby players (and indeed many athletes) during a competitive season are never truly at 'rest' bringing about methodological questions about the timing during a training period when RMR should be measured to accurately predict energy requirements. A protocol according to best practice and adhering strictly to a minimum rest time, fasted measurement, and proper outpatient protocols as per resting metabolic rate are crucial for reliability but this measure may need to be categorised differently (Fullmer et al., 2015; Bone and Burke, 2018a). In the applied world, the term 'Morning Metabolic Rate' may be a more accurate description of what is being measured and future studies may choose to adopt this terminology.

4.5.2 Conclusions

In conclusion, the present study has for the first time assessed the resting metabolic rate of elite rugby union players across a competitive match week using indirect calorimetry. Please refer to figure 4.4 for a summary of the research rationale and findings. We report a significant increase in the RMR of these contact sport athletes in the days after match play. There were also significant shifts in RER at two and three days after competition. We propose these changes could be attributed to the collisions experienced in match play rather than the internal and external loads the athletes are exposed to throughout the microcycle. The metabolic perturbations associated with these muscle damaging actions need to be researched further to help guide athletes as how best to feed their recovery after competition. To this end, we propose that the utilisation of a systems-based approach viewing the whole-body response to training and match play may provide the insight required.

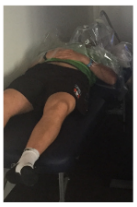
Rationale

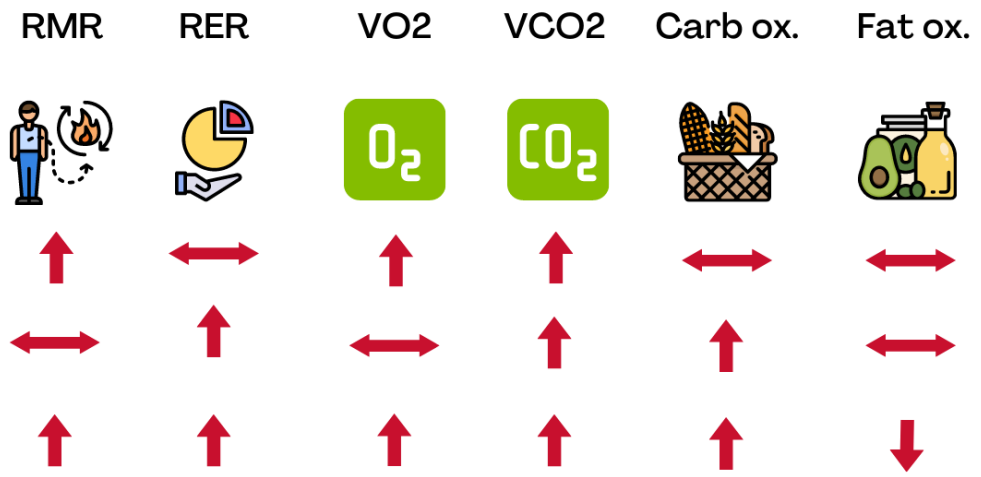
Day-to-day variations in metabolic requirements of athletes can be investigated using indirect calorimetry under strict outpatient conditions

Objective

Investigate whether exposure to elite rugby union training and match play alters resting metabolism on a day-to-day basis throughout a competitive match week

Results





- The whole group experienced a significant mean increase of 231kcal, a 10% increase at GD+1 and significant changes in RER at GD+2 & GD+3
- The backs did not experience any significant changes to any measures whilst in the forwards we saw significant changes in RMR and substrate oxidation in the days after match play
- There are no significant correlations between number of contacts and changes in RMR or RER, we cannot accurately measure the force of these collisions currently.

Conclusion

The collisions inherent with rugby union match play are responsible for the significant changes changes to resting metabolism in recovery from elite rugby union match play

Next Steps

Can the metabolome be investigated to determine how the metabolic requirements of recovery may be met

Figure 4.4. Infographic overview of the chapter to summarise research rationale and findings.

Chapter Five

Untargeted metabolomics investigation of blood serum in elite rugby union: Acute match play and the time course of recovery.

The research and findings within this chapter were published in the journal Metabolites in 2021.

(Hudson et al., 2021) Hudson, J. F., M. M. Phelan, D. J. Owens, J. P. Morton, G. L. Close and C. E. Stewart (2021). ""Fuel for the Damage Induced": Untargeted Metabolomics in Elite Rugby Union Match Play." Metabolites **11**(8).

5.1 Abstract

INTRODUCTION: The metabolic perturbations caused by competitive rugby are not well characterised. Our aim was to utilize untargeted metabolomics to develop appropriate interventions, based on the metabolic fluctuations that occur in response to this collision-based team sport.

METHODS: Seven members of an English Premiership rugby squad consented to provide blood samples daily, over a competitive week including gameday (GD), with physical demands and dietary intake also recorded. Sample collection, processing, and statistical analyses (univariate and multivariate) were performed in accordance with best practice set out by the metabolomics standards initiative for studies employing 700 MHz NMR spectroscopy.

RESULTS: Acute energy needs of this high intensity sport are met via glycolysis, the TCA cycle and gluconeogenesis evidenced by significant increases in serum citrate ($p= 0.032$) and alanine ($p= 0.017$) immediately post-match with significantly ranked pathways of glucose-alanine cycle ($p= 0.0019$), glycolysis ($p=0.005$), and BCAA degradation ($p= 0.030$). The recovery period after cessation of match play and prior to training recommencing sees a re-entry to gluconeogenesis denoted by a significant increase in serum alanine at GD+2 ($p= 0.019$) and the glucose-alanine cycle highly ranked ($p=0.0005$) coupled with pathways of oxidative stress such as glutathione metabolism ($p= 0.031$), glycine-serine metabolism ($p=0.042$), and alterations to fatty acid metabolism.

CONCLUSIONS: This novel insight leads us to propose the increased metabolic requirements in recovery from muscle damaging collisions after elite rugby union match play is dependent upon the availability of glucose. An adjustment in the periodisation of carbohydrate on

GD+1, to increase provision, may prevent the oxidation of amino acids for fuel. Should we expand the 'Fuel for the work required' paradigm in collision-based team sports to include 'Fuel for the damage induced'?

5.2 Introduction

The exceptionally high energy expenditures of rugby league athletes during competitive match weeks (5374± 644 kcal) (Morehen et al., 2016) far exceed those of non-contact team sport athletes (3566± 585 kcal) (Anderson et al., 2017). This disparity, combined with increases in TEE in young rugby players attributed to collisions (Costello et al., 2018) lead to the investigation of the metabolic requirements after match play in elite rugby union. Our findings of significant changes in RMR throughout a competitive match week (Hudson et al., 2020) were reported in chapter 4 of this thesis. We demonstrated that both RMR and carbohydrate oxidation in the fasted state increased significantly in the days following elite rugby union match play and proposed this was due to the muscle damage caused by the collisions inherent with the sport (Hudson et al., 2020).

Despite a greater understanding of glycogen utilisation during match play (Bradley et al., 2016) and implications for the timing of feeding in recovery (Bradley et al., 2017), our understanding of the metabolic perturbations caused by competitive rugby are not well characterized and warrants further investigation. Metabolites within cells, biofluids, tissues or the whole organism are known as the metabolome and are the product of complex interactions occurring within the genome, transcriptome, and proteome of the cellular compartment, combined with environmental influences outside the cell (Macel, Van Dam and Keurentjes, 2010; Patti, Yanes and Siuzdak, 2012). Whilst a systems biology approach may include a multi-omics investigative framework, all potential causal mechanistic pathways may be captured by the metabolome (Hoffman, 2017). The rapid and lasting changes to energy metabolism, anabolism and catabolism are amplified by intense exercise (Hawley et al., 2014). Recent insights from exercise metabolome studies showed a total of

196 metabolites significantly changed within 24 h of a bout of endurance or resistance exercise in human blood, sweat, urine, and saliva (Schranner et al., 2020). Significantly altered metabolites in blood samples after these exercise bouts mapped to alterations in energy production, amino acid metabolism, and indicators of oxidative stress (Peake et al., 2014; Berton et al., 2017).

In addition to the analyses of blood serum acutely around match play, it is also crucial to investigate the recovery period beyond 24 hours, as no exercise metabolomics research has examined this timeframe to date (Schranner et al., 2020). It is also paramount in this rugby population because the secondary muscle damage, experienced as delayed onset muscle soreness (DOMS), and accompanied by inflammation and satellite cell activity, peaks between 24–48 h post-match (Owens et al., 2019). Previous work investigating inflammatory cell signalling molecules and immuno-endocrine responses have gained some insight into the responses to rugby match play occurring by measuring specific analytes at these extended timepoints (Cunniffe et al., 2010; McLellan, Lovell and Gass, 2010; Cunniffe et al., 2011; McLellan, Lovell and Gass, 2011a; Morehen et al., 2020).

Despite these reports of individual immunoendocrine and inflammatory markers, changes to TEE and RMR around rugby match play, we require a greater understanding to enable more specific recommendations to be made on meeting metabolic needs throughout recovery. The complex and integrated nature of the whole body exercise response means the use of metabolomics as an unbiased systems approach may be appropriate to fill the critical gaps in our understanding (Hoffman, 2017).

Our overarching objective is to capture the metabolic perturbations that occur in response to elite rugby union match play prior to, and in the days after the cessation of the match,

which will allow us to generate further explanation of the metabolic requirements of recovery.

5.3 Methods

5.3.1 Participants and research design

Seven healthy elite rugby union players were recruited for this study, all members of an English Premiership squad (mean ± SD, age; 22.0 ± 2.7 years, body mass; 102.5 ± 13.7 kg). All participants gave full written consent prior to commencing the study. Ethical approval (19/SPS/039) was granted by the university research ethics committee at Liverpool John Moores University (Liverpool, UK).

Venous blood was collected throughout a competitive match week during the early part of the competitive season. Time points throughout the study are described relative to game day (GD) using +/−symbols for the days preceding (−) and days after (+) GD. Selection of the team by coaches to play the competitive fixture was finalised on GD-4. Due to the timing of this selection defining when recruitment could occur, the first measurement was taken at GD−2. Venous blood samples were collected in the morning in the fasted state each day apart from the GD sample which was collected immediately after match play. Figure 5.1 shows the study design and workflow.

5.3.2 Training and match demands

External demands of all rugby training sessions and match play were recorded using micro-technological units worn by players containing GPS (10Hz) and accelerometer (100Hz) as detailed in section 3.4.1. The corresponding internal loads were assessed by session rating of perceived exertion (sRPE) as explained I section 3.4.2 of this thesis.

5.3.3 Subjective ratings of wellness, recovery, and sleep

Each morning participants provided ratings of perceived muscle soreness, vigour, non-training stress, and sleep quality using a 1-7 Linkert scale. The players also reported sleep

duration in hours slept each day. The participants were familiar with this process, and it was part of their normal requirements each day. For further detail please see section 3.4.3.

5.3.4 Dietary intake

Dietary intake was recorded using the participants mobile phone device incorporating the 'Snap'n'Send' method (Costello et al., 2017). A wide range of meals and snacks designed by the team nutritionist were provided at the training facility. Their choices and portions in the club dining facility and whilst away from there were self-selected. The composite dishes where several ingredients were included are the most challenging to analyse (Stables et al., 2021). To ensure accuracy the recipes were dictated by the team nutritionist (lead researcher) and prepared by the team's performance chef as per the instructed amounts. The dining facility then utilised cup measuring scoops to allow the players to record their portions, e.g. 2 scoops of a choice accompanying the photographic recording. Other multi-ingredient dishes such as lasagne for example, were analysed per tray and divided up into portions prior to player choice to again increase the accuracy of overall dietary intake. The dietary analysis software Nutritics (Nutritics Ltd., Dublin, Ireland) was used by a registered sports and exercise nutritionist (SENr) to analyse food intake over the match week.

5.3.5 Blood sample collection

Blood samples were collected in a fasted state apart from the GD time point when the samples were taken post-match play within 30 minutes of the final whistle. Whole blood samples (10ml) were drawn using standard venepuncture techniques and collected using serum tubes (Vacutainer Systems, Becton Dickinson) which did not contain clotting gels or additives as these may interfere with metabolomics analysis (Phelan and Lian, 2016).

Samples were allowed to clot at room temperature (18-22C°) for 40 minutes prior to centrifugation at 1600g for 15 minutes. For further detail please see section 3.6.1.

5.3.6 NMR Sample preparation

500 µL aliquots of serum were thawed and diluted in deuterium oxide (^2H_2O, Sigma-Aldrich) and 1.2 mM sodium azide (NaN_3, Sigma-Aldrich), then transferred into 5 mm outer diameter NMR tubes (Bruker, Coventry, UK). Please see section 3.7.1 for further detail.

5.3.7 NMR Spectroscopy

Non-targeted 1D ^1H NMR spectra were acquired at 37 °C using a 700 MHz Bruker Advance IIIHD spectrometer equipped with a TCI cryoprobe and chilled Sample-Jet autosampler. For further detail please see section 3.7.2.

5.3.8 Spectral processing and annotation

All spectra were analysed to ensure conformity with the recommended minimum reporting standards set out by the Metabolites Standard Initiative (MSI) (Sumner et al., 2007),(Considine et al., 2017). Serum spectra were aligned to glucose anomeric peak at 5.24 ppm. Serum spectra were integrated into 160 bins with 104 (65%) annotated corresponding to 38 metabolites and 56 unknown metabolite bins. For further detail please see section 3.7.3.

5.3.9 Data analysis

Spectral analysis is described in full in section 3.7.4. The statistical analysis of the physical loads and dietary intake were performed using SPSS (Version 26 for Windows, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (Version 8.4.3 for Windows, GraphPad Software, San Diego, CA, USA). All data are presented as mean (±SD), and a one-way repeated measures

ANOVA was used to compare all measures across the week. The test of within subjects' effects provided values for Mauchly's test for sphericity. If this was violated, then a Greenhouse-Geisser correction was used. The difference between means was tested at a significance level of $p < 0.05$. A Tukey correction post hoc was used to compare specific time points when the ANOVA revealed a significant difference between measures over the week.

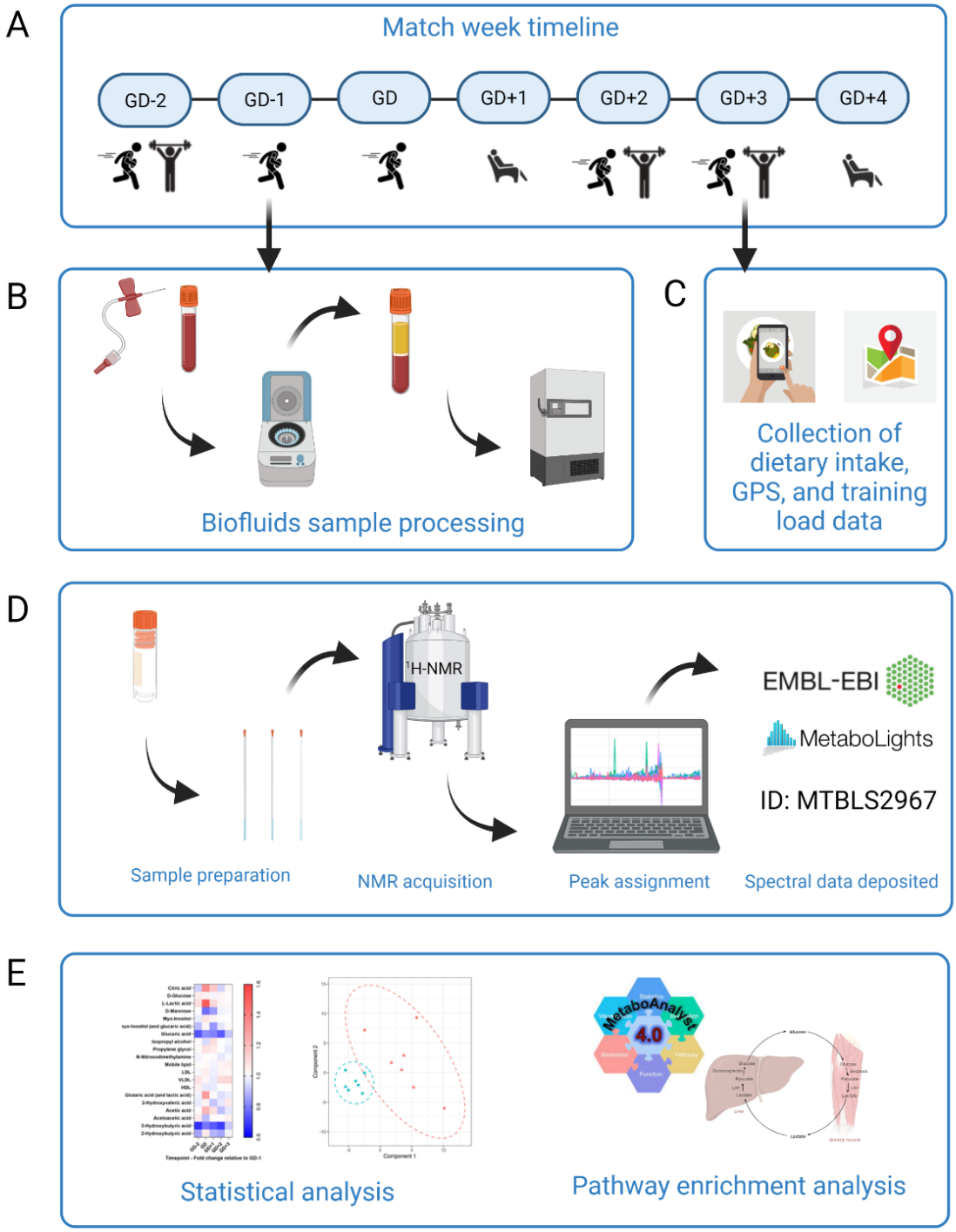


Figure 5.1 Schematic overview of the study design. (**A**) Participants (*n* = 7) began the study at GD-2 and completed a whole match week schedule of rugby specific sessions, resistance training and rest. (**B**) Biofluids sample processing. Participants provided a sample of venous blood every morning apart from the GD sample which was taken immediately post-match play. Samples were processed immediately with timings rigorously repeated each day. Serum was aliquoted to cryovials then frozen for later analysis. (**C**) Dietary intake for all seven days using the Snap'n'Send method, with all GPS and load data were collated. This was all analysed to further translate changes to the metabolome. (**D**) Sample preparation and analysis by ^{1}H-NMR. Spectra were acquired and then peaks assigned using Chenomx. Full spectrum parameter sets are available with the data deposited at MetaboLights public repository (ID number MTBLS2967). (**E**) Statistical analysis. Univariate and multivariate data analysis was performed in R to elucidate key metabolites between all sample timepoints. MetaboAnalyst 4.0 pathway enrichment analysis was then carried out with those statistically significant and key discriminatory metabolites. Created with BioRender.com

5.4 Results

This section will display the training schedule with internal and external loading data, results of the dietary intake, wellbeing monitoring, and the blood serum metabolomics analyses. The metabolomics analyses are divided into the 'acute' changes to the metabolome analysed utilizing the samples gathered immediately after match play, and the 'recovery' period which compares the fasted samples from the day preceding (GD−1) with the days after match play.

5.4.1 Training and match demands

The training schedule for the match week is detailed in table 5.1 with the external and internal load data presented in table 5.2 below.

Table 5.1 The in-season training schedule including session content and physical objectives.

Time Point	GD−3	GD−2	GD−1	GD	GD+1	GD+2	GD+3	GD+4
Purpose	Rest & Recovery	Intensity Execute tactical specifics at high intensity	Team Run Low intensity rehearsal of game plan	Match Play Maximal physical performance	Rest & Recovery	Installation Tactical learning	Overload run volume	Rest & Recovery
Resistance Training Content	None	Upper Limb Strength (45 min)	None	None	None	Lower Limb Strength (45 min)	Upper Limb Strength (45 min)	None
Physical Rugby Content	None	Specific Game Prep (45 min) Unit Split (20 min)	Execution of specific game plan at a low intensity (35 min)	Individual & Team prep. Rugby Match Play (80 min).	None	Low intensity attack shapes and defensive system installation. (50 min)	High Intensity throughout rugby specific drills. (75 min)	None

Table 5.2 In-Season physical metrics from training sessions and game day throughout the match week. * Significant difference in pairwise comparison with Gameday (GD) metrics after one-way repeated measures ANOVA and Tukey post-hoc correction.

Time Point	GD−2	GD−1	GD	GD+2	GD+3	ANOVA (*p*-Value)
Player Load (sRPE x Time)	600.71 ± 69.72	78.00 ± 13.90 *	533.14 ± 120.32	253.57 ± 173.00 *	512.14 ± 211.79	$p < 0.0001$
HSR Distance (m)	164.00 ± 71.65	72.57 ± 25.13 *	198.43 ± 80.05	100.43 ± 86.42	150.29 ± 97.21	$p = 0.0327$
HSR Efforts (*n*)	13.57 ± 5.07	7.00 ± 2.27	12.57 ± 3.42	7.57 ± 5.80	8.57 ± 5.04	$p = 0.0513$
VHSR Distance (m)	17.00 ± 13.28	0.57 ± 1.40	16.29 ± 21.62	4.86 ± 7.85	17.86 ± 13.14	$p = 0.0733$
VHSR (*n*)	1.71 ± 1.28	0.14 ± 0.35 *	1.29 ± 0.88	0.43 ± 0.73	1.29 ± 1.03	$p = 0.0007$
Accelerations > 3 ms (*n*)	9.00 ± 2.45	2.00 ± 1.41 *	6.43 ± 0.90	2.71 ± 3.15	3.14 ± 3.31	$p < 0.0001$
Decelerations > 3 ms (*n*)	10.29 ± 6.94	3.29 ± 1.67	10.71 ± 5.75	4.29 ± 2.96	5.14 ± 4.52	$p = 0.0289$

The daily load the rugby union players were exposed to throughout the match week varied significantly ($p<0.0001$). The physical load on GD-1 (78.00 ± 13.90) was significantly lower than GD-2 (600.71 ± 69.72) ($p<0.0001$), GD (533.14 ± 120.32) ($p<0.0001$), and GD+3 (512.14 ± 211.79) ($p<0.0001$). This was also true of the load on GD+2 (253.57 ± 173.00) being significantly lower than GD-2 ($p=0.0008$), GD ($p=0.0076$), and GD+3 ($p=0.0149$).

The daily High-speed running (HSR) distance covered by the participants was significantly different across the seven days ($p=0.0327$). HSR metres were significantly lower on GD-1 (72.57 ± 25.13m) compared to GD (198.43 ± 80.05m) ($p=0.0335$). The very high-speed running distance did not significantly change throughout the match week ($p=0.0733$).

There were no significant differences in the number of HSR efforts throughout the week ($p=0.0513$). There were significant differences in the number of VHSR efforts ($p=0.0007$). Specifically, the number of VHSR efforts were significantly lower on GD-1 (0.14 ± 0.35) compared with GD-2 (1.71 ± 1.28) ($p=0.0013$), GD (1.29 ± 0.88) ($p=0.0247$), and GD+3 (1.29 ± 1.03) ($p=0.0247$). The number of VHSR efforts were also significantly lower on GD+2 (0.43 ± 0.73) compared with GD-2 ($p=0.0095$).

The number of accelerations >3ms were significantly different ($p<0.0001$). Specifically, there were significantly fewer performed on GD-1 (2.00 ± 1.41) compared with GD-2 (9.00 ± 2.45) ($p<0.0001$), and GD (6.43 ± 0.90) ($p=0.0135$). Finally, there were significant differences in the number of decelerations >3ms performed across the days of the match week ($p=0.0289$), but there were no significant pairwise comparisons after correction.

Overall, this demonstrates these athletes were exposed to a training sessions repeatedly throughout the match week, containing high-speed running (HSR), very high-speed running (VHSR), high velocity accelerations, and decelerations comparable to the demands of game

day. They are accustomed to the volume and intensity of dynamic high-speed movements as per match play but not exposed to full collisions in training.

5.4.2 Self-reported wellness measures

The measures of all elements of the daily wellness questionnaire are detailed in table 5.3 and presented graphically in figure 5.2 below. Vigour was significantly lower on GD+1 than all other days. Vigour at GD+1 (p= 0.0001), and GD+2 (p= 0.0330) was significantly lower than scores recorded the morning of gameday. Muscle soreness was significantly greater at both GD+1 and GD+2 compared with timepoints prior to the match GD-2 (p= 0.0009 and p=0.0330), GD-1 (p= 0.0009 and p=0.0061), and GD (p= 0.0002 and p= 0.0056). The measure at GD+3 (p= 0.0403) was still significantly higher than GD. After this peak of soreness at GD+1 the reported measures were not significantly lower at GD+2 (p=0.2103).

There was no significant change in reported measures of non-training stress (p=0.3334), sleep quality (p= 0.0981), and sleep duration (p= 0.0750) across the match week.

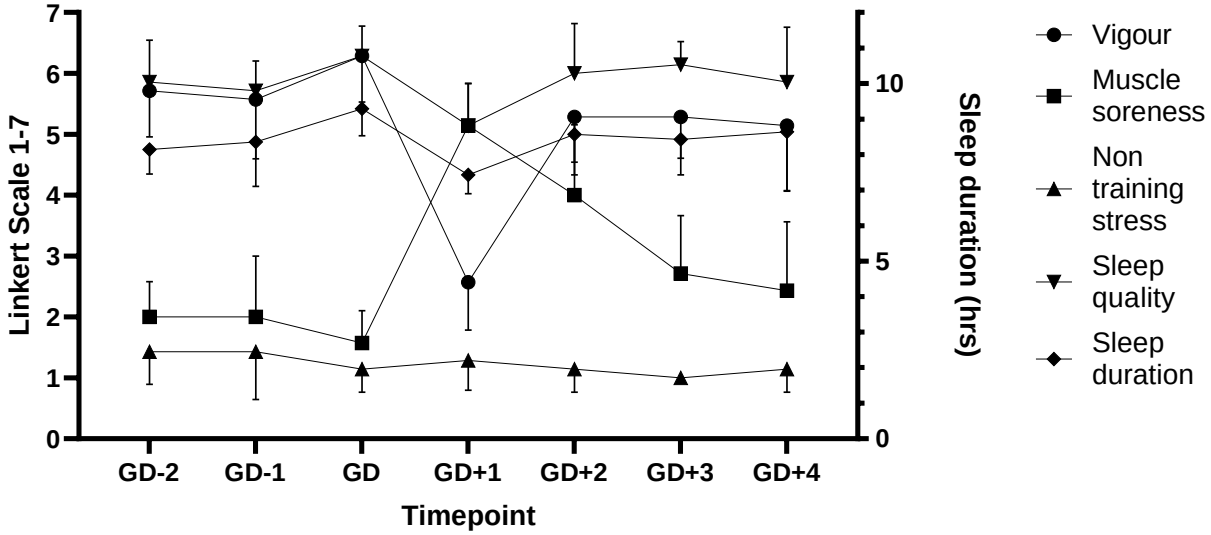


Figure 5.2 Self-reported wellness markers submitted each morning, presented chronologically as a score 1-7 Linkert scale or Time (hrs) for sleep duration.

5.4.3 Dietary intake

Daily dietary intake for the participants is displayed in table 5.4 with macronutrients relative to body mass (kg) and absolute energy intakes (kcal). There were no differences across the match week in daily protein ($p=0.3743$) and fat ($p=0.3666$) intake. Daily carbohydrate intake was significantly different across the week ($p<0.0001$), with intake on GD (5.62± 0.85g·kg^{-1}) being higher than all other days and intake on GD-1 (4.32 ±0.89g·kg^{-1}) higher than all days apart from gameday itself. This pattern was mirrored in total energy with a mean intake of 3323± 630 kcal·day^{-1} across the week. There was no difference in this analysis when reviewing absolute macronutrient and energy intakes or relative to body mass.

Table 5.3 Self-reported wellness measures for each day of the match week. *Denotes significantly different than GD after one-way repeated measures ANOVA and Tukey post-hoc correction. 1-7 Linkert scale used for subjective ratings and time (hrs) used for sleep duration.

Measure	GD-2	GD-1	GD	GD+1	GD+2	GD+3	GD+4	ANOVA
Vigour	5.71 ±0.70	5.57 ±0.90	6.29 ±0.70	2.57 ±0.73*	5.29 ±0.88*	5.29 ±0.88	5.14 ±0.99	p<0.0001
Muscle Soreness	2.00 ±0.53	2.00 ±0.93	1.57 ±0.49	5.14 ±0.64*	4.00 ±1.07*	2.71 ±0.88*	2.43 ±1.05	p<0.0001
Non-training stress	1.43 ±0.49	1.43 ±0.73	1.14 ±0.35	1.29 ±0.45	1.14 ±0.35	1.00 ±0.00	1.14 ±0.35	p = 0.3334
Sleep Quality	5.86 ±0.64	5.71 ±0.45	6.29 ±0.45	5.14 ±0.64	6.00 ±0.76	6.14 ±0.35	5.86 ±0.83	p = 0.0981
Sleep duration (hrs)	8.14 ±0.64	8.36 ±1.16	9.29 ±0.70	7.43 ±0.49	8.57 ±0.73	8.43 ±0.49	8.64 ±1.55	p = 0.0750

Table 5.4. Macronutrient intake for each day of the match week displayed relative to body mass ($g \cdot kg^{-1}$) and overall energy intake as total kilocalories (kcal). *Denotes significantly lower than GD, † denotes significantly lower than GD-1 after one-way repeated measures ANOVA and Tukey post-hoc correction.

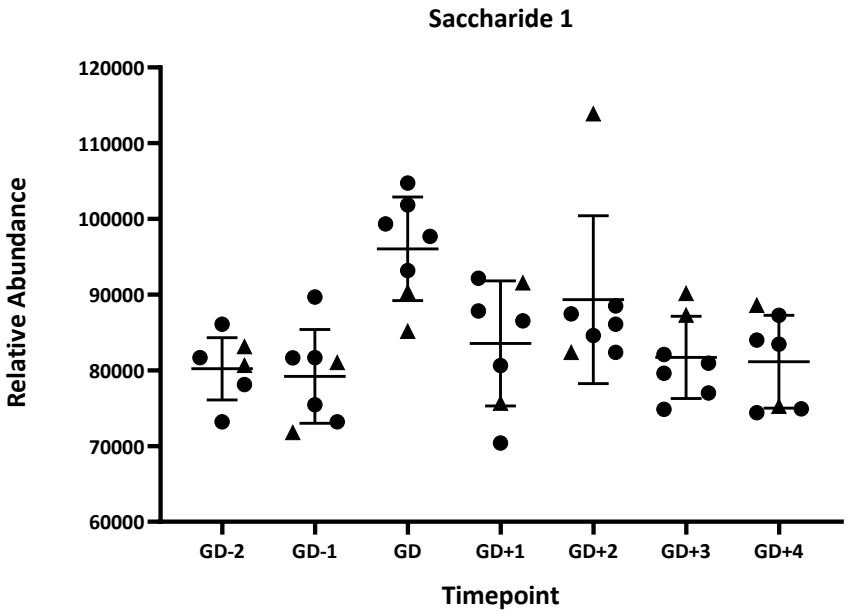
Time Point	GD-2	GD-1	GD	GD+1	GD+2	GD+3	GD+4	ANOVA (p-value)
Carbohydrate ($g \cdot kg^{-1}$)	2.52 ±0.30*†	4.32 ±0.89*	5.62 ±0.85	2.93 ±0.64*†	2.11 ±0.42*†	2.42 ±0.51*†	2.25 ±0.68*†	p<0.0001
Protein ($g \cdot kg^{-1}$)	2.55 ±0.39	2.37 ±0.48	2.20 ±0.24	2.15 ±0.62	2.62 ±0.30	2.45 ±0.30	2.37 ±0.85	p=0.3743
Fat ($g \cdot kg^{-1}$)	1.09 ±0.33	1.17 ±0.18	1.05 ±0.35	1.34 ±0.28	1.04 ±0.24	1.19 ±0.11	1.25 ±0.40	p=0.3666
Energy (kcal)	3042 ±326*†	3770 ±235	4288 ±624	3272 ±379*	2856 ±151*†	3060 ±216*†	2971 ±625	p=0.0021

6.4.4 Acute changes to the blood serum metabolome

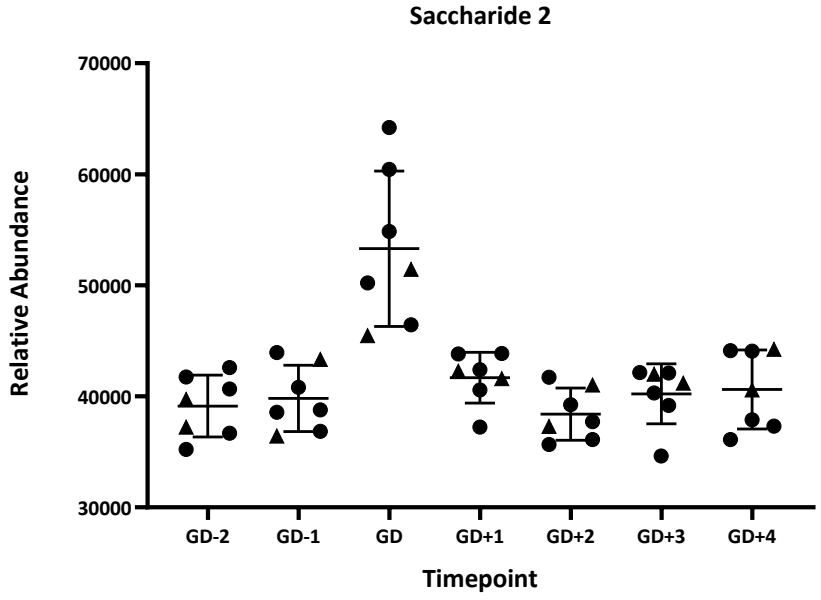
Univariate analyses revealed four significant metabolites (alanine, citrate, and two unidentified saccharides) in the GD blood serum samples. The relative abundance of these metabolites is represented graphically in figure 5.3. Adjusted p values for the four identified metabolites were saccharide 1 ($p= 0.030$), saccharide 2 ($p<0.0001$), L-Alanine ($p= 0.017$), and Citrate ($p= 0.032$). When investigating the time point comparisons *post hoc*; saccharide 1 was significantly greater at GD compared to all other time points apart from GD+2 ($p= 0.587$). Saccharide 2 was significantly greater at GD compared with all other time points ($p<0.001$ for all comparisons). Citrate was significantly increased at GD compared to all other time points apart from GD+1 ($p=0.487$) where it remained elevated. L-Alanine is significantly greater at GD compared with all other time points; GD-2 ($p=0.018$), GD-1 ($p<0.0001$), GD+1 ($p=0.019$), GD+3 ($p=0.030$), and GD+4 ($p=0.0074$), apart from GD+2 ($p= 0.487$). L-Alanine is also significantly greater at GD+2 compared with GD-1 ($p= 0.019$).

Multivariate analyses of the blood serum samples generated four high quality PLS-DA models which identified the key discriminatory metabolites which went forward to pathway enrichment analyses and are displayed in table 5.5.

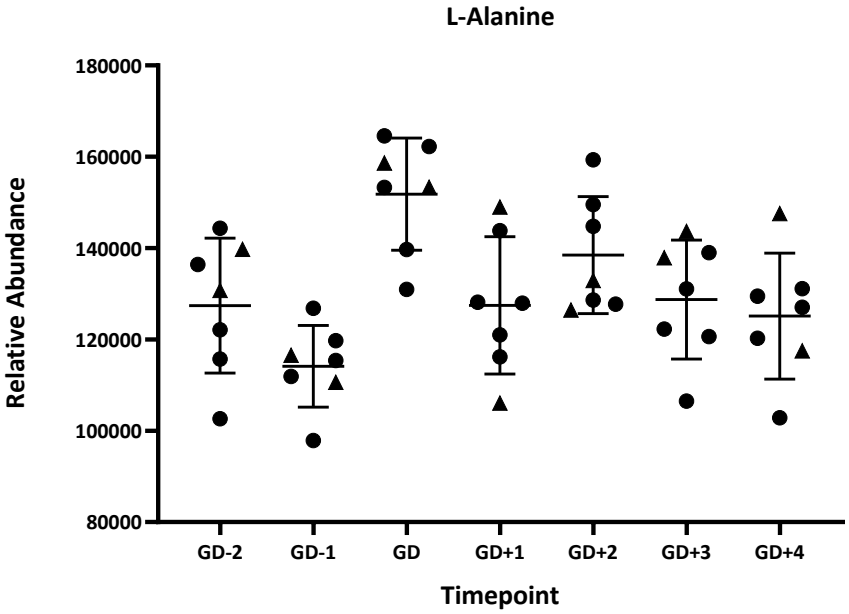
5.3a)


Saccharide 1
Relative Abundance
120000
110000
100000
90000
80000
70000
60000
GD-2
GD-1
GD
GD+1
GD+2
GD+3
GD+4
Timepoint

5.3b)


Saccharide 2
Relative Abundance
70000
60000
50000
40000
30000
GD-2
GD-1
GD
GD+1
GD+2
GD+3
GD+4
Timepoint

5.3c)



5.3d)

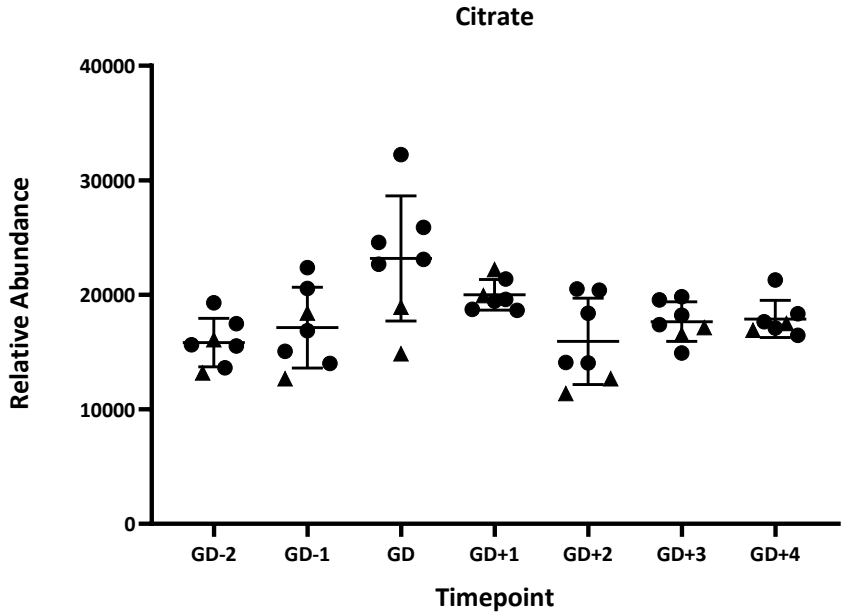


Figure 5.3 Blood serum levels of the four metabolites identified via univariate analysis across the match week. Individual relative abundance is included with mean ±SD for the participants (n=7). The metabolites identified are a). Saccharide 1, b). Saccharide 2, c). Alanine, and d). Citrate. Black filled triangles denote the backs positional players and the black filled circles denote the forwards positional players.

Table 5.5 Metabolites identified as key discriminators between samples collected immediately post-match play (GD) and the GD−1, GD+1, and GD+2 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The unadjusted ranked *p*-values are displayed here.

Acute pathways	**Timepoint Comparison (Unadjusted *p*-value)**				**Metabolites Included**
	GD-2 vs. GD	GD-1 vs. GD	GD vs. GD+1	GD vs. GD+2	
Glucose-Alanine Cycle	0.0028	0.0019	0.0019	0.0025	D-Glucose, L-Glutamic acid, L-Alanine
Urea Cycle	0.0037	0.0022	0.0193	0.0032	L-Glutamic acid, L-Alanine, L-Arginine, L-Glutamine
Warburg Effect (aerobic glycolysis)	0.0091	0.0050	0.0050	0.0075	Citrate, D-Glucose, L-Glutamic acid, Lactate, L-Glutamine
Valine, Leucine, and Isoleucine Degradation	0.0468	0.0301	0.0301	0.0087	L-Glutamic acid, L-Isoleucine, L-Leucine, L-Valine, Acetoacetate
Phenylalanine and Tyrosine Metabolism			0.0175	0.0225	Acetoacetate, L-Glutamic acid, L-Tyrosine
Arginine and Proline Metabolism				0.0272	Creatine, L-Glutamic acid, L-Proline, L-Arginine
Ammonia Recycling	0.0359	0.0252	0.0252	0.0321	L-Glutamic acid, L-Histidine, L-Glutamine
Glycine and Serine Metabolism				0.0386	Creatine, Glutamic acid, L-Alanine, L-Arginine
Aspartate Metabolism	0.0453	0.0319		0.0405	L-Glutamic acid, L-Arginine, L-Glutamine
Galactose Metabolism		0.0395			D-Glucose, D-Mannose, Myo-inositol
Alanine Metabolism		0.0453	0.0453		L-Glutamic acid, L-Alanine

The high metabolic demands of match play

The high energy demands of this sport are demonstrated by the inclusion of glycolysis (p= 0.0050), glucose-alanine cycle (p= 0.0019), and pathways associated with amino acid degradation (p= 0.0301) being highly ranked in serum samples immediately post-match (Table 5.5).

Two unidentified saccharides were significantly elevated in the GD serum samples ($p = 0.030$ and $p < 0.0001$) most likely due to the ingestion of carbohydrate. Conversion of pyruvate into lactate ensures glycolysis can continue, explaining the serum lactate peak immediately post-match (Figure 5.4). Univariate analysis identified serum citrate ($p = 0.032$) as significantly increased at the GD sample, together with increases in serum acetate, the presence of these TCA cycle intermediaries explains the key pathways identified for energy provision during the match.

Serum alanine, using univariate analysis was significantly increased at GD compared to GD−1 ($p < 0.0001$). The pathway enrichment for serum has the glucose-alanine cycle ranked most highly, indicating gluconeogenesis as required to meet the total energy needs.

The key discriminatory metabolites associated with the ketone body metabolism pathway identified are serum acetoacetate and 3-hydroxybutyrate. Both metabolites were reduced immediately post-match compared with GD-1 levels. This may indicate a reduced fatty acid oxidation during match play.

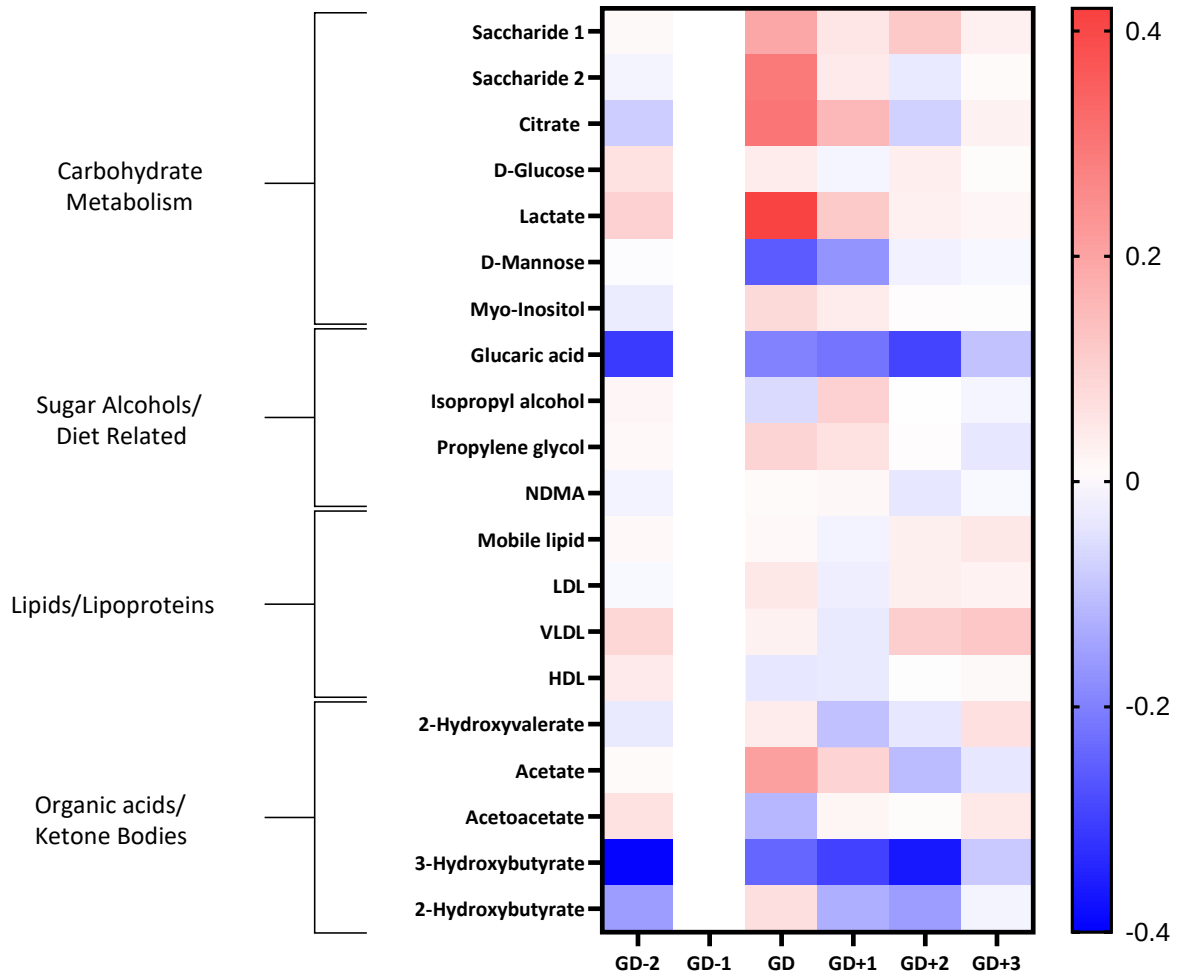


Figure 5.4 Heatmap (part 1 of 2) of metabolites identified as significant via univariate analysis and as key discriminators between timepoints via PLSDA modelling in blood serum samples. These include metabolites associated with carbohydrate and fat metabolism. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increased (greater than 0, red) or decreased (less than 0, blue).

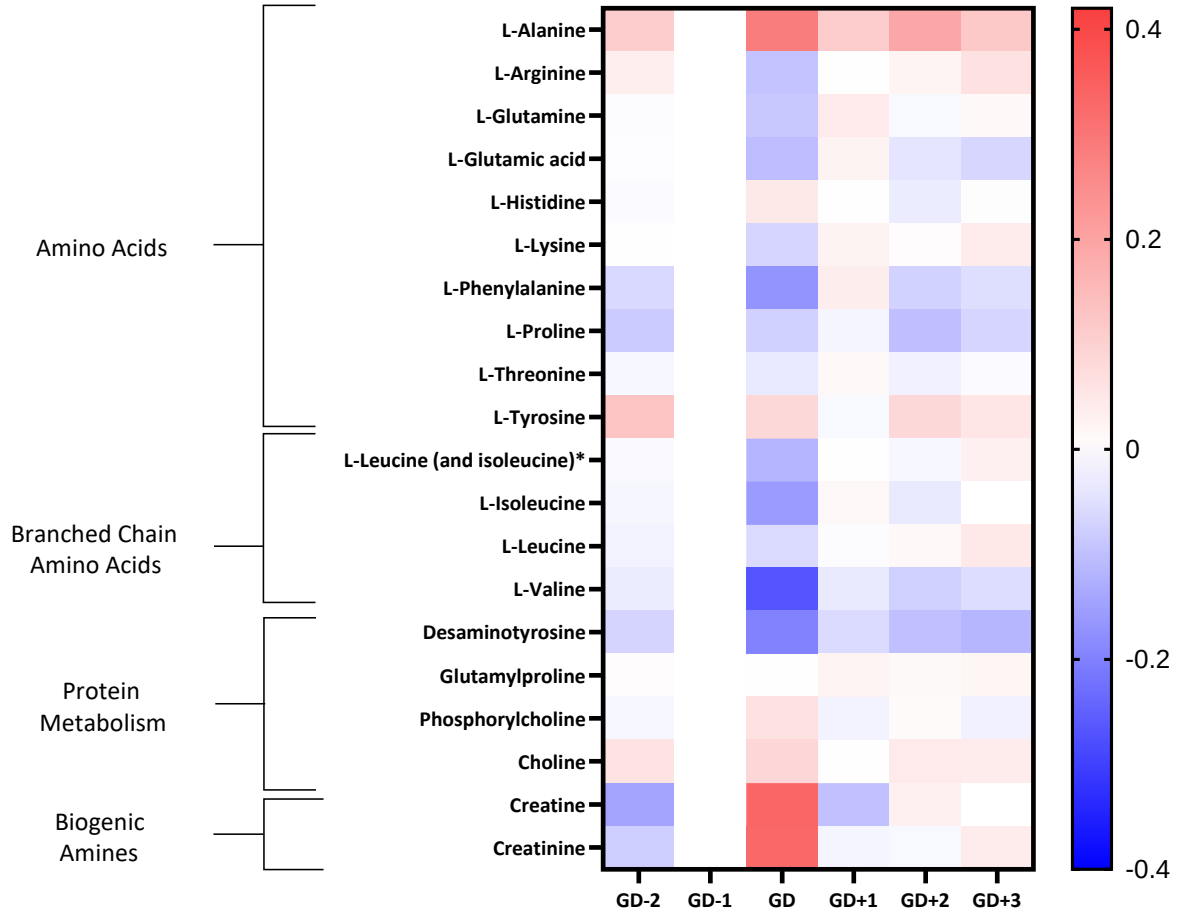


Figure 5.5 Heatmap (part 2 of 2) of metabolites identified as significant via univariate analysis and as key discriminators between timepoints via PLSDA modelling in blood serum samples. These includes biogenic amines, amino acids and metabolites associated with protein metabolism. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increased (greater than 0, red) or decreased (less than 0, blue).

Amino acid metabolism

Both the urea cycle (p= 0.0022) and ammonia recycling (p= 0.0252) are highly ranked pathways in serum acutely post-match together with BCAA degradation (p= 0.0301) (Table 5.5). All serum amino acids apart from alanine, histidine and tyrosine are reduced in the GD samples (Figure 5.5).

Metabolic stress

The organic acid 2-hydroxybutyrate peaks acutely post-match in serum (Figure 5.4). The serum levels of creatine and creatinine reach a marked peak in the GD sample also (Figure 5.5).

5.4.5 Changes in the serum metabolome in recovery from match play

Univariate analysis identified one metabolite, alanine as significantly (p = 0.0019) increased at GD+2, in the recovery period. Multivariate analysis of blood serum generated four high quality models (ROC > 0.75) via PLSDA. The metabolites identified as key discriminators (VIP > 1) between samples were put forward for pathway enrichment analysis, the results of which are displayed in Table 5.6.

Table 5.6 Metabolites identified as key discriminators between samples collected the day prior to match play (GD-1) and the GD+1, GD+2, and GD+3 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The unadjusted ranked *p*-values are displayed here.

Recovery pathways	**Timepoint Comparison (Unadjusted p-value)**				**Metabolites Included**
	GD-1 vs. GD+1	GD-1 vs. GD+2	GD+1 vs. GD+2	GD+2 vs. GD+3	
Glucose-Alanine Cycle	0.0182	0.0005	0.0182	0.0225	D-Glucose, L-Alanine
Galactose Metabolism	0.0224				D-Glucose, D-Mannose, Myo-inositol
Transfer of Acetyl Groups into Mitochondria	0.0493		0.0493		Citrate, D-Glucose
Propanoate Metabolism		0.0170			2-Hydroxybutyrate, L-Glutamic acid, L-Valine
Alanine Metabolism		0.0210			L-Glutamic acid, L-Alanine
Glutathione Metabolism		0.0314			L-Glutamic acid, L-Alanine
Glycine and Serine Metabolism		0.0420			L-Glutamic acid, L-Alanine, L-Threonine
Valine, Leucine, and Isoleucine Degradation		0.0438			L-Glutamic acid, L-Isoleucine, L-Valine.
Pyruvate Metabolism			0.0414		Acetic acid, Lactate, Propylene glycol
Urea Cycle				0.0146	L-Glutamic acid, L-Alanine, L-Arginine, L-Glutamine

Energy and amino acid metabolism

Levels of serum amino acids appear to normalize at GD+1 (Figure 5.5). Alanine remains above pre-match levels with citrate, lactate, and acetate. However, between the morning after the match at GD+1, and the GD+2 timepoint there is a shift denoted by pathways of glucose-alanine cycle (p= 0.0182), mitochondrial activity (p= 0.0493), and pyruvate metabolism (p= 0.0414) being ranked highly in serum (Table 5.6, Figures 5.4 and 5.5).

Serum levels of alanine are significantly higher again (p = 0.019) at GD+2 compared with GD−1, and the levels of all glucogenic amino acids are reduced. The ketogenic amino acids leucine and lysine remain at pre-match levels in blood serum.

Metabolic stress

Pathway enrichment of the key discriminatory metabolites between GD-1 and GD+2 also identified glutathione metabolism (p= 0.0314), and, glycine and serine metabolism (p= 0.0420) in serum samples in recovery.

Fatty acid metabolism

Acetoacetate in serum normalizes in recovery with 3-hydroxybutyrate levels staying well below pre-match concentrations throughout. Serum lipoprotein fractions shift with reductions in HDL at GD and GD+1, whilst VLDL rises in recovery at GD+2.

5.5 Discussion

This is the first research to investigate the metabolome in a collision sport population and to provide characterization of the metabolic perturbations caused by competitive elite rugby union. Our prior work investigating changes to resting metabolic rate in this same population (Chapter 4), revealed a significant increase in metabolism in recovery post-match play together with significant shifts in substrate oxidation at rest. The aim of this study was to explain these changes and ultimately understand how best to guide any nutritional interventions. The acute energy needs of this high intensity sport are met via glycolysis, the TCA cycle and gluconeogenesis. The recovery period after cessation of match play and prior to training recommencing sees a re-entry to gluconeogenesis, coupled with markers of oxidative stress, and potential alterations to fatty acid metabolism. This complex and integrated whole-body response allows us to discuss how best to meet the increased metabolic requirements of recovery after collision-based team sports for the first time.

A novel part of our exercise metabolomics research design was the simultaneous analysis of dietary intake to account for the influence of nutrition on the metabolomic responses to rugby match play. This was particularly important as we analysed the metabolome beyond the previously reported 24 h time point post exercise (Schranner et al., 2020). Monitoring dietary intake is essential to ensure our data can be translated in to the world of applied sports nutrition (Close, Kasper and Morton, 2019). Carbohydrate intake of the players here was periodized with training load influenced by the 'Fuel for the work required' paradigm framework (Impey et al., 2018) and prioritising a high intake at GD−1 to ensure high glycogen levels for performance (Bradley et al., 2016). A target of 6 g/kg carbohydrate may be appropriate on GD−1 to ensure sufficient glycogen and may be more appropriate than 3

g/kg (Bradley et al., 2016). Carbohydrates were also consumed during the match as per ingestion guidelines of 60 g/h (Baker et al., 2015) in the form of drinks and gels, which yielded the two significant unidentified saccharides in serum. These athletes did not meet the upper target of carbohydrate on GD−1 but at 4.32 ± 0.89 g/kg we would not expect this combined with the high intake on GD pre-, and peri-match to have limited glucose availability. Nevertheless, this novel insight provides further evidence regarding the importance of carbohydrates for performance. Glycolysis, together with the TCA cycle and glucose-alanine cycle are the predominant pathways accounting for energy production during match play. Serum acetate levels do peak at GD and the beta-oxidation of fatty acids could contribute to this. However, there is no accumulation of acetoacetate or 3-hydroxybutyrate in serum which would indicate the saturation of β-oxidation as seen previously in prolonged endurance exercise (Stander et al., 2018; Bester et al., 2021). This leads us to propose the high intensity nature of elite rugby union reduces the utilization of fatty acids for energy provision.

Rather, pyruvate conversion to lactate ensures glycolysis can continue if aerobic re-oxidation of NAD to NADH is not possible, and pyruvate can also enter the TCA cycle to further generate ATP (Berg et al., 2015). Lactate is transported to the liver and converted to glucose to be transported back to the muscle or peripheral tissues (Felig and Wahren, 1971). Pyruvate can also be converted to alanine in the muscle via the aminotransferase enzyme, in turn also converting glutamate to alpha-ketoglutarate (Berg et al., 2015). The significant increase in serum alanine as reported here, has previously been observed within 30 min of exercise (Schranner et al., 2020), specifically after high intensity training (HIT) rather than isoenergetic, moderate intensity exercise (Peake et al., 2014). This gluconeogenesis is accompanied by amino acid degradation and the upregulation of the urea cycle and

ammonia recycling, evidenced in serum acutely. It is important to note that the origin of these amino acids is unclear, as they may be entering the bloodstream via the gut as the GD samples were not fasted and protein sources were consumed at breakfast and pre-match meal (Jungas, Halperin and Brosnan, 1992). Glutamate in the muscle can be converted to glutamine, transported in the blood to the liver to be converted back into glutamate to aid in the ammonia recycling to supply the alanine aminotransferase reaction for alanine to pyruvate conversion, and then the ammonia feeds into the urea cycle for detoxification (Felig and Wahren, 1971). The rise in serum tyrosine levels can be explained by the conversion of phenylalanine into tyrosine during exercise (Van Hall, Saltin and Wagenmakers, 1999). Amino acid metabolism during prolonged exercise appears in the early phases to create a net consumption of glutamate from the muscle to replenish TCA cycle intermediates (van Hall et al., 1995). The formation of α-oxoglutarate, succinyl-CoA, and oxaloacetate from glutamate, valine, and isoleucine are mechanisms for anaplerosis in exercise which may explain a number of the reductions in serum amino acids witnessed here (Van Hall, Saltin and Wagenmakers, 1999).

When reviewing prior metabolomics research, blood, urine and sweat levels of all three BCAAs appear to drop in the early (0-30 minutes) and intermediate (30 minutes-3 hours) sampling times (Schranner et al., 2020). After resistance training, they have been described as metabolites of slow response, with levels reducing to below pre-exercise at 60 minutes post training (Berton et al., 2017). Even though we would expect a drop in the levels of these amino acids after resistance training due to protein synthesis (Jackman et al., 2017), the previously highlighted pathways of amino acid degradation are more likely to account for the lower levels here. In tandem with BCAA degradation we may expect to see metabolites such as the keto-acids; ketoisovalerate, ketoisocaproate, ketomethylvalerate in

the post-match samples (Holecek, 2018). These were not identified using NMR spectroscopy and may be due to interactions with albumin in the samples or swift metabolism to other downstream metabolites may have occurred. There is little appearance of these keto-acids in blood samples in the published literature. Ketoisovalerate and ketoisocaproate have been detected in blood samples of female adults with reference to specific nutrient intakes (Pallister et al., 2016). Ketoisocaproate appears in the blood metabolites of adolescent in adult females presenting with autism disorders (Novarino et al., 2012). The only study with blood samples containing all three of the keto-acids contained male and female adult participants used early gas chromatography mass spectrometry (GC-MS) for identification (Hoffmann et al., 1993). It is possible that examination of other biofluids such as saliva (Gardner, Carpenter and So, 2020) and urine (<Guide_to_Hidden_Sugars.pdf>; Siopi et al., 2017) may provide more insights into the degradation of BCAAs post exercise. Alterations in BCAA metabolism when measured in the fasted state are associated with changes to glucose tolerance and insulin sensitivity (Holecek, 2018). Detrimental changes to glucose tolerance have been attributed to elevated fasted levels of BCAA in the blood (She et al., 2007) as opposed to the lower levels reported here.

Overall, the intensity and duration of elite rugby union match play cause acute metabolite perturbations indicative of both oxidative and metabolic stress whilst fulfilling the high energy demands. The increase in serum 2-hydroxybutyrate post-match is indicative of the cumulative stress upon energy systems (Landaas and Pettersen, 1975) and may be due to increased catabolism of L-threonine (Bui, Ravasz and Chinopoulos, 2019) and glutathione synthesis (Lu, 2013) in response to oxidative stress. Transient increases have also been witnessed after HIT exercise (Peake et al., 2014) and proposed as a marker of dysglycemia (Ferrannini et al., 2013). In non-diabetic populations it is an early biomarker for both insulin

resistance and impaired glucose regulation (Gall et al., 2010). This is particularly interesting as the changes to the metabolome in recovery that follow, implicate changes in glucose regulation and gluconeogenesis. The pathways of glutathione metabolism, and glycine and serine metabolism are ranked highly in recovery, with reductions in serum L-threonine indicative of increased demands for hepatic glutathione production (Lu, 2013) as this oxidative stress continues in the days after match play.

As we have shown previously in chapter 4 of this thesis and in our publication (Hudson et al., 2020), in training, these athletes are exposed regularly to the high intensity activities of the sport, but without full collisions. In the days following match play they are therefore recovering from muscle damage due to unaccustomed activities and the collisions inherent with tackling, carrying, and contesting the ball which result in EIMD and IIMD (Naughton, Miller and Slater, 2018a), and it is the resulting metabolic perturbations we are examining herein. Whatever the primary mechanism of ultrastructural damage, the cascade of events comprising the secondary mechanism is triggered by an acute inflammatory response due to the action of immune cells such as neutrophils and macrophages (Peake et al., 2017).

The subjective measures of wellness support this, with vigor decreased significantly for two days and muscle soreness significantly increased for three days when compared to measures prior to match play. Despite intense training in the days preceding match play there was no significant increase in soreness prior to the game. This has been reviewed across both codes of rugby with soreness peaking between 12 and 36 hours post-match (Naughton et al., 2021). Interestingly we did not see any significant deprivation of sleep duration or reduction in quality across the week, and no potentially confounding non-training stress in the participants.

The increased cytokine concentrations of IL-6 (Cunniffe et al., 2010; Cunniffe et al., 2011), IL-8 and IL-10 (Morehen et al., 2020) have been profiled in this population post-match play. The inflammation may also be responsible for the reductions in serum HDL levels at GD and GD+1, whilst VLDL rises in the GD+2 and GD+3 samples are also associated with cytokine activity (Khovidhunkit et al., 2004). The increase in VLDL production and secretion is a result of: increased hepatic fatty acid synthesis, increase in transport of fatty acids to the liver and a decrease in fatty acid oxidation in the liver (Khovidhunkit et al., 2004). There may therefore be alterations to fatty acid oxidation in the recovery period associated with the previously observed inflammation after rugby match play.

Previous work has implicated the inflammatory cytokine IL-6 released by the working muscle to alter substrate oxidation across body tissues (Pedersen et al., 2003; Knudsen et al., 2017) with evidence it stimulate endogenous glucose production (Febbraio et al., 2004). The associated immune response may also explain an increased demand for glucose.

Examination of the immune response post elite rugby union has revealed significant increases in total leukocytes, specifically neutrophils and monocytes peaking acutely post-match play and remaining significantly elevated compared to baseline measures (Cunniffe et al., 2010; Cunniffe et al., 2011). Increases in lymphocytes, specifically significant increases in $CD4^+$ have also been witnessed at the GD+1 timepoint (Cunniffe et al., 2010). Any reducing or blocking of this initial immune cell response can interfere in regeneration and subsequent adaptive remodeling of muscle tissue (Peake et al., 2017). Neutrophils respond to stimuli by enhancing their glucose uptake and increasing expression of glucose transporters suggesting a functional dependence of glucose in modulating their function, especially phagocytic

events (Kumar and Dikshit, 2019). Activated $CD4^+$ and $CD8^+$ T cells both display elevated glycolysis in vivo, critical for rapid growth and proliferation (Macintyre et al., 2014).

The reduced serum levels of glucogenic amino acids in recovery lead us to propose that this requirement for glucose due to the secondary response to muscle damage has not been met via dietary intake. Similar dietary carbohydrate periodisation has been reported previously (Bradley et al., 2015a) with a mean weekly intake of 3.4 g/kg compared to our 3.2 g/kg here, within the intakes across the literature for this population (Black, Black and Baker, 2018). The intake of carbohydrate on GD+1 was below the mean for the week at 2.9 g/kg, again like the earlier work in an elite rugby union population in-season reporting 3.1 g/kg (Bradley et al., 2015a). This earlier work recorded similar energy intakes to those observed here and reported a predicted balance of energy utilizing wearable devices to measure TEE (Bradley et al., 2015a). There are limitations to these measures of TEE, but our findings here suggest that energy availability generally may not be as crucial for recovery as carbohydrate availability specifically. Revisiting our work in chapter 4 of this thesis, we can calculate the significant increase in fasted carbohydrate oxidation at rest to be from 228 $g \cdot day^{-1}$ the morning prior to match play, up to 319 $g \cdot day^{-1}$ at GD+2 (Hudson et al., 2020). We propose increasing dietary carbohydrate intake at GD+1 to account for this increased resting requirement, normal daily activity, and any light recovery modalities, would result in a significant increase on the intake observed here and in previous observations of this population. The hypothesis if this carbohydrate requirement is met, being a reduction in amino acid degradation and gluconeogenesis activity as reported here in the blood serum metabolome.

5.5.1 Conclusion

In conclusion, novel insight is provided into how energy systems cope with the metabolic demands acutely around rugby match play, but also in the recovery days, and may explain the shifts in RER reported in chapter 4. Rather than the availability of energy being the priority, a potential reduction in the ability to oxidize fatty acids, coupled with glucogenic amino acid degradation with upregulated gluconeogenesis leads us to propose that the effective recovery from muscle damaging collisions during elite rugby union match play, is dependent upon the availability of glucose. This would translate into the periodisation of carbohydrate throughout the competitive microcycle in contact sports to be re-evaluated to prioritize carbohydrate in recovery whilst balancing energy intake to maintain optimal body composition and performance throughout a season. Should we expand the 'Fuel for the work required' paradigm in collision-based team sports to include 'Fuel for the damage induced'?

The ramifications of insufficient carbohydrate availability during the recovery period are not clear though. The origin of the amino acids oxidized to meet the requirements of the system are unknown as the blood serum metabolome offered novel insight as to the energy systems and oxidative stress induced by elite rugby union match play, but not some potential further downstream metabolites. We propose that complementary metabolomics investigation using saliva and urine may corroborate the hypothesis generated from the blood serum investigation. These two biofluids are much less invasive to collect and may provide another window into the metabolic requirements following match play and throughout recovery. As summary of the major findings can be found below at figure 5.6 in an infographic format.

Rationale

Metabolomics as an unbiased systems based approach is able to capture the complex and integrated whole-body response to exercise

Objective

Investigate the metabolic perturbations associated with elite rugby union match play acutely and throughout recovery in blood serum

Results

Acute pathways

Glycolysis,
Glucose-alanine cycle,
Urea cycle,
Ammonia recycling,
BCAA degradation,
AA metabolism.

Recovery pathways

Glucose-alanine cycle,
Glutathione, glycine & serine metabolism,
Urea cycle,
BCAA degradation,
Pyruvate metabolism.

Energy provision during match play appears to be via glycolysis, TCA cycle, and gluconeogenesis. Fatty acid oxidation appears relatively low due to the intensity of the match play. Metabolites indicative of a cumulative metabolic stress were apparent.

After the normalisation of many perturbations at GD+1, the re-entry to gluconeogenesis at GD+2 is accompanied by pathways associated with oxidative stress, amino acid degradation, and a potential reduction in fatty acid oxidation.

Conclusion

We propose that the effective recovery from muscle damaging collisions during elite rugby union match play, is dependent upon the availability of glucose.

Next Steps

Investigating multiple biofluids may provide congruency in pathway analysis with findings from serum and identify further significant metabolites

Figure 5.6 Infographic overview of the chapter to summarise the major findings.

Chapter Six

Untargeted metabolomics investigation of urine and saliva in elite rugby union: Acute match play and the time course of recovery.

The research and findings within this chapter were published in the journal Metabolites in 2021.

(Hudson et al., 2021) Hudson, J. F., M. M. Phelan, D. J. Owens, J. P. Morton, G. L. Close and C. E. Stewart (2021). ""Fuel for the Damage Induced": Untargeted Metabolomics in Elite Rugby Union Match Play." Metabolites **11**(8).

6.1 Abstract

INTRODUCTION: Investigating the serum metabolome around match play generated the hypothesis that the metabolic requirements of recovery are reliant upon carbohydrate. The identified pathways of gluconeogenesis, amino acid degradation, and oxidative stress in serum may be investigated further utilising the minimally invasive body fluids urine and saliva. Whilst metabolites between biological samples may not be used interchangeably, there is evidence for congruency in pathway analysis between multiple biofluids. Multiple metabolomes have never been investigated in Elite athletes previously.

METHODS: Seven members of an English Premiership rugby squad consented to providing urine and saliva samples daily, over a competitive week including gameday (GD), with physical demands and dietary intake also recorded. Sample collection, processing and statistical analyses were performed in accordance with best practice set out by the metabolomics standards initiative (MSI) employing 700 MHz NMR spectroscopy (Considine et al., 2017).

RESULTS: There were no metabolites identified as significantly different across the match week after univariate analysis. Multivariate PLS-DA analysis provided 12 high quality models with receiver operator characteristic scores (ROC > 0.75) and metabolites identified as key discriminators if the variable importance projection scores (VIP>1) between samples entering pathway enrichment. Pathways most highly ranked in saliva were aligned with findings from the serum metabolome. The glucose-alanine cycle (p= 0.008) and gluconeogenesis (p= 0.028), together with pathways of amino acid degradation (arginine & proline metabolism p= 0.042, methionine metabolism p= 0.015) were identified in recovery. The metabolism of tryptophan via the kynurenine pathway was identified in urine samples

as being significantly altered, both acutely (p= 0.005) and in recovery (p= 0.035), providing further evidence for how the system copes with the acute high energy demands of the sport and recovery. Both biofluids supported markers of oxidative stress in purine metabolism. Markers of muscle protein and connective tissue breakdown were identified as increased in the days following competitive match play.

CONCLUSIONS: This complementary biofluid analyses reveals congruency in pathway enrichment analysis between blood serum and saliva, together with downstream metabolites supporting earlier serum insights. We propose these findings reinforce the concept of upregulated gluconeogenesis in the recovery period, post-match, due to the requirement of carbohydrate. If not provided, via dietary intake, the degradation of amino acids may be derived from muscle protein and connective tissue due to the collision-based activities the athletes are exposed to. This also accentuates the need for carbohydrate periodisation in team collision sports to account for the recovery period and to 'fuel for the damage induced'.

6.2 Introduction

Chapter 5 provided novel insight into the acute metabolic fluctuations immediately after elite rugby union match play as well as in the days afterwards. The relationship between the blood metabolome and other biofluids has not been studied widely in exercise research, but a network based approach to investigating intra- and inter-fluid metabolite associations has been carried out (Do et al., 2015). A multi biofluid approach has been effectively utilised in dietary standardisation practices (Walsh et al., 2006), and in disease states such as type one diabetes (Balderas et al., 2013), in HIV patients (Munshi et al., 2013), and in biomarker selection for drug-induced toxicity (Adourian et al., 2008).

Substances, ultimately excreted through urine, are first transported to the kidneys via the bloodstream and filtered into the bladder prior to forming urine, so a relationship between these two biofluids may be anticipated (Bouatra et al., 2013). Saliva is produced in the oral cavity by the submandibular and parotid glands where molecules from the systemic circulation may enter via blood capillaries (Dame et al., 2015; Gardner, Carpenter and So, 2020). However, the salivary metabolome also provides an insight to oral health, the oral microbiome and dietary intake (Dame et al., 2015; Gardner, Carpenter and So, 2020). The network-based approach revealed significant occurrence of amino acids, carbohydrates and xenobiotics in at least two biofluids indicates a strong exchange between fluids which may generate congruency in pathway analysis (Do et al., 2015). Blood sampling in any athlete population is challenging and so addressing the utility of the minimally invasive body fluids: urine and saliva, should be investigated to validate future use in these populations (Lindsay and Costello, 2017; Gardner, Carpenter and So, 2020).

Saliva and urinary metabolome analyses have been employed to investigate performance testing in soccer players (Santone et al., 2014), pre- and post- match play (Pitti et al., 2019) and during a season, to examine fatigue (Ra et al., 2014) or physical load (Quintas et al., 2020) as well as in basketball throughout match play (Khoramipour et al., 2020). These single biofluid studies have provided valuable insight to energy systems utilised at the sport specific intensity, together with potential pathways associated with fatigue such as metabolism of purines and markers of protein degradation. Work in youth professional soccer analysed plasma, urine, and saliva samples to investigate the effect of short-term physical activity upon the metabolome (Alzharani et al., 2020). Whilst plasma provided the more comprehensive picture, there were complementary insights of the other biofluids in purine and amino acid metabolism (Alzharani et al., 2020).

Significant challenges remain in determining the suitability of such easily accessible biofluids, and it has been warned that the concentration of biomarkers in saliva and blood cannot be used interchangeably in healthy adults (Williamson et al., 2012). Whilst the comparison of lactate levels for example, in both biofluids is better in trained athletes, they do exhibit a differing response to maximal exercise (Tekus et al., 2012). The review of exercise studies carried out by Schranner and colleagues illustrated this point well in that metabolites from blood and urine associated with carbohydrate metabolism, TCA cycle, and amino acid metabolism, could differ greatly in response to exercise (Schranner et al., 2020). Metabolite changes in lactate and pyruvate for example, were a similar positive fold change whilst citrate and succinate could be seen to have opposite changes with increases in blood mirrored with decreases in urine. The converse responses were also true of some amino acids (Schranner et al., 2020). We would propose the gathering of multiple biofluids at the

same sample timepoints serves to investigate congruences in metabolic pathway regulation after the exercise stimulus (Schranner et al., 2021).

The objective is to investigate the metabolic perturbations in the metabolomes of both urine and saliva samples in response to elite rugby union match play. This analysis may provide novel insight as to the congruency of pathway enrichment analysis between multiple biofluids. There may also be downstream metabolites identified as key discriminators between samples to support or refute the finding of chapter 5 herein.

6.3 Methods

6.3.1 Participants and research design

The participants were the same cohort, and the research design is as detailed in section 5.3.1. Figure 6.1 shows the study design and workflow.

6.3.2 Training and match demands

Internal and external loads were recorded for both training and match play throughout the week as per section 3.4. Tables 5.1 and 5.2 detail the physical content of the match week.

6.3.3 Dietary intake

Dietary intake was recorded as described in section 3.4.4. with results displayed in table 5.4.

6.3.4 Biofluid sample collection

Urine was collected in 15 mL urine centrifuge tubes (Sarstedt, Leicester, UK) which contained no citrate or other stabilizers. Saliva samples were collected using the previously validated Salivette swabs (Salivette Sarstedt, Nubrecht, Germany) without additives. For further detail please see section 3.6.2.

6.3.5 NMR Sample preparation

Urine and saliva samples (500 µL) were thawed at room temperature before addition of buffers and sodium azide. The final concentration in the NMR tube was 50% urine or saliva, 10% $^{2}H_2O$, 1.2 mM sodium azide and 100 µM TSP. Please see section 3.7.1 for further detail of sample preparation.

6.3.6 NMR Spectroscopy

Urine and saliva 1D ^{1}H-NMR spectra were acquired at 25 °C using a 700 MHz Bruker Advance IIIHD spectrometer to facilitate analysis via Chenomx Standard library. For further detail on how spectra were acquired please refer to section 3.7.2.

6.3.7 Spectral processing and annotation

Saliva spectra were integrated into 251 bins with 134 (53%) annotated corresponding to 82 metabolites and 117 unknown metabolite bins. Chenomx v8.2 software was used to perform metabolite annotation on the individual urine spectra using automated fit all metabolites routine Full spectral analyses is described in section 3.7.3.

6.3.8 Data analyses

Spectral analysis is described in full in section 3.7.4. Please refer to section 5.3.8 for the metabolomics data analysis methodology and statistical analysis utilised.

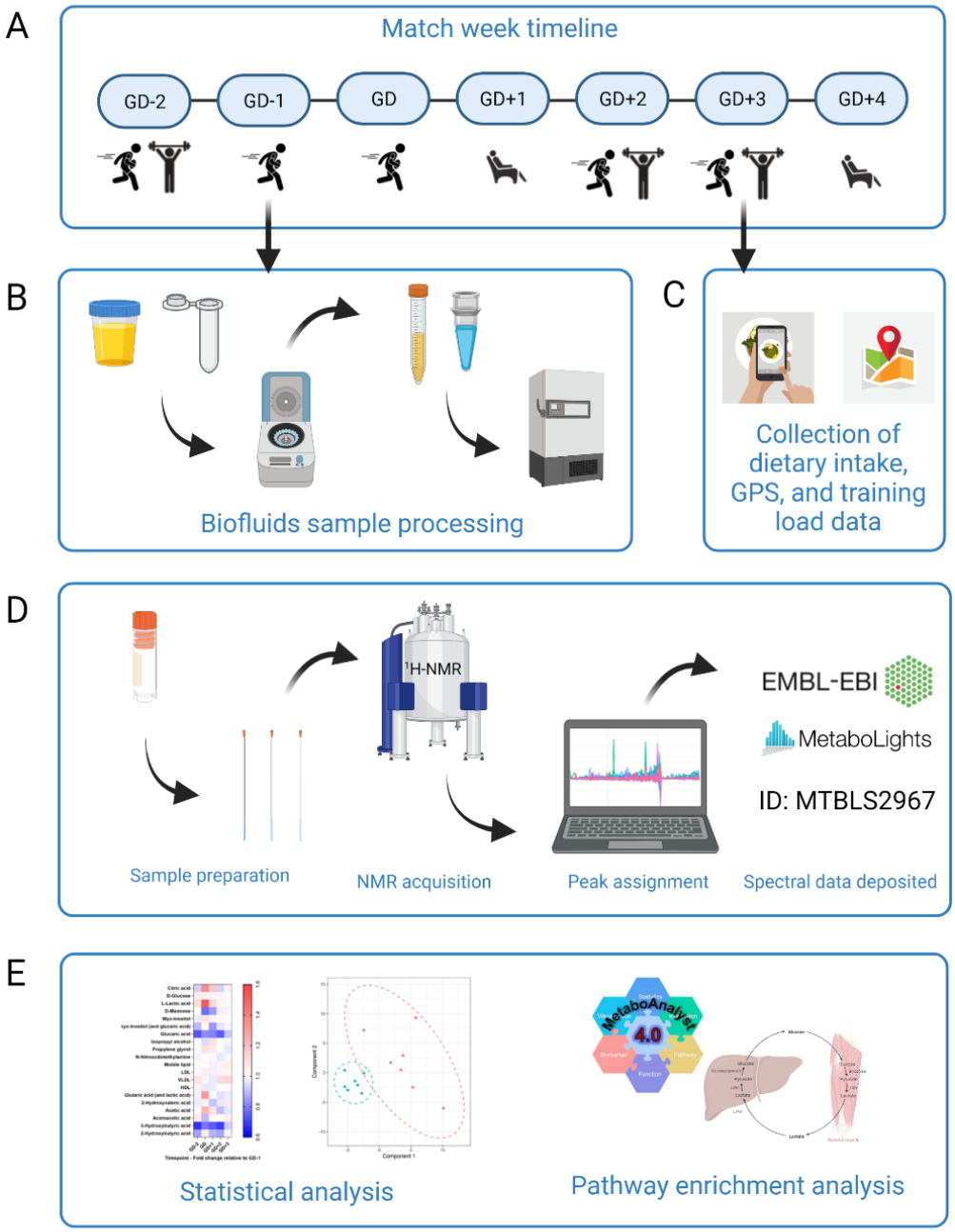


Figure 6.1 Schematic overview of the study design. (**A**) Participants (*n* = 7) began the study at GD-2 and completed a whole match week schedule of rugby specific sessions, resistance training and rest. (**B**) Biofluids sample processing. Participants provided a sample of urine and saliva every morning apart from the GD samples which were taken immediately post-match play. Samples were processed immediately with timings rigorously repeated each day. Samples were aliquoted to cryovials then frozen for later analysis. (**C**) Dietary intake for all seven days using the Snap'n'Send method, with all GPS and load data were collated. This was all analysed to further translate changes to the metabolome. (**D**) Sample preparation and analysis by ^{1}H-NMR. Spectra were acquired and then peaks assigned using a combination of Chenomx and in-house libraries. Full spectrum parameter sets are available with the data deposited at MetaboLights public repository (ID number MTBLS2967). (**E**) Statistical analysis. Univariate and multivariate data analysis was performed in R to elucidate key metabolites between all sample timepoints. MetaboAnalyst 4.0 pathway enrichment analysis was then carried out with those statistically significant and key discriminatory metabolites. Created with BioRender.com

6.4 Results

Please refer to sections 5.4.1 training and match demands, 5.4.2 subjective measures of wellness, and 5.4.3 dietary intake, for the detailed results of these areas of data collection. The urinary and salivary metabolomes shall be reported here again referring to the 'acute' changes to the metabolome utilising the samples gathered immediately after match play, and the 'recovery' period comparing fasted metabolome in the days prior to match play with days after.

6.4.1 Acute changes to the metabolome after match play

Univariate analysis of both the saliva and urine metabolomes yielded no significant metabolites in the GD samples when analysed. Multivariate analyses of both biofluids generated five high quality PLS-DA models which identified key discriminatory metabolites which went forward to pathway enrichment analysis. The two saliva model pathway results are displayed in table 6.1 and the three urine model pathway results are displayed in table 6.2.

Table 6.1 Saliva metabolites identified as key discriminators between saliva samples collected immediately post-match play (GD) and the GD+1, and GD+2 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The ranked *p*-values are displayed here.

Acute Pathways	**Timepoint Comparison (Unadjusted *p*-value)**		**Metabolites Included**
	GD vs GD+1	GD vs GD+2	
Urea Cycle	0.025		L-Glutamic acid, L-Alanine, L-Aspartic acid, L-Glutamine
Glucose-Alanine Cycle	0.013		D-Glucose, L-Alanine, L-Glutamic acid
Lactose Degradation	0.004	0.045	D-Glucose, D-Galactose, Alpha-Lactose
Ketone Body Metabolism	0.013	0.012	Acetoacetic acid, Succinic acid, Acetone
Ammonia Recycling	0.035		L-Glutamic acid, L-Aspartic acid, Urocanic acid, L-Glutamine
Glutamate Metabolism	0.042		L-Glutamic acid, L-Alanine, L-Aspartic acid, Succinic acid, L-Glutamine
Aspartate Metabolism	0.047		Acetic acid, L-Glutamic acid, L-Aspartic acid, L-Glutamine

Table 6.2 Urine metabolites identified as key discriminators between samples collected immediately post-match play (GD) and the GD-1, and GD+2 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The ranked *p*-values are displayed here.

Acute Pathway	**Timepoint Comparison (Unadjusted p-value)**			**Metabolite List**
	GD-1 vs. GD	GD vs. GD+2	GD vs. GD+3	
Tryptophan Metabolism	0.005	0.013		Formic acid, L-Alanine, Serotonin, L-Kynurenine, Kynurenic acid, 5-Hydroxyindoleacetic acid, Xanthurenic acid, L-Tryptophan, N-Acetyl serotonin.

The high metabolic demands of match play

Acute saliva pathways include the glucose-alanine cycle (p= 0.013), lactose degradation (p= 0.004), and ketone body metabolism (p= 0.013). Key discriminatory metabolites associated with carbohydrate metabolism and TCA cycle intermediates such as pyruvate, lactate, citrate, and succinate in the GD saliva samples (Figure 6.2). Lactose metabolism is identified due to key discriminatory metabolites alpha-lactose, galactose, and glucose (Figure 6.2).

Increases in the metabolites glucose, alanine, and glutamic acid at GD compared the GD-1 explain the inclusion of the glucose-alanine cycle being highly ranked in acute saliva samples contributing to the energy needs of the system during match play (Figures 6.2 and 6.3).

Levels of acetic acid and acetoacetic acid both rise at GD, but salivary acetone is reduced compared with pregame levels (Figure 6.4) indicating a potential decrease in ketone body formation.

Amino acid metabolism

The pathways of ammonia recycling (p= 0.035) and the urea cycle (p= 0.025) are highly ranked from the acute GD salivary metabolome changes, as are the pathways of glutamate (p= 0.042) and aspartate metabolism (p= 0.047) (Table 6.1). Salivary branched chain amino acid levels reduce at GD with phenylalanine, whilst all other amino acids increase in the acute samples (Figure 6.3).

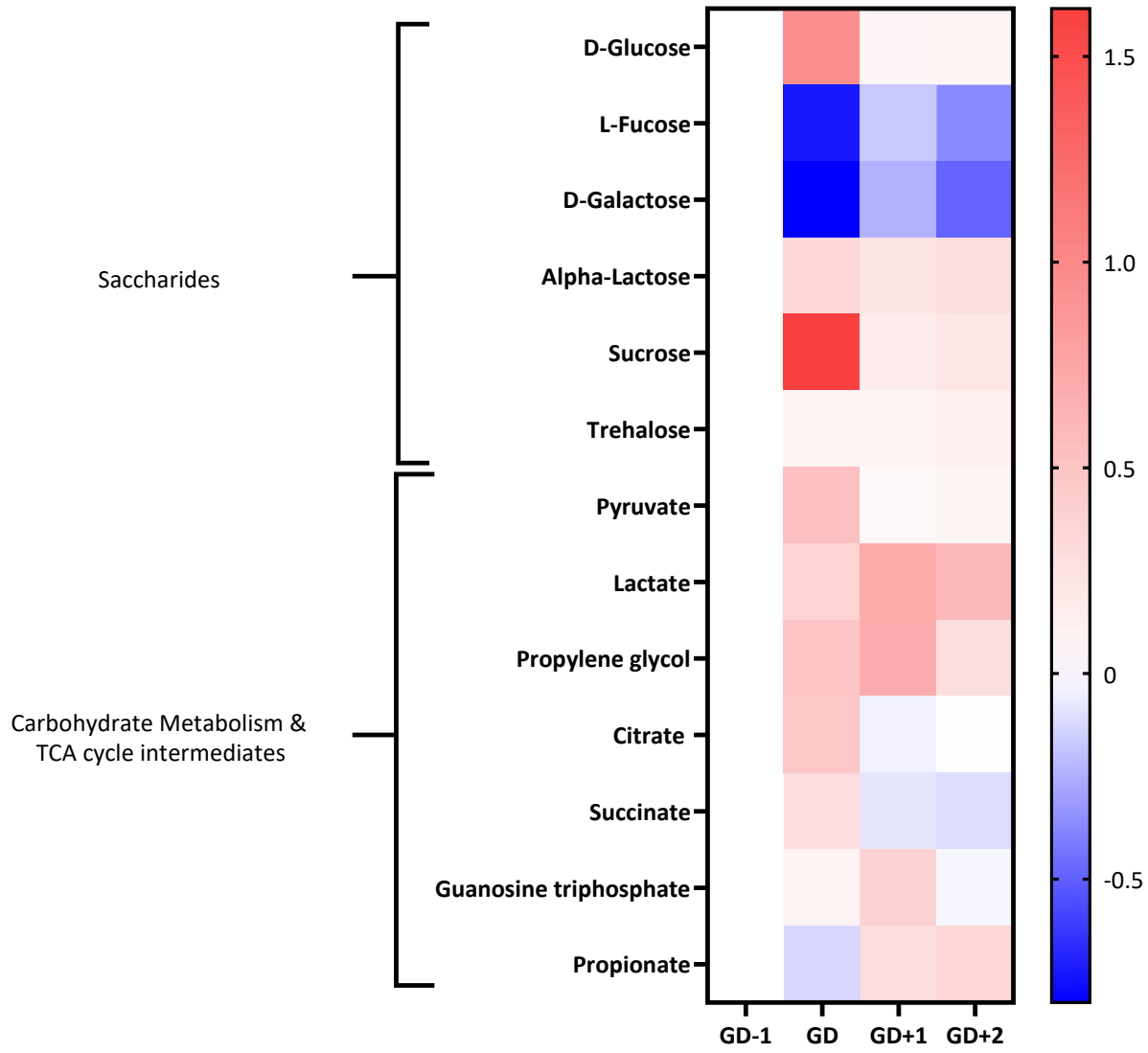


Figure 6.2 Heatmap of saliva metabolites identified as key discriminators between timepoints via PLSDA modelling. These include metabolites of carbohydrate metabolism, TCA cycle intermediaries, and saccharides. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

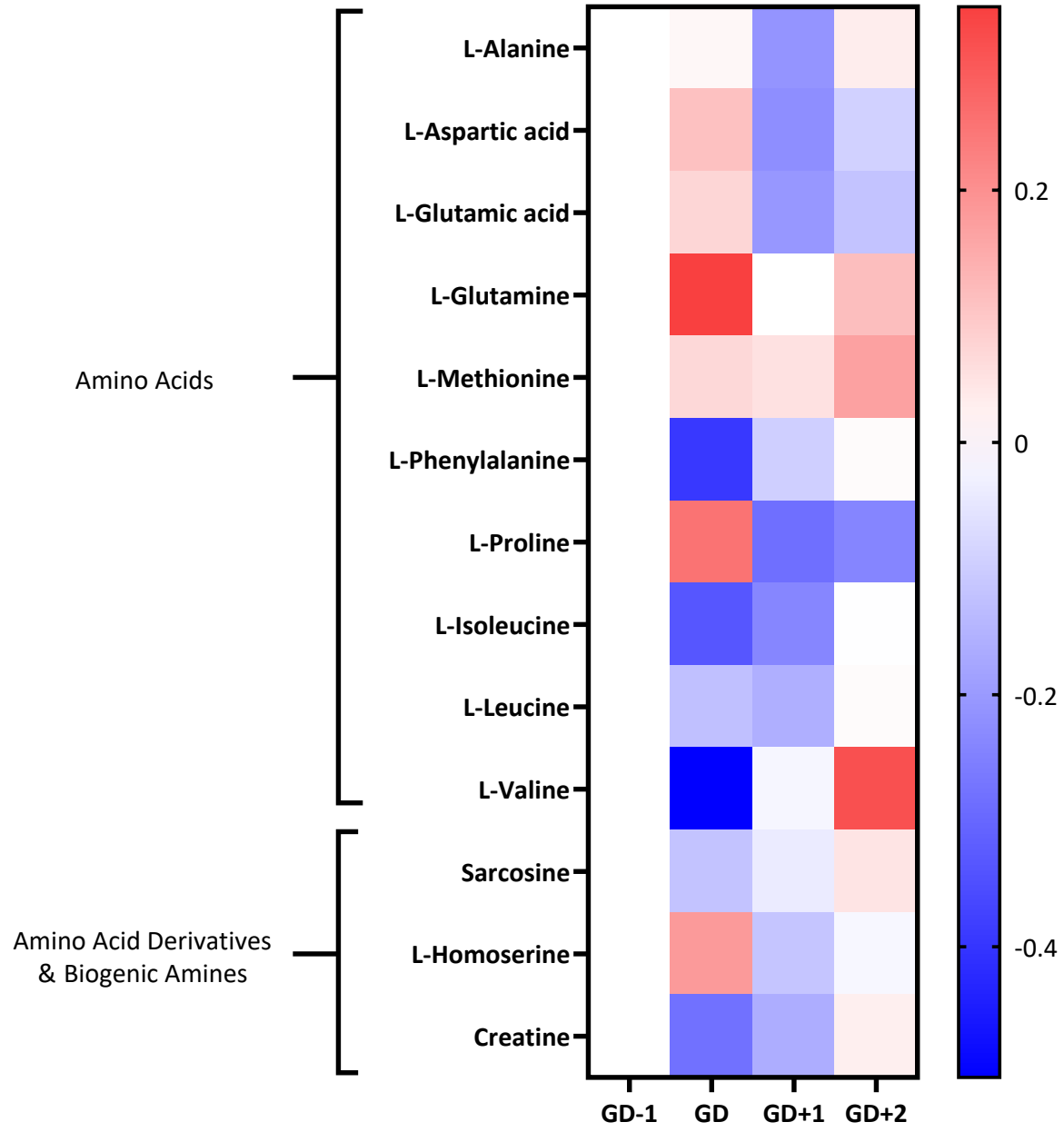


Figure 6.3 Heatmap of saliva metabolites identified as key discriminators between timepoints via PLSDA modelling. These includes biogenic amines, amino acids, and derivatives of amino acids. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

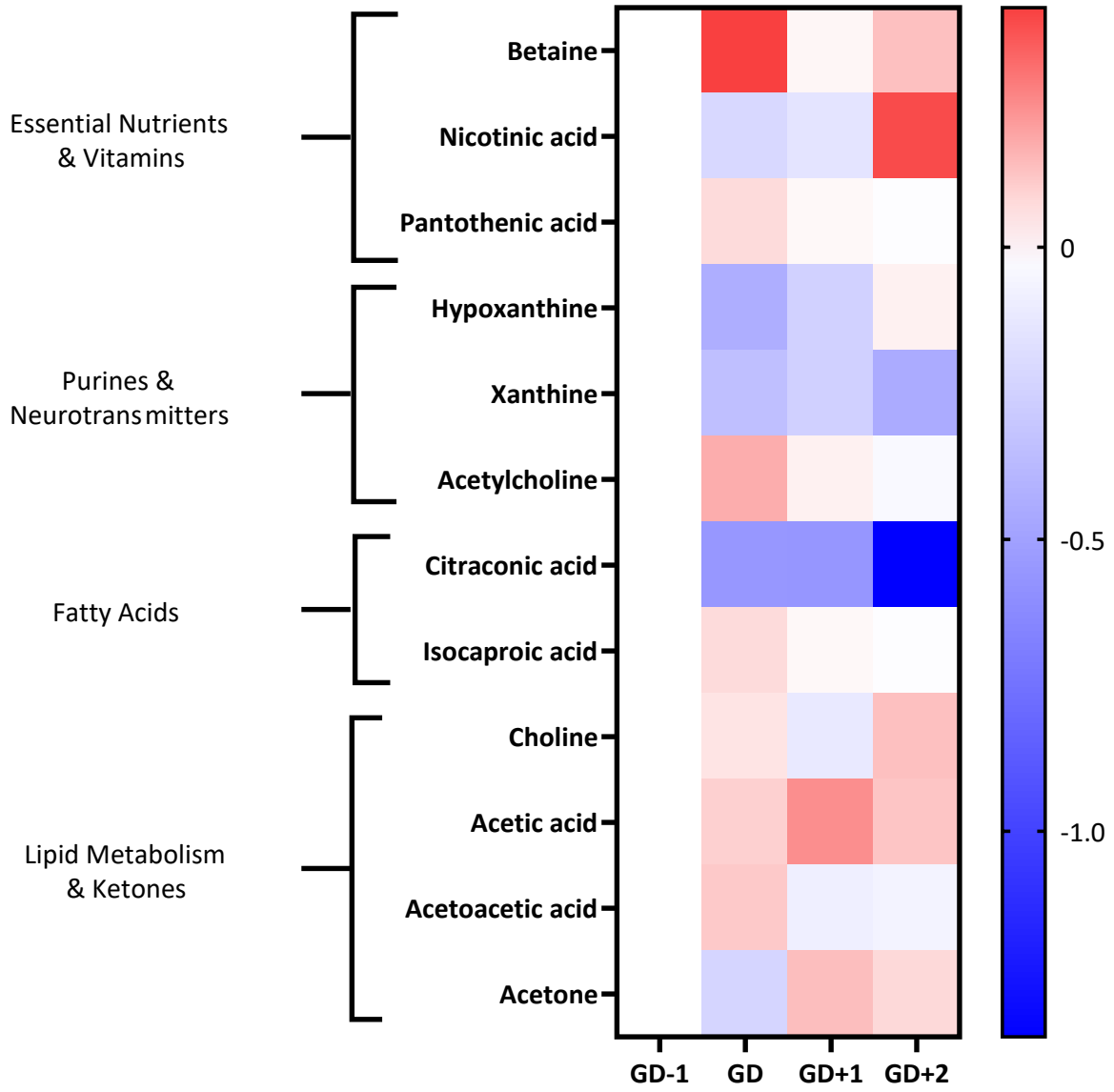


Figure 6.4 Heatmap of saliva metabolites identified as key discriminators between timepoints via PLSDA modelling. These include essential nutrients & vitamins, purines & neurotransmitters, fatty acids, lipid, and ketone metabolites. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

The downstream metabolites of leucine; 2-hydroxyisocaproate, 3- hydroxyisovalerate, and 3-hydroxy-3-methylglutarate are also identified in urine as key discriminators in the GD samples, indicating the degradation of this branched chain amino acid (BCAA) (Figure 6.6).

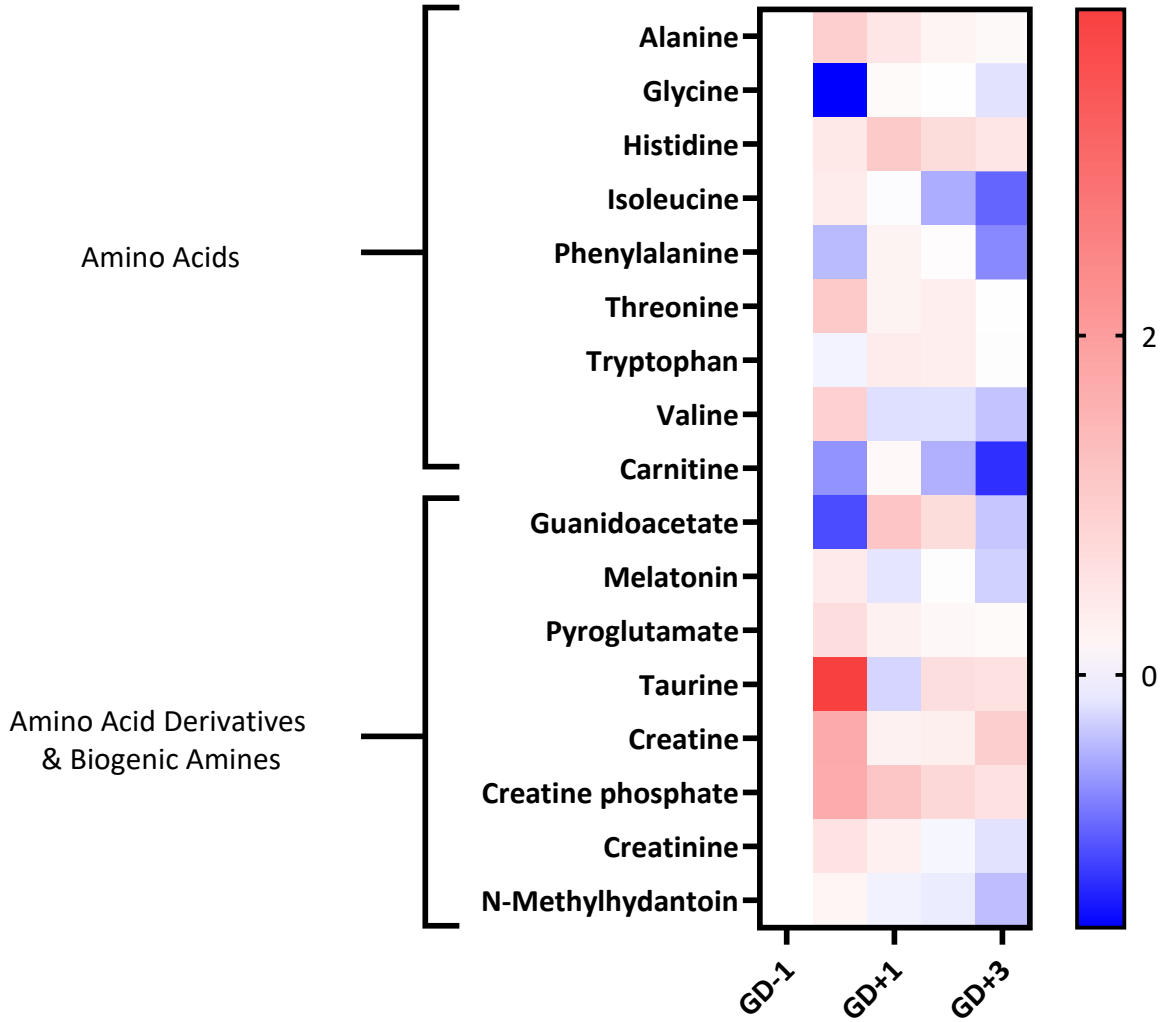


Figure 6.5 Heatmap of urine metabolites identified as key discriminators between timepoints via PLSDA modelling. These includes biogenic amines, amino acid derivatives, and amino acids. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

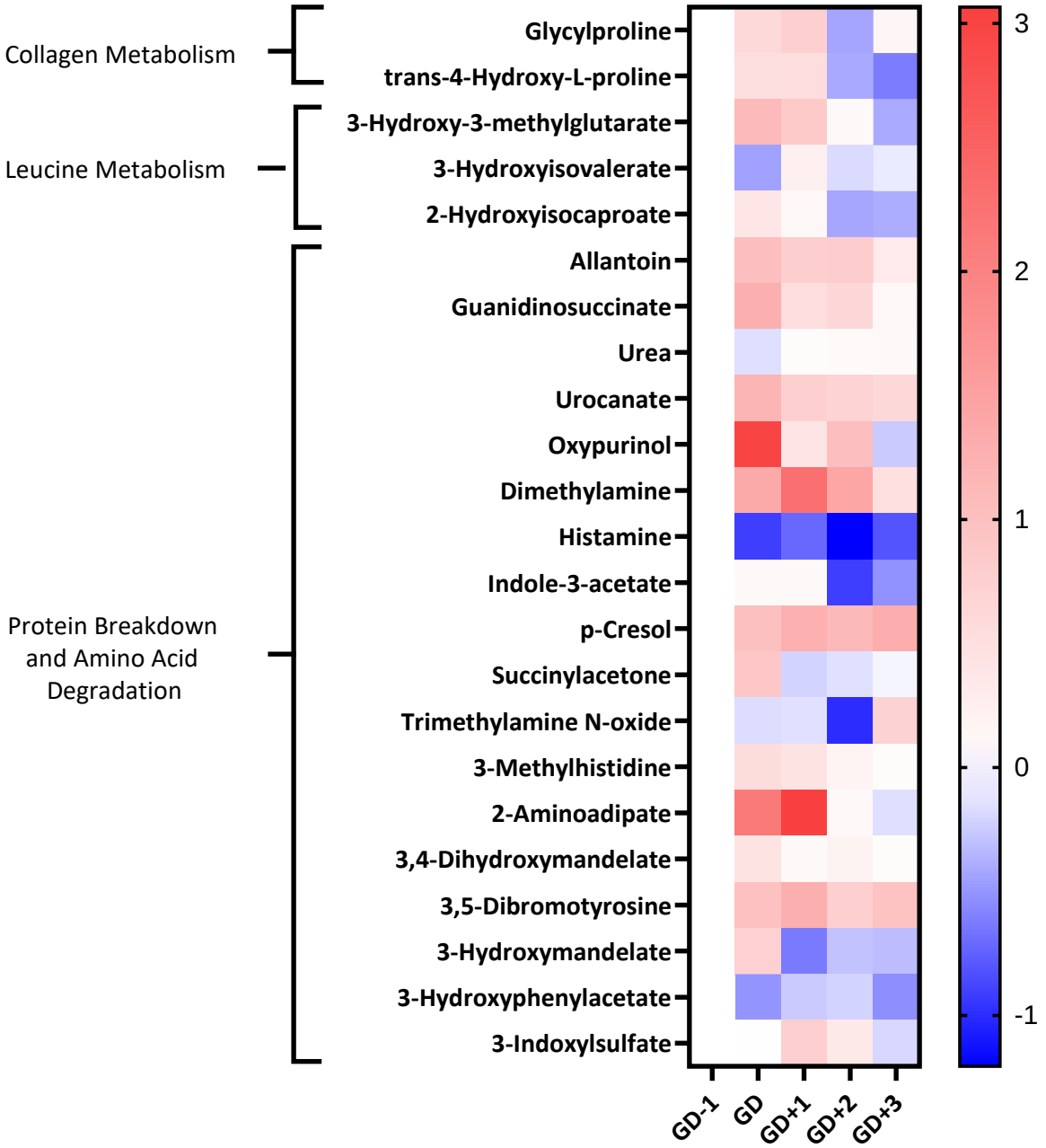


Figure 6.6 Heatmap of urine metabolites identified as key discriminators between timepoints via PLSDA modelling. This includes metabolites of collagen metabolism, leucine metabolism, and the degradation of amino acids and protein breakdown. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

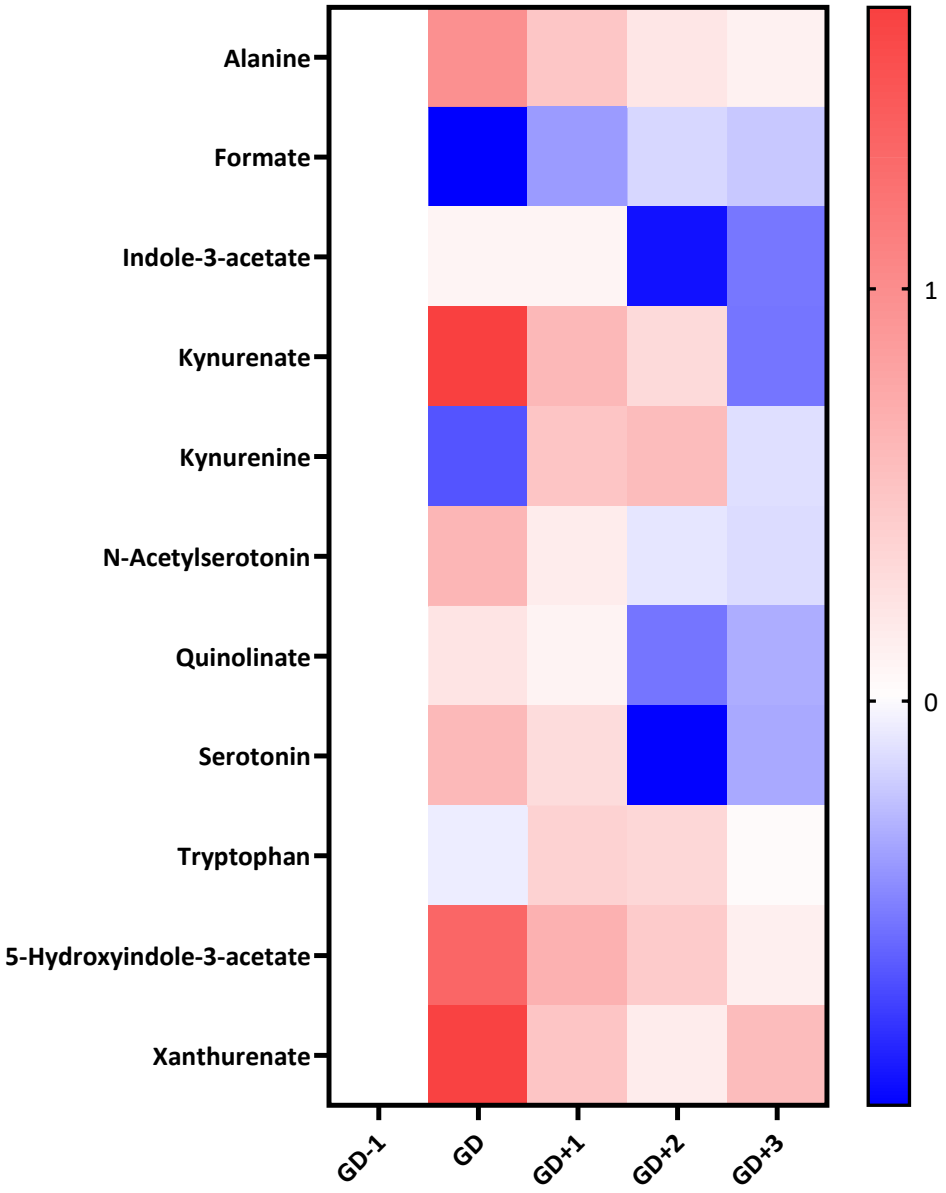


Figure 6.7 Heatmap of urine metabolites identified as key discriminators between timepoints via PLSDA modelling in samples specifically involved in the pathway of tryptophan metabolism (also depicted in figure 7.13 below). The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

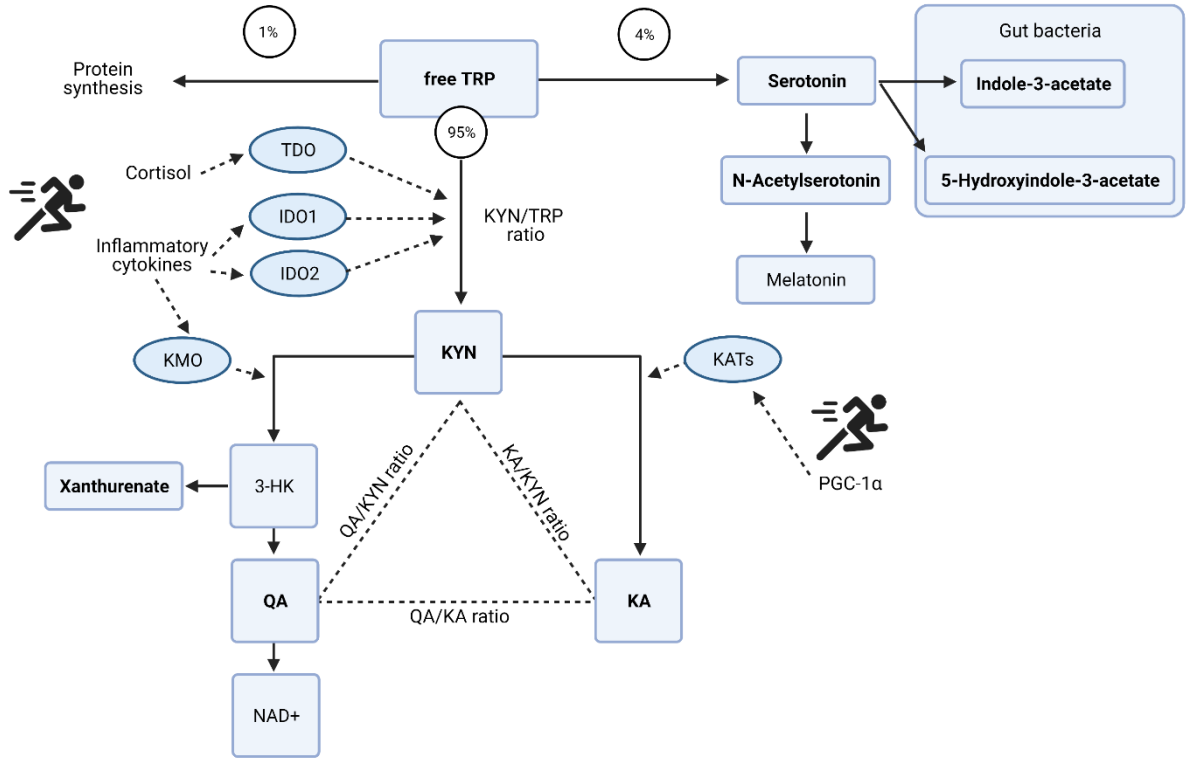


Figure 6.8 Potential mechanisms of tryptophan (TRP) metabolism and the kynurenine (KYN) pathway induced by exercise. Redrawn from (Joisten et al., 2020a) with additional information and the specific metabolites identified as key discriminators via PLSDA modelling in the acute post-match and recovery period. Kynurenate (KA); quinolinate (QA); oxidized form of nicotinamide adenine aminotransferase (NAD+); 3-hydroxykynurenine (3-HK); tryptophan 2,3-dioxygenase (TDO); indolamine 2,3-dioxygenase (IDO); kynurenine 3-monooxygenase (KMO); kynurenine aminotransferase (KATs); proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α). Urinary metabolites identified via multivariate analysis are in bold. Figure created with BioRender.

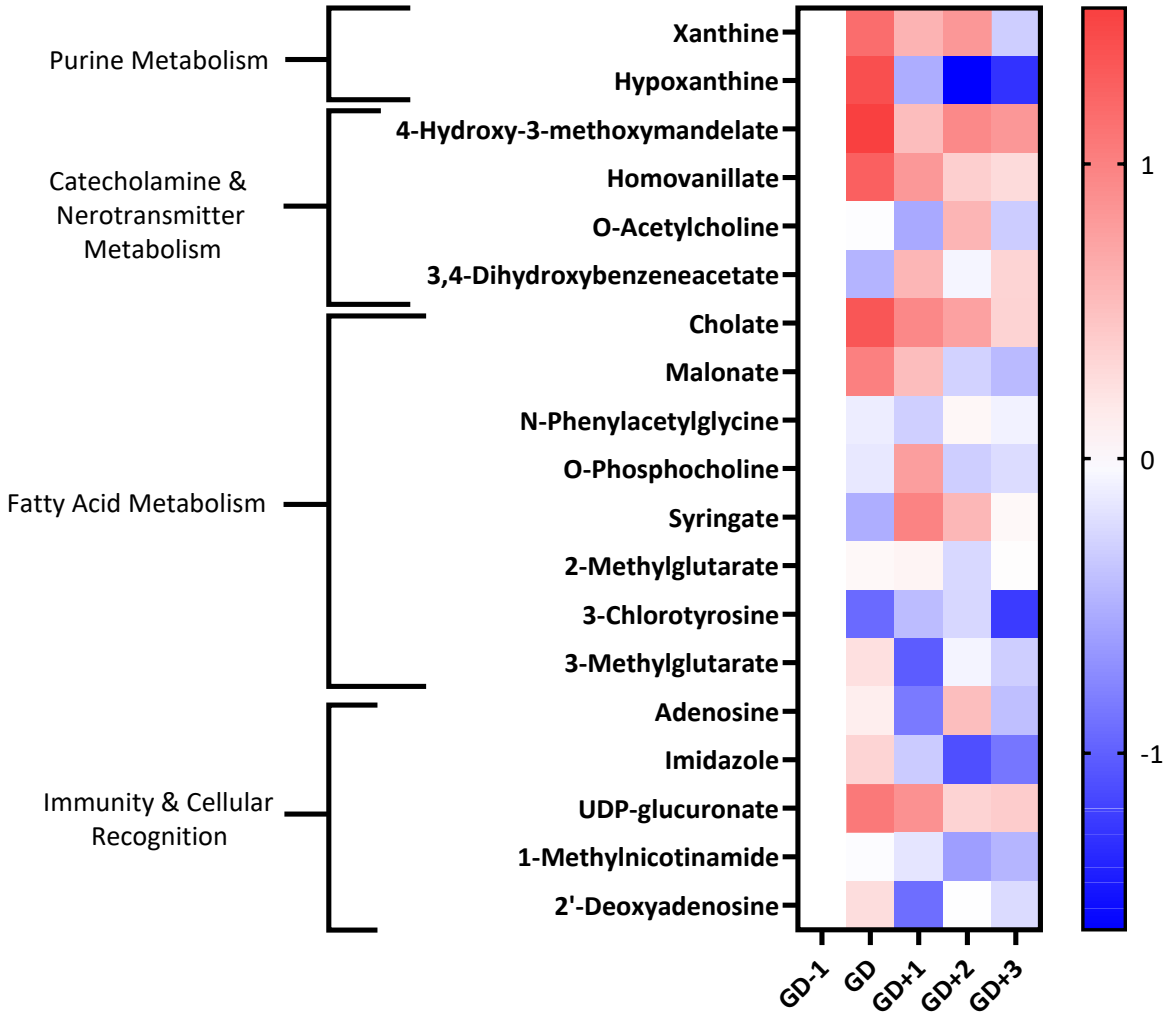


Figure 6.9 Heatmap of urine metabolites identified as key discriminators between timepoints via PLSDA modelling. These are grouped in associations with purine metabolism, catecholamine & neurotransmitter metabolism, fatty acid metabolism, and immunity & cellular recognition. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

The urine enrichment analysis identified tryptophan metabolism as a key pathway (p= 0.005) (Table 6.2). The changes in associated metabolites are visualized in Figure 6.7 and the potential mechanisms induced by exercise shown in Figure 6.8. There is an acute shift for downstream metabolites of the kynurenine pathway such as kynurenate (KA), quinolinate (QA), and xanthurenate to increase, with a marked reduction in kynurenine (KYN).

Acute oxidative and metabolic stress

The salivary purines, xanthine and hypoxanthine are at their lowest levels in the GD samples (Figure 6.4). Urine samples at GD also contained purines as key discriminators with 3-methylxanthine reduced, xanthine and xanthosine both increased above GD-1 levels (Figure 6.9).

6.4.2 Changes in the metabolome in recovery from match play

Univariate analysis did not identify any metabolites as significantly altered during the recovery period in either saliva or urine samples. Multivariate analysis generated seven high quality models (ROC > 0.75) via PLSDA, three saliva and four urine models. The metabolites identified as key discriminators (VIP > 1) between samples were put forward for pathway enrichment analysis, the results of which are displayed in Table 6.3 and 6.4.

Energy metabolism in recovery

The pathways of the glucose alanine cycle (p= 0.010) and gluconeogenesis (p= 0.028) are highly ranked in saliva samples in the recovery days. Aerobic glycolysis is also identified in comparing GD+2 with pre match levels at GD-1 (p= 0.048) due to salivary glutamine, succinic acid, and glutamic acid being key discriminatory metabolites (Figure 6.2).

The levels of salivary metabolites associated with the TCA cycle such as pyruvate and citrate normalise by GD+2 to pre-match levels, although lactate remains elevated (Figure 6.2).

Metabolites of lactose degradation (p= 0.003) and galactose metabolism (p= 0.040) rank highly in saliva (Figure 6.2) and urine (p= 0.011) throughout the recovery period.

Table 6.3 Saliva metabolites identified as key discriminators between saliva samples collected the day prior to match play (GD-1) and the GD+1, and GD+2 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The unadjusted ranked *p*-values are displayed here.

Recovery Pathway	Timepoint Comparison (Unadjusted p-value)			Metabolites Included
	GD-1 vs GD+1	GD-1 vs GD+2	GD+1 vs GD+2	
Urea Cycle	0.003			L-Glutamic acid, L-Alanine, L-Aspartic acid, Urea, Glutamine
Aspartate Metabolism	0.008	0.031		Acetic acid, L-Glutamic acid, L-Aspartic acid, L-Glutamine, Guanosine triphosphate
Glucose-Alanine Cycle	0.010		0.008	D-Glucose, L-Glutamic acid, L-Alanine, Pyruvic acid
Ammonia Recycling	0.028	0.023		L-Glutamic acid, L-Aspartic acid, Urocanic acid, L-Glutamine
Lactose Degradation	0.041	0.003		D-Glucose, Alpha-Lactose, D-Galactose
Arginine and Proline Metabolism	0.042			Creatine, L-Glutamic acid, L-Proline, L-Aspartic acid, Urea
Methionine Metabolism		0.015		Betaine, Choline, Sarcosine, L-Methionine, L-Homoserine
Betaine Metabolism		0.034		Betaine, Choline, L-Methionine
Galactose Metabolism		0.040		D-Glucose, D-Galactose, Alpha-Lactose, Sucrose
Malate-Aspartate Shuttle		0.045		L-Glutamic acid, L-Aspartic acid
Warburg Effect (aerobic glycolysis)		0.048		D-Glucose, L-Glutamic acid, Succinic acid, L-Glutamine
Gluconeogenesis			0.028	D-Glucose, L-Lactic acid, Pyruvic acid, Guanosine triphosphate

Table 6.4 Urine metabolites identified as key discriminators between samples collected the day prior to match play (GD-1) and the GD+1, GD+2, and GD+3 timepoints via PLSDA modelling were then put forward for pathway enrichment analysis using MetaboAnalyst 4.0. The unadjusted ranked *p*-values are displayed here.

Recovery Pathway	Timepoint Comparison (Unadjusted p-value)				Metabolite List
	GD-1 vs. GD+1	GD-1 vs. GD+2	GD+1 vs. GD+2	GD-1 vs. GD+3	
Galactose Metabolism		0.011	0.018		D-Galactose, Alpha-Lactose, Sorbitol, Uridine diphosphate glucose, Uridine diphosphate galactose, Glucose-6-phosphate, Glucose-1-Phosphate.
Nucleotide Sugars Metabolism		0.017	0.008		D-Galactose, Uridine diphosphate glucose, Uridine diphosphate galactose, Glucose-6-phosphate, Glucose-1-Phosphate.
Tryptophan Metabolism			0.035		Indoleacetic acid, Quinolinic acid, Serotonin, L-Kynurenine, Kynurenic acid, L-Tryptophan.
Lactose Synthesis			0.048		Alpha-Lactose, Uridine diphosphate glucose, Uridine diphosphate galactose.

Fatty acid metabolism

The urinary metabolome in recovery exhibits a 21-fold increase of 2-aminoadipic acid (2-AAA) (Figure 6.6).

Amino acid metabolism in recovery

Levels of salivary amino acids appear to return to or drop below pre match values at GD+1 except for methione which stays raised at immediately post-match (Figure 6.3). There is, however, a shift again at GD+2 with alanine, glutamine, methionine, and valine levels exceeding those prior to match play (Figure 6.3). The pathways of the urea cycle, ammonia recycling, the metabolism of many specific amino acids is highly ranked in saliva in recovery.

There are also increases in the products of amino acid breakdown methylsuccinic acid and urocanic acid (Figure 6.11) during recovery in saliva.

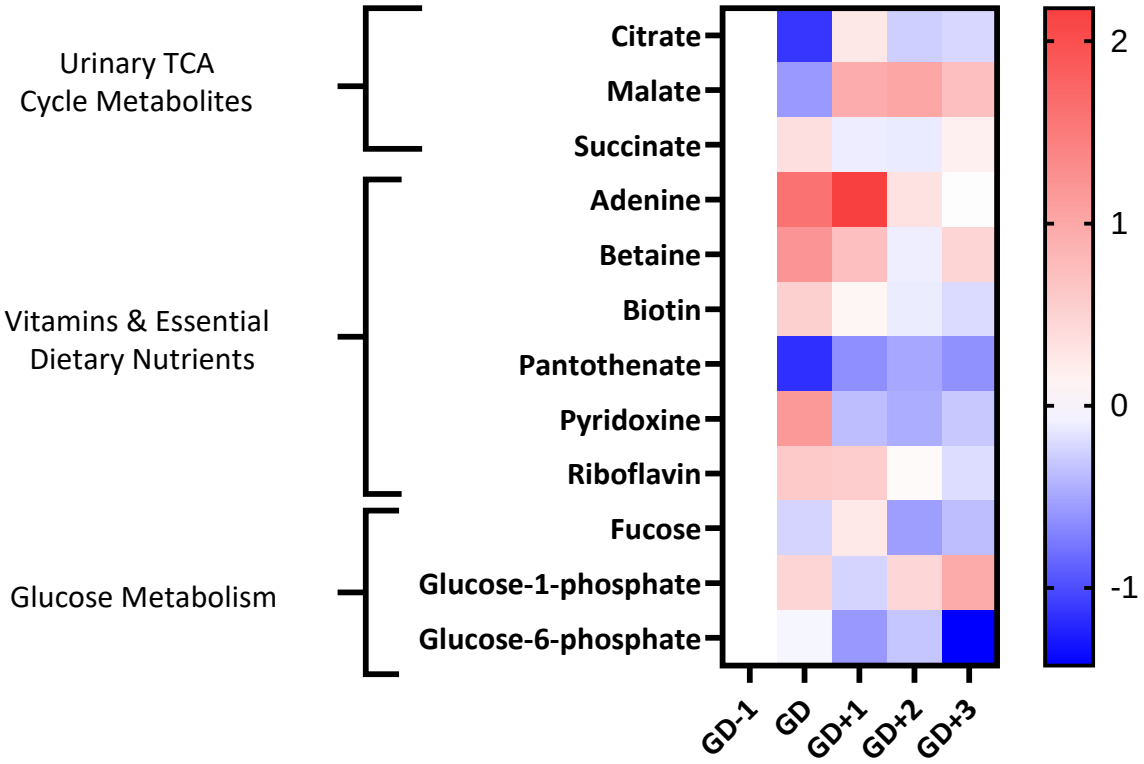


Figure 6.10 Heatmap of urine metabolites identified as key discriminators between timepoints via PLSDA modelling. These include urinary metabolites of the TCA cycle, glucose metabolism, and vitamins & essential dietary nutrients. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

Markers of structural protein degradation

3-Methylhistidine (3-MH) is a key discriminatory metabolite in both urine and saliva (Figures 6.6 and 6.11). Levels in both biofluids peak at GD+1 with elevated levels in urine at GD and in saliva at GD+2 also. Collagen metabolites, glycylproline and 4-hydroxyproline are both elevated in urine post-match and into the recovery period (Figure 6.6).

Metabolic and oxidative stress

In urine, 3,5-dibromotyrosine peaks in recovery at GD+1 and is above pre-match abundancies in all comparison timepoints. Urinary tryptophan metabolism is again significantly ranked (p= 0.035) in recovery, specifically the kynurenine pathway displayed in Figure 6.7 and the mechanisms in Figure 6.8. Methionine metabolism is also highly ranked in saliva pathway enrichment, specifically at GD+2 compared with GD-1. The salivary purine levels of xanthine and hypoxanthine remain reduced at GD+1 and xanthine remained reduced compared to pre-match levels at GD+2 also (Figure 6.4). Urinary Xanthine is elevated throughout GD+1 and GD+2 whilst the acutely elevated levels of hypoxanthine are reduced in recovery below pre-match measures (Figure 6.9).

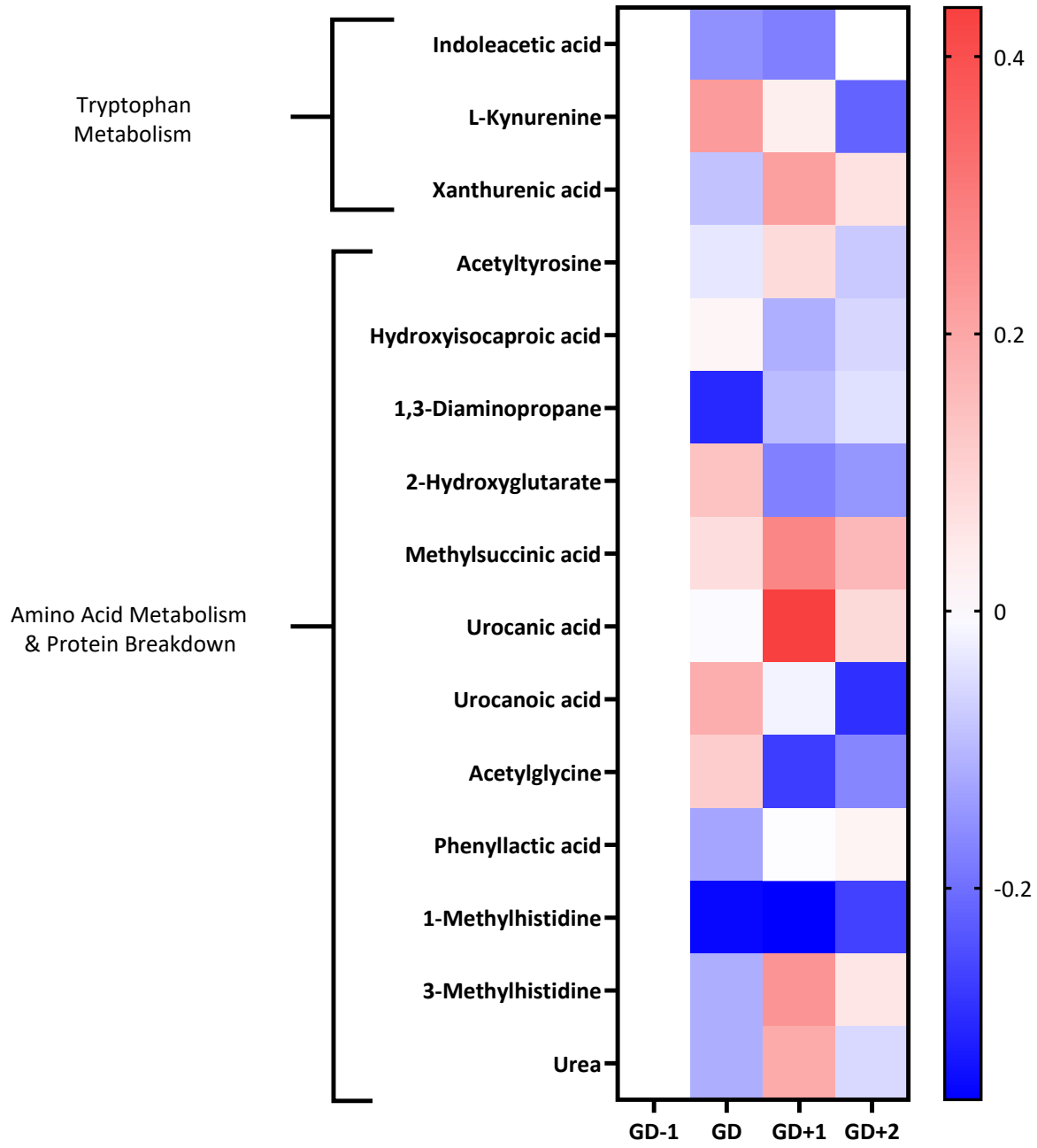


Figure 6.11 Heatmap of saliva metabolites identified as key discriminators between timepoints via PLSDA modelling. These include metabolites of protein metabolism, breakdown, and tryptophan metabolism. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

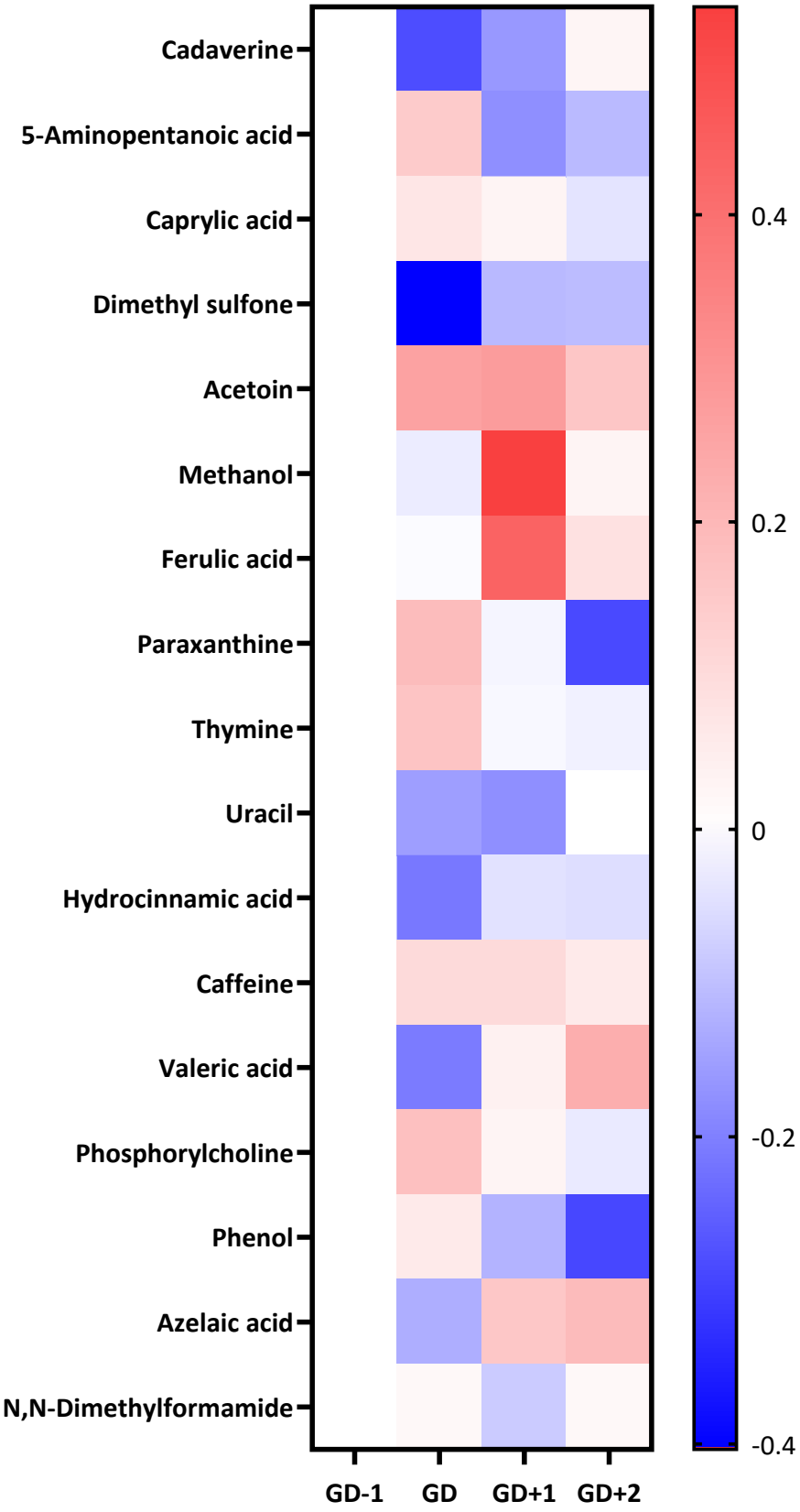


Figure 6.12 Displays the heatmap for the saliva metabolites identified via PLSDA modelling that were attributed to dietary intake or environmental influence causing them to appear in samples. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

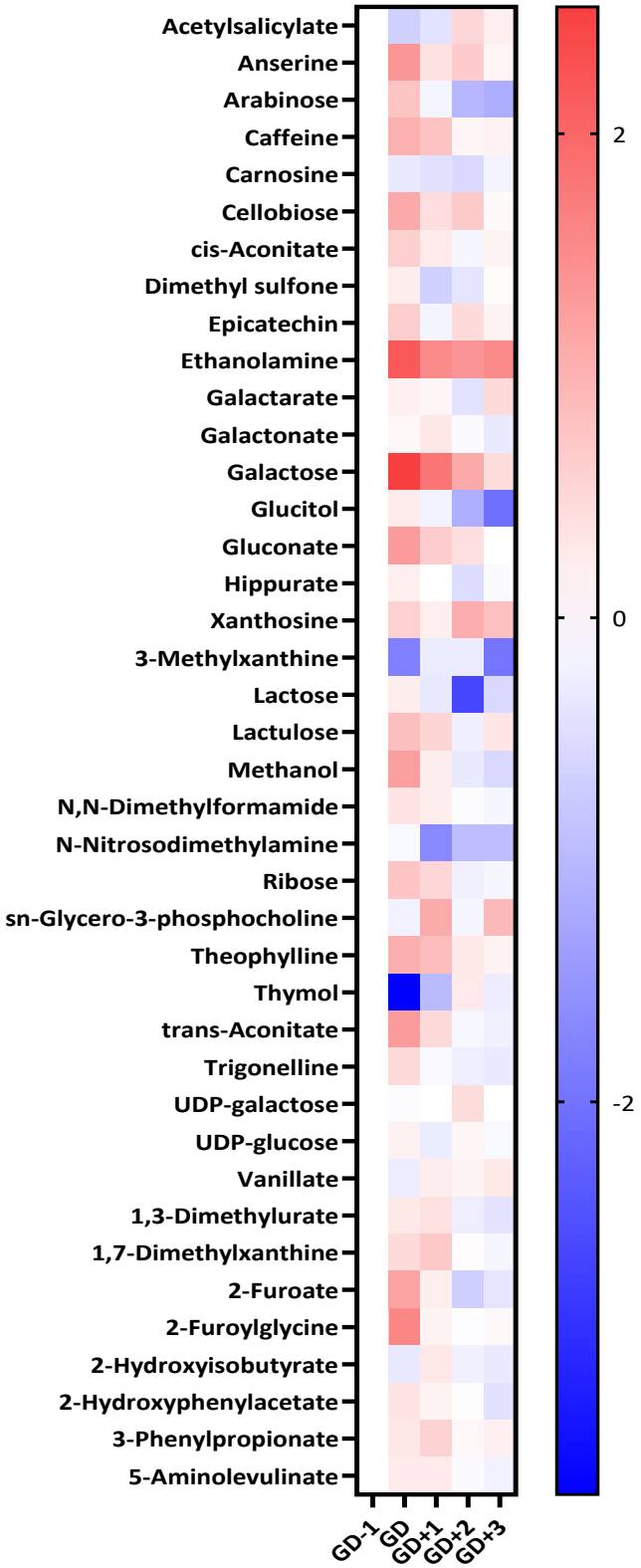


Figure 6.13 Displays the heatmap for the urine metabolites identified via PLSDA modelling that were attributed to dietary intake or environmental influence causing them to appear in samples. The fold change calculated from the GD−1 sample is displayed as the natural logarithm to indicate an increase (greater than 0, red) or decreased (less than 0, blue).

6.5 Discussion

This is the first research in an elite collision sport population to examine the metabolome in response to competitive match play across multiple biofluids. The findings in Chapter 5 of the serum metabolome are supported here examining the changes to the saliva and urine metabolomes. Both biofluids provide further insight into how the body meets the high energy needs both acutely and in recovery from match play. The pathways associated with each phase are evidenced but also the identification of downstream metabolites in these biofluids provides greater evidence to support the hypotheses generated after analyses of the blood serum metabolome herein.

The acute changes to the salivary metabolome support those witnessed in serum, with the glucose-alanine cycle, aspartate and glutamate metabolism ranked highly. These support the proposal that the high intensity nature of the match play requires these pathways to allow glycolysis to continue and for TCA cycle intermediaries to be replenished. The identification of ketone body metabolism including a reduction in salivary acetone may also support the observations in serum that the lower levels of 3-hydroxybutyrate lead us to conclude the high intensity nature of the sport at the elite level reduces the utilisation of fatty acids for energy provision.

The urinary metabolome identified tryptophan metabolism as the sole pathway significantly ranked acutely. The changes specifically to metabolites in this pathway can be seen in Figure 6.7 with the pathway mapped in Figure 6.8 highlighting those metabolites identified in multivariate analysis. The essential amino acid tryptophan can be either used for protein synthesis or used as a substrate to generate several bioactive compounds (Cervenka,

Agudelo and Ruas, 2017). The majority (95%) of tryptophan (TRP) is metabolised through the kynurenine (KYN) pathway, with only one percent used for protein synthesis, and a relatively small amount also used to synthesise serotonin and melatonin (Badawy, 2017). Various chronic disease states have been linked to dysregulation in this kynurenine pathway, such as cancer and diabetes (Cervenka, Agudelo and Ruas, 2017). These also include diseases involving the central nervous system as KYN can be converted to the neuroprotective kynurenate (KA) or quinolinic acid (QA) which is linked to neuronal excitotoxicity (Campbell et al., 2014). Importantly here, the potentially preferred end product is the *de novo* synthesis of NAD+ from QA. NAD+ is an essential cofactor for cellular energy metabolism and there is clear rationale for the high energy demands resulting from exercise via the mechanical activation and resulting humoral activity as being improved energy homeostasis (Joisten et al., 2020b), explaining the marked shift in QA:KYN ratio post-match.

The novel aspect of this research in recording and analysing dietary intake throughout, allowed specific foods to be accounted for (Walsh et al., 2006; Pallister et al., 2016; Playdon et al., 2016; Collins, McNamara and Brennan, 2019). Multivariate analysis of the urine samples revealed the presence of metabolites involved in galactose metabolism, and lactose synthesis to be key discriminators in the fasted recovery comparisons. Saliva analysis also identified lactose degradation and galactose metabolism as pathways associated with the acute and recovery periods, and those metabolites as key discriminators between the GD-1 and GD+1/GD+2 sample timepoints. This may be due to the inclusion of dairy products in the participants diet, especially in the evening prior to sleep. This is a well-researched strategy in sports nutrition with 40g of dairy protein consumed providing support for muscle recovery and adaptations to strength and hypertrophy training (Snijders et al., 2019).

Despite the fasting period overnight and prior to sample collection each morning being adhered to strictly, this is likely the explanation for these metabolites being present in samples.

This serves as a reminder also that the gameday samples were not fasted and therefore the appearance of some metabolites will be due to the ingestion of caffeine as an ergogenic aid for example (Wellington, Leveritt and Kelly, 2017; Cornelis et al., 2018), and these with other dietary or environmental metabolites are included in Figures 6.12 & 6.13.

Although some metabolites of purines such as xanthosine and paraxanthine are associated with coffee consumption (Cornelis et al., 2018; Kim et al., 2019) and these are observed to increase acutely here in saliva and urine, there are reductions in salivary xanthine (XA) and hypoxanthine (HX) both metabolites associated with oxidative stress. These are naturally occurring purine derivatives from purine nucleotides (adenine and guanine). Their appearance is associated with ATP consumption (Finsterer, 2012) and the formation of reactive oxygen species due to exercise (Radak, Chung and Goto, 2008). Blood plasma levels of HX and XA significantly increased in response to isokinetic exercise (Speranza et al., 2007) and elevated urinary abundances of these oxypurines are associated with increased levels of ATP degradation in disease states (Turgan et al., 1999). In saliva, levels of both oxypurines were reduced immediately and 1hr post exhaustive cycling exercise and they returned to pre-test levels after 24 hours (Arthur et al., 2018). In Chapter 5, neither of these metabolites were identified after univariate or multivariate analysis in serum, but they do appear as key discriminators in saliva and urine. The salivary metabolome demonstrates a reduction in both oxypurine levels immediately post-match and at the GD+1 timepoint and xanthine remains below pre-match levels at the GD+2 timepoint also. The increases in the urine

samples of both purines, is immediately post-match, whilst xanthine remains elevated throughout recovery to GD+2.

Overall, these observations support the pathways associated with oxidative stress and the metabolism of glutathione after rugby match play from the serum metabolome changes detailed in chapter 5. It is novel that the salivary reduction in xanthine and increased urinary xanthine levels are prolonged to GD+2. This may indicate the extended period of oxidative stress after collision activities. When compared with the lab-based cycling studies (Arthur et al., 2018) that have indicated these metabolites maybe a useful marker to observe recovery from exhaustive exercise protocols, the levels of mechanical damage and IIMD may cause the extended observations here.

The collisions experienced on gameday, rather than throughout the training week, cause this ultrastructural damage leading to the cascade of events comprising the secondary mechanism triggered by an acute inflammatory response due to the action of immune cells such as neutrophils and macrophages (Peake et al., 2017). The increased cytokine concentrations of IL-6 (Cunniffe et al., 2010; Cunniffe et al., 2011), IL-8 and IL-10 (Morehen et al., 2020) have been profiled in this population post-match play. The realization of this potential inflammatory response on the urinary metabolome is in the tryptophan metabolism pathway which was again significantly ranked in the recovery analysis. Local and systemic inflammatory stimuli cause an increase in IDO1 mediated conversion of TRP to KYN, specifically, elevated levels of interferon-gamma (IFN-γ) (Werner et al., 1987; Taylor and Feng, 1991), and increases in pro-inflammatory cytokines interleukin (IL)-6 and tumour necrosis factor (TNF)-α (Connor et al., 2008; Divanovic et al., 2012). Importantly, IDO activity is also inhibited by anti-inflammatory cytokines (Chaves et al., 2001). The metabolite KYN

can also alter immune response via suppression of cytotoxic T-cells and the differentiation of regulatory T-cells (Chung et al., 2009; Mandi and Vecsei, 2012). A chronic reduction in TRP may result in lowered serotonin production and a pathological mechanism of depression and mood disorders (Marx et al., 2020). This could have been reflected in the perception of vigour reported by the participants which was significantly lower at GD+2 than the morning of GD, with serotonin levels concurrently at their lowest on GD+2. Even without the presence of apparent inflammatory conditions there is a clear relationship between TRP catabolism and immune system biomarkers such as neopterin and IL-10 in healthy young adults (Deac et al., 2016).

To our knowledge there are no metabolomics studies in exercise where this number of kynurenine metabolites has been identified during analysis. In the urine of professional football players when examining the relationship between load and the metabolome, tryptophan metabolism was identified as a pathway of interest comparing urine metabolome samples collected in the pre-season and again in the middle of the season (Quintas et al., 2020). There were, however, no metabolites associated with this pathway identified as associated with external training load in the authors analysis (Quintas et al., 2020).

Prior research has shown exercise can change kynurenine metabolism in skeletal muscle and significantly alter metabolites of the pathway in peripheral tissues and the CNS (Martin, Azzolini and Lira Ruas, 2020). The peroxisome proliferator-activated receptor-γ family of transcriptional coactivators which are important for the adaptation of skeletal muscle to exercise, influence this pathway (Correia, Ferreira and Ruas, 2015). Of this family, PGC-1α especially, whose expression is induced by acute and chronic exercise, is thought to

upregulate the expression of kynurenine aminotransferase (KAT) which catalyses the conversion of KYN to KA (Mudry et al., 2016). In a comparison of resistance (RE) and endurance type (EE) exercise in serum, EE induced a stronger effect than RE in increasing IL-6 and cortisol levels acutely. However, this did not translate into significant changes in the conversion of TRP to KYN but did see the formation of KA causing a significant shift in the KA:KYN ratio (Joisten et al., 2020a). In a more demanding exhaustive EE protocol in trained athletes the increase in KYN:TRP ratio was significant, and this was positively associated with the marker of immune system activation, neopterin (Strasser et al., 2016).

The proposal that myoglobin increases in plasma samples due to the loss of structural integrity after the collisions and damage caused by rugby union match play has received much attention (Takarada, 2003; Lindsay et al., 2015; Lindsay et al., 2016a). Neopterin is a marker of immune system activation, associated with oxidative stress and indicative of a proinflammatory immune status (Gieseg, Baxter-Parker and Lindsay, 2018). The primary pterin, 7,8-dihydroneopterin is generated by monocyte-derived macrophages during immune activation. Then γ-interferon mediates the oxidation of 7,8-dihydroneopterin to neopterin because of the inflammation and oxidative environment (Gieseg, Baxter-Parker and Lindsay, 2018). Myoglobin, increased after rugby match play, is capable of oxidising 7,8-dihydroneopterin to neopterin and other pterin forms (dihydroxanthopterin and xanthopterin) (Lindsay et al., 2016a).

Here neither pterin metabolite was identified in urinary samples, and they have not been detected previously utilising NMR spectroscopy (Bouatra et al., 2013). However, research in trained athletes after exhaustive EE demonstrated a significant increase in neopterin levels (159% increase) and a significant correlation between neopterin and the KYN/TRP ratio in

blood samples (Strasser et al., 2016). In early *in vitro* and *in vivo* murine models, it was demonstrated that the cytokine stimulated metabolism of tryptophan via the kynurenine pathway is independent of pterin synthesis (Sakai et al., 1993). They are therefore associated by the inflammation and oxidative stress present after exercise but independent of each other it would appear.

These insights provided by the urinary kynurenine pathway provide a link to prior work in collision research exploring the potential for pterins and myoglobin to be indicative of the inflammation and immune responses due to EIMD and IIMD. What is novel in the analysis presented here are the markers of structural damage in both the urine and salivary metabolomes. The increased urinary 3,5-dibromotyrosine observed herein, peaking at GD+1 is indicative of eosinophil activity and may indicate protein oxidation of injured tissue (Wu et al., 1999). The 2-aminoadipic acid (2-AAA) which increased 21-fold at GD+1 in urine, is a product of lysine degradation, and can appear in circulation due to degradation of whole tissue or plasma proteins. There are also evidence 2-AAA levels can rise when free lysine residues are degraded, derived from collagen (skin) (Sell et al., 2008). The collagen metabolites glycylproline and 4-hydroxyproline are elevated immediately post-match and at GD+1 potentially providing the residues for degradation leading to the large rise in urine 2-AAA.

2-AAA is also a metabolite biomarker that predicts the development of diabetes in normoglycemic individuals. High blood plasma concentrations are indicative of a 4-fold higher risk of future diabetes (Wang et al., 2013). It has been proposed that 2-AAA acts to compensate in early insulin resistance by an upregulation of insulin secretion to maintain glucose homeostasis (Wang et al., 2013). 2-AAA is associated with adipogenesis, and this

study also suggests increased levels are associated with insulin resistance which induces abnormal gluconeogenesis (Lee et al., 2019). *In vitro* experiments revealed that the presences of 2-AAA impair insulin signalling via reductions in the phosphorylation of AKT and insulin receptors in adipose tissue cultures (Lee et al., 2019).

The large fold change increase in urine of 2-AAA is especially interesting when we consider the gluconeogenesis upregulation observed in the analysis of the serum metabolome. The reduced levels of branched chain amino acids post-match play in serum are mirrored in the saliva metabolome. The BCAA degradation pathway finding is also supported in the metabolites of leucine; 2-hydroxyisocaproate, and 3-hydroxy-3-methylglutarate identified in urine to increase in the GD samples. The degradation of glucogenic amino acids between the GD+1 and GD+2 timepoints in blood serum to support gluconeogenesis was an important observation in the recovery period, but the impact of this was unknown. We also hypothesised that fatty acid metabolism, which appeared reduced in competitive match play, was also reduced in the recovery days due to continued lower levels of 3-hydroxybutyrate and alterations to the lipoprotein profile. We proposed the inflammation due to match play may have contributed to this in chapter 5. The acute reduction in salivary acetone agreed with the observations derived from serum and has also been observed after competitive soccer (Pitti et al., 2019) . However, in our observations, there is an increase in saliva acetone in the days after match play which cannot be explained alongside the continued reduced blood levels of ketone bodies, as the increased levels of blood ketone bodies in a diabetes disease state have been shown to correlate with salivary acetone levels (Fujii et al., 2014). This may be due to the fact that saliva accounts for not only the changes in metabolites generated and perfused from blood capillaries in the mouth, but also oral microbiome contributes to the overall metabolome investigated (Gardner, Carpenter and

So, 2020). It may be that the peak in acetoacetic acid post-match is broken down via non-enzymatic reactions to form the acetone which appears increased in saliva at GD+1 (Ruzsanyi and Peter Kalapos, 2017). The catabolism of leucine is another potential route to acetone formation and can be achieved by bacteria of the oral microbiome (Hausinger, 2007). Saliva leucine does not mirror serum and despite both metabolomes having reduced leucine at GD, only the saliva leucine levels remain reduced at GD+1 alongside the increased acetone.

The observations from the serum metabolome in chapter 5 led us to propose the reductions in serum glucogenic amino acids together with the upregulation of pathways associated with gluconeogenesis, translate to insufficient glucose being provided from dietary intake to meet elevated requirements due to the secondary response to muscle damage in recovery from match play. The questions remained though what the impact of this might be in addition to the immediate capacity to recover. Both the urine and saliva metabolome identified 3-methylhistidine as a key discriminatory metabolite.

This metabolite has been purported as a marker of muscle protein breakdown, as degradation of contractile proteins leads to the release of 3-methylhistidine (3-MH) (Viru, 1987). This amino acid cannot be re-utilised and so is excreted, with early work identifying it in urine (Bilmazes et al., 1978). Later work has demonstrated that it directly represents myofibrillar degradation (Bird, Tarpenning and Marino, 2006). Exercise studies in participants with strength or resistance training experience demonstrate no change in 3-MH (Wilson et al., 2013; Wilson et al., 2014) whilst untrained individuals have shown a significant increase post resistance training (Cornish et al., 2009).

It is important to regard the relationship between lean body mass, dietary meat intake and 3-MH excretion when quantifying the metabolite (Lukaski et al., 1981) especially when we are dealing with rugby union athletes. A 3-day meat free diet has been shown to return 3-MH levels to a baseline to then investigate changes due to exercise (Lukaski et al., 1981). Useful clinical information can then be gleaned with dietary control (Elia et al., 1981). When it comes to dietary contribution the short term effects demonstrate a linear relationship between protein intake and 3-MH urinary excretion, with the conclusion that the intake of meat, either be accounted for, or absent from the diet, to accurately quantify muscle protein catabolism from 3-MH (Marliss, Wei and Dietrich, 1979). A novel aspect of our research is the recording of dietary intake throughout which demonstrated no significant difference in protein intake across the match week. As we are investigating the change in relative abundance within the samples after normalisation and scaling, rather than quantifying the levels of metabolites, we feel this provides sufficient evidence the appearance of this metabolite as a key discriminator between GD-1 and GD+1/+2 can be linked with muscle protein catabolism. We are obviously cautious in using the result of the GD sample as the participants had eaten animal protein prior to match play that day, whereas the fasted samples where these differences in 3-MH occurred should be regarded. Urine levels rose at the GD timepoint but also at GD+1 indicating muscle protein breakdown may be occurring in the day after the match.

Saliva 3-MH has been reported in the final quarter of basketball match play (Khoramipour et al., 2020) and in the saliva of young professional football players after training (crucially they do not differentiate between the 1- and 3- forms of MH) (Alzharani et al., 2020). Similarly, after a 3-day consecutive game program in male footballers, those identified as fatigued via subjective mood scores, significant reduction in body mass, and changes to heart rate at rest

provided saliva samples for analysis. These fatigued players displayed elevated 3-MH levels in fasted morning saliva (Ra et al., 2014). Acute changes to the metabolome in female professional footballers did not identify 3-MH as a marker relative to immediately post-match play (Pitti et al., 2019). Our findings here do not show a rise in 3-MH immediately after match play relative to the GD-1 abundance, but the GD+1 and GD+2 levels are higher, again indicating these fasted values of 3-MH are reflective of increased muscle protein breakdown.

6.5.1 Conclusion

Whilst our serum metabolome revealed this process of gluconeogenesis in recovery from match play coupled with changed to fatty acid metabolism which we attributed to the increased requirement for glucose not being met through dietary intake, the full impact if this was not appreciated. Here the evidence presented from these two complementary biofluids leads us to conclude that the markers of connective tissue and muscle protein degradation may be the source of amino acids degraded in the upregulation of gluconeogenesis. Therefore, the sufficient provision of dietary carbohydrate in recovery at GD+1 should be the primary aim. The summary of major findings of the chapter can be found in infographic form in figure 6.14 below.

Chapter 4 of this thesis has provided evidence for increased metabolic rate at rest on GD+1 with shifts in RER implicating carbohydrate oxidation to be increased in the days following match play. This novel investigation of the metabolome of three biofluids reinforced this need and we propose specifically carbohydrate is required to meet these metabolic needs. Collision-based team sports need to 'fuel the work required' but to maximise recovery between fixtures they also need to 'fuel the damage induced'.

The implication that gluconeogenesis in recovery may be associated with myofibrillar protein breakdown poses the question as to what the cumulative effects are if energy and carbohydrate needs are not met in the days after match play throughout an entire season.

Rationale

A multi-biofluid approach has been effective in untargeted investigations. The use of minimally invasive biofluids may be important to further the use of metabolomics in elite athletes

Objective

Investigate the metabolic perturbations associated with elite rugby union match play acutely and throughout recovery in urine and saliva

Results

	Acute pathways	Recovery pathways
	Glucose-alanine cycle, Urea cycle, Ammonia recycling, Lactose degradation, Ketone body metabolism.	Glucose-alanine cycle, Glycolysis, Gluconeogenesis Urea cycle, Ammonia recycling, arginine/proline/methionine metabolism.
	Tryptophan metabolism	Tryptophan metabolism,

Congruent pathway identification between saliva and serum metabolomes. Urine metabolome identified tryptophan metabolism kynurenine pathway which also supports the whole body attempting to cope with high energy demands

Downstream metabolites of leucine support serum findings together with metabolites of muscle protein breakdown and connective tissue degradation. A 21-fold increase in 2-AAA a metabolite denoting abnormal gluconeogenesis a key descriminant from urine metabolome

Conclusion

The use of minimally invasive biofluids provided a congruent and complementary analysis of the multiple metabolomes. The apparent re-entry to gluconeogenesis in recovery and degradation of amino acids when insufficient glucose is provided from the diet may result in muscle protein breakdown

Next Steps

The increased metabolic requirements in recovery from elite rugby union match play appear to be increased energy in the form of carbohydrate. The outcome of many repeated bouts of match play and recovery upon the athlete remains to be elucidated

Figure 6.14 Infographic overview of chapter 6 to summarise the major findings.

Chapter Seven

Changes in Body Composition and predicted energy balance in a Premiership Rugby Union Squad over the In-season period.

7.1 Abstract

INTRODUCTION: Snapshots of energy expenditures (EE) and energy intakes (EI) in rugby have been determined and have alerted practitioners to the potential energy deficits these athletes may be in. Data are, however, sparse and do not necessarily represent chronic habits of energy balance (EB). The objective here was to monitor changes in body composition and to estimate longitudinal energy imbalance in an English Premiership rugby union squad throughout a competitive season.

METHODS: 46 professional Premiership rugby union players were recruited. Body composition was measured using a dual-energy-X-ray absorptiometry (DXA) fan beam scanner (Hologic Horizon W, Hologic, Bedford, MA) at the start, midpoint, and end of the competitive season. EB was calculated using the validated equation (de Jonge et al., 2007) . Internal loads for training sessions and match play exposures were tracked using the session rating of perceived exertion (sRPE).

RESULTS: Over the whole season from start-end there were significant increases in body mass 1.38 ± 2.28kg (p=0.0004)) and fat mass 1.26 ± 1.56kg (p=0.0001), but no significant change in lean mass 0.16± 1.77kg (p=0.5767). However, a loss of lean mass was observed from start-mid (0.72± 1.55kg; p=0.0055), followed by an increase in lean mass (0.88± 1.40kg; p=0.0003) from mid-end. The loss of lean mass from start-mid season was significantly correlated with an estimated self-reported mean daily energy surplus, r^2= 0.17 (p=0.0087). High match exposure was significantly correlated with increases in fat mass over the whole season, r= 0.6245 (p= 0.0014).

CONCLUSIONS: Despite lean mass being maintained over the course of the competitive season, unfavourable shifts in body composition with reductions in lean mass and increases

in fat mass may occur during periods of intense match play. Despite the reported losses in lean mass being accompanied by a positive mean daily energy balance, if the increased metabolic demands of recovery from match play are not met, transient energy deficits in recovery could account for these negative reductions in lean mass. This is the first insight as to how changes in body composition relate to mean daily energy balance throughout the competitive season in elite rugby union players and provides more evidence that in elite rugby we must now consider 'fuelling for the damage induced'.

7.2 Introduction

A season playing in the English Rugby Union Premiership competition constitutes 24 matches and for some teams competing in the European competition, a further 9 European and domestic cup matches. This number may rise by as many as 7 games if a team is successful in knockout stages of the cup competitions creating a gruelling 9-month season. This calendar requires careful planning to both maintain performance levels and for the wider protection and welfare of the players.

The health and welfare of elite athletes requires an integrated approach across the multi-disciplinary team (Dijkstra et al., 2014) with sports nutritionists primarily aiming to ensure energy balance (EB) is appropriate for specific training and performance periods (Melin et al., 2019). EB during the preseason period in elite rugby union male athletes may include purposeful energy deficits to restore or improve body composition after the off-season (Bradley et al., 2015b). However, the in-season period aims to optimise recovery, maintain excellent body composition, and meet the overall fuel requirements of training and match play by achieving chronic energy balance (Bradley et al., 2015a). Sustained energy deficits are associated with a host of unfavourable consequences such as altered endocrine function (Elliott-Sale et al., 2018), suppressed resting metabolic rate (RMR) (Thompson, Manore and Skinner, 1993), sub-optimal shifts in body composition (Deutz et al., 2000) and impaired physical performance (Murphy et al., 2018). Periods of negative energy balance either due to increases in exercise energy expenditure or reductions in energy intake may also result in unfavourable metabolic adaptations associated with low energy availability (LEA) (Wasserfurth et al., 2020).

Promising screening tools have been developed for female athletes (Logue et al., 2020), but this has been more challenging in a male cohort with few effective self-reported symptoms and further clinical assessment still required to identify LEA (Lundy et al., 2022). Concerns over team sport athletes meeting energy and macronutrient recommendations (Jenner et al., 2019) coupled with an increased prevalence in relative energy deficiency in sport (RED-S) in male athletes (Burke et al., 2018) dictates the monitoring of energy balance (EB) in male team sport athletes is crucial. This need for further research is only heightened by the limited data available demonstrating a significant shortfall in EI compared with TEE over a 14-day period in elite rugby league athletes (Morehen et al., 2016) combined with the increases in the metabolic demands of the recovery period after match play reported in chapter 4 of this thesis.

Assessment of energy intake (EI) is notoriously challenging with self-reported intake revealing an underestimation by 19% when energy expenditure (EE) is assessed concurrently using doubly labelled water (DLW) (Capling et al., 2017). Even with less burden placed on the athlete via the remote food photographic methods such as 'snap-n-send' (Costello et al., 2017), it is demanding for even experienced practitioners to assess the records especially when complex meals are eaten (Stables et al., 2021). The EE of rugby athletes has been measured using wearable devices (Bradley et al., 2015a), and the DLW technique (Morehen et al., 2016; Costello et al., 2018; Smith et al., 2018) but unreliability and/or high costs often mean small sample sizes and short 7-14 day sampling periods, thus limiting the practical value. Furthermore, these isolated, acute, observations of TEE of athletes may not be representative of their chronic habits throughout a season.

It is possible however, to utilise measures of body composition generated via dual energy x-ray absorptiometry (DXA), together with the time between scans, to calculate estimations of energy balance (de Jonge et al., 2007). This method has provided valuable information for military personnel as to how energy deficits elicit declines in lower body power and strength (Murphy et al., 2018) and negative effects on body composition and physical performance (Fortes et al., 2011). Using this technique in athletic populations has evidenced that periods of negative EB were due to intensified training or competition and increased EE rather than purposeful restriction of EI (Silva et al., 2017). Over a whole season of Australian rules football this method demonstrated that lean mass (LM) can be accrued in states of energy surplus, balance, and deficit (Bartlett et al., 2019). Utilising changes to the body composition compartments of LM and FM may provide the desired insight as to the chronic energy balance of elite rugby union athletes.

To date, research has neither observed changes in body composition throughout a competitive season, nor has energy balance been investigated for an extended period in elite rugby union players. Therefore, the primary aim of the current study was to assess how body composition and EB change across the in-season competitive period in elite rugby union athletes. We also investigated the relationship between any changes in body composition and the training loads and match play exposures.

We hypothesised that due to the significantly increased metabolic requirements of recovery reported in preceding chapters of this thesis, combined with the relatively low energy and carbohydrate intakes observed in chapter 5 and previously (Bradley et al., 2015a; Posthumus et al., 2021), players will be in a mean energy deficit during periods of repeated

match play. This will result in losses of body mass during the season, derived from the lean mass compartment.

7.3 Methods

7.3.1 Participants

Table 7.1 details the 46 members of an English Premiership rugby union squad who provided informed consent to participate in the study. Ethics were granted by Birmingham City University ethics committee. Of the 46 participants who completed scans at the start and end of the season, 6 were unable to attend a scan at the mid-point also. For that reason, the analysis of body composition across the three timepoints is n= 40. Training and match load data were collected for all 46 participants, however, if a player was unavailable for more than 4 consecutive weeks due to injury causing inactivity or immobilisation, those players were excluded from the analysis.

Table 7.1 Participant characteristics (n=46) divided up into positional groups for the English Premiership rugby union squad taking part in the study.

Positional group	Position (n)	Body mass (kg)	Height (cm)	Age (yrs.)
Forwards	Prop (9)	119.3 ± 5.5	186.4 ± 3.7	24.5 ± 4.8
	Hooker (4)	98.9 ± 6.4	181.7 ± 3.5	22.2 ± 2.7
	Lock (5)	114.5 ± 3.0	198.2 ± 2.6	25.3 ± 4.4
	Backrow (8)	106.3 ± 3.3	189.3 ± 3.3	25.0 ± 3.3
Backs	Halfbacks (6)	83.0 ± 6.2	176.8 ± 5.9	22.9 ± 2.4
	Centre (3)	100.0 ± 6.4	189.7 ± 5.0	29.2 ± 0.5
	Back 3 (11)	90.6 ± 5.6	184.7 ± 2.8	22.6 ± 4.1

7.3.2 Study Design

Three DXA scans were undertaken across the season. These were at the beginning of the competitive season in September (Start), the midpoint of the season in January (Mid), and the end of the competitive fixtures in May (End), dividing the in-season period into three distinct periods for calculations of energy balance and changes in body composition. Start-Mid was 148± 12 days and was characterised by a dense fixture calendar of 21 matches with many players experiencing high chronic match load. Mid-End was 103±12 days with 12 matches played, allowing more rest weeks without fixtures. Start-End, the total period of the in-season was 251± 17 days.

7.3.3 Training and match load

Internal loads for each training session, both resistance and rugby based, were assessed by the session rating of perceived exertion (sRPE) recorded using a modified 10-point Borg scale as per section 3.4.2 together with match play exposure in minutes.

7.3.4 Body composition analysis

Height and mass were recorded as per section 3.3.1.

7.3.4.1 Dual-energy-X-ray absorptiometry (DXA) scanning

Body composition was measured using a dual-energy-X-ray absorptiometry (DXA) fan beam scanner (Hologic Horizon W, Hologic, Bedford, MA), with scanning and analysis performed by the same trained individual using Apex software version 13.5.3.1 (Hologic, Bedford, MA). Measurements were taken in the morning prior to eating or exercise and protocols implemented to maximise reliability of positioning (Nana et al., 2015). The scanning venue was approximately a 45 minute drive for the participants. Due to this travel the players were

asked to limit fluid intake to 500ml and repeat this for each visit. This guidance on fluid intake allowed for the players to meet the desire to drink something after waking and during travel but prevented the over consumption of fluids which may alter the DXA scan results. Hydration testing utilising urine osmolality upon arrival, ensured participants were in a euhydrated state. The same day of the week was used for every visit, corresponding to a rest day in the middle of a training week. There were at least 18 hours rest after the prior days training before scanning was performed.

The Hologic Horizon has been precision tested and the coefficient of variation (CV) measured for total body fat mass (FM) 0.89% and lean mass (LM) 0.51% (Cheung, Roff and Grossmann, 2020). Two machines of the same model when tested in parallel were found to have CV of 0.78% and 0.77% for FM, with 0.52% and 0.40% for LM (Nowitz and Monahan, 2018). The CV for the period of scanning within this study was 0.253% as recorded by the individual machine used for all scans. This CV was calculated using the spine and step phantom scanning as part of the daily calibrations prior to testing.

7.3.4.2 Skinfold measurements

Subcutaneous skinfold measurements were measured after each participant had been DXA scanned by the same qualified and trained individual with as per the International Society for the Advancement of Kinanthropometry (ISAK) guidelines. Participants were landmarked using a hypoallergenic eyeliner pen, measuring tape (Bodymorph, Portsmouth, England) and segmometer (Bodymorph, Portsmouth, England). Skinfold measurements were taken using calibrated skinfold callipers (Harpenden, Baty Intl, England) in accordance with prescribed methods (Norton, Marfell-Jones and Whittingham, 2000). Measurements included eight sites (Bicep, Tricep, Subscapular, Supra-iliac, Supra-spinale, Abdominal, Thigh, and Calf).

Duplicate measures were recorded at each site and a third recorded if the variance of the first two measures exceeded 5%. The mean of the two measures was used as the final measure for each site with the median recorded if a third measure was required. The cumulative score of the eight sites (mm) was recorded. This method was utilised to corroborate any changes observed in DXA derived body composition as there has been shown to be very large agreement between sum of skinfold measures and DXA fat mass previously (Zemski et al., 2018).

7.3.5 Energy balance estimation

EB was calculated using the previously validated equation (de Jonge et al., 2007; Pieper et al., 2011) based upon the known energy densities producing a change in body tissues of 1.0 kcal·g^{-1} for fat free soft tissue mass (FFSTM) and 9.5 kcal·g^{-1} for fat mass (FM) (Dulloo and Jacquet, 1999) :

$$EB\ (\mathrm{kcal}\cdot\mathrm{day}^{-1}) = 1.0\ \frac{\Delta\mathrm{FFSTM}}{\Delta\mathrm{t}} + 9.5\ \frac{\Delta FM}{\Delta t}$$

EB is energy balance per day when Δt is the number of days in between scans. ΔFFSTM and ΔFM are changes in grams between scan timepoints. Please note that although the terms FFSTM and LM refer to the same compartment of body composition, for consistency with prior research, the term lean mass (LM) will be used going forward in this chapter.

7.3.6 Statistical analyses

Data was tested for normality, as determined by the Shapiro-Wilk test. When investigating the changes across the three scan time points; start, mid, and end, parametric data was tested with a one-way repeated measures of ANOVA with comparisons post hoc made using Fisher's least significant difference (LSD). Non-parametric data was tested with a Friedman

test with uncorrected Dunn's test for comparison of means post-hoc. The difference between means were tested at a significance level of $p<0.05$.

Determining relationships between changes in body composition compartments with EB, measures of load and between skinfold data incorporated Pearson's correlation coefficient with an r^2 value generated. A Spearman's rank correlation was used if data sets were non-parametric, generating an r value. The significance of any relationships found was tested at $p<0.05$.

7.4 Results

7.4.1 Skinfold measures and DXA derived bodyfat

The relationship between all changes in skinfold measures and the corresponding DXA derived bodyfat compartment is displayed in figure 7.1. The changes in DXA derived fat mass (kg) and sum of eight skinfold measures (mm) correlated significantly together ($p< 0.0001$) ($r = 0.804$). When this was also examined for each phase of the data collection there were significant positive relationships from Start-Mid ($p< 0.0001$, $r = 0.800$), Mid-End ($p< 0.0001$, $r^2 = 0.584$), and Start-End ($p< 0.0001$, $r = 0.765$). These significant agreements in changes to the fat mass compartment between techniques support the DXA findings used to estimate energy balance in the group.

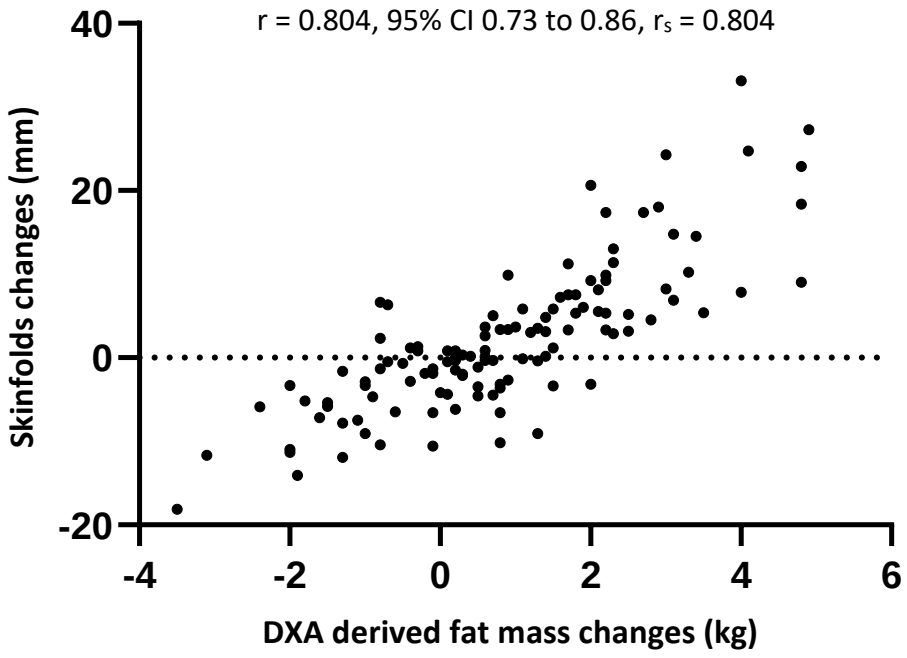


Figure 7.1 Changes in fat mass (kg) derived from DXA scanning and the corresponding changes to sum of eight sites skinfolds measurements (mm) throughout the season (n= 40).

7.4.2 Changes in body composition

The changes in body mass and body composition compartments throughout the in-season period derived from all three scans are shown in figure 7.2 (n=40). Values for the body composition compartments displayed as mean (± S.D) are presented in table 7.2 with results of analysis of variance between the in-season measures.

The significant variance in body mass across the season (p= 0.0001) was due to a significant gain (p< 0.0001) of 1.10± 1.56kg in body mass from the start to mid timepoints. There was no significant change in body mass between mid to end. From start to end there was a significant gain in body mass (p=0.0004) of 1.38 ± 2.28kg of body mass in the whole group.

The significant variance in lean mass (p< 0.0001) was specifically, a loss of lean mass (p=0.0055) in the start-mid period of 0.72 ± 1.55kg, followed by a significant increase in lean mass (p=0.0003) of 0.88± 1.40kg from mid-end. However, viewing the season from start (75.2± 8.68kg) to end (75.3± 8.16kg) there was no significant change in lean mass (p=0.5767).

There was a significant variance in fat mass across the season (p<0.0001). A significant increase of 1.67 ± 1.45kg was seen from start-mid (p<0.0001), followed by a reduction of 0.41 ± 1.39kg which was not significant. Overall fat mass increased significantly (p=0.0001) 1.26 ± 1.56kg from start-end.

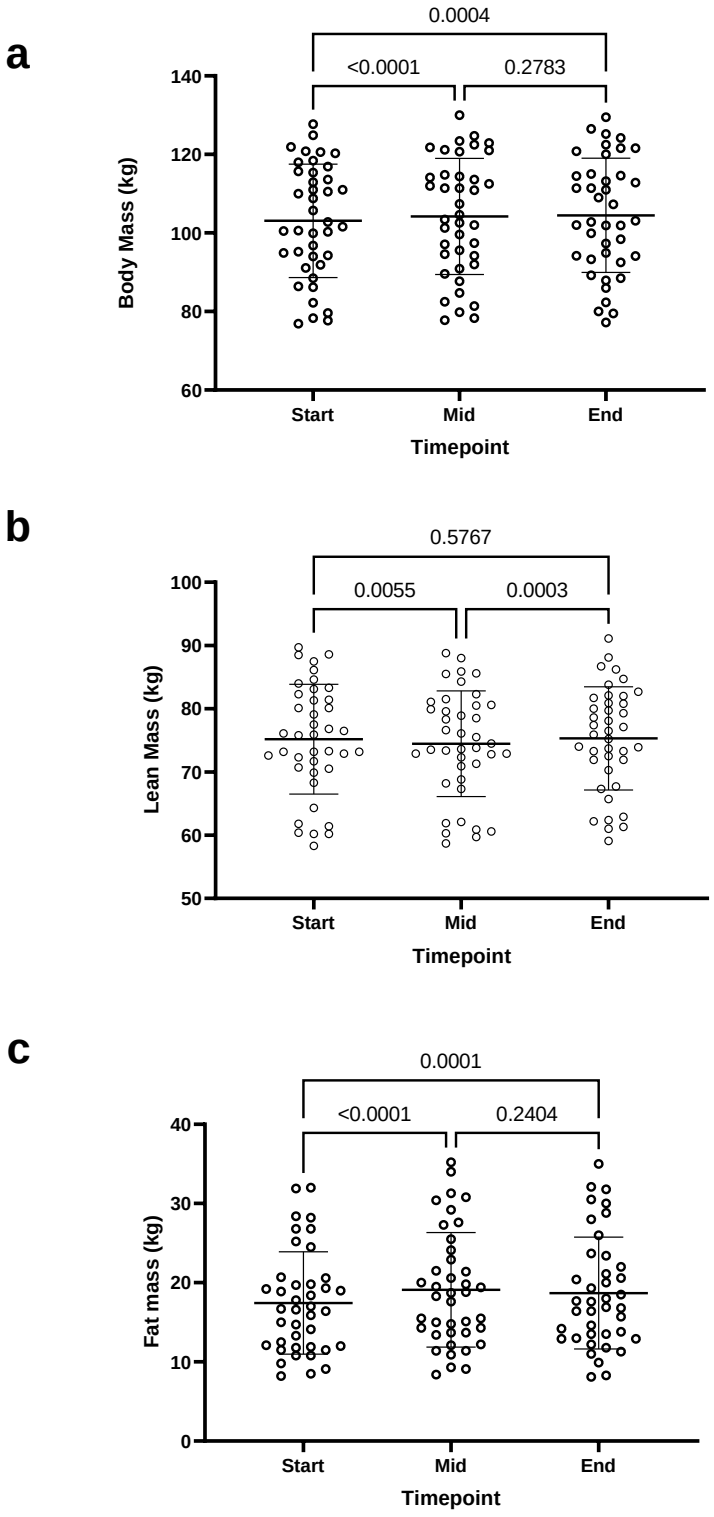


Figure 7.2. Changes in a). body mass, b). lean mass and c). fat mass throughout the in-season period derived from three DXA scan timepoints season start, mid-point, and end (n=40). The p-values for specific timepoint comparisons are included.

Table 7.2 Positional subgroups forwards (n=26) and backs (n=14) changes in body mass and body composition across the in-season periods with three DXA scans undertaken.

Group	Body composition compartment	Timepoint			ANOVA (p value)
		Start	Middle	End	
Whole squad (n= 40)	Body mass (kg)	103.1 ± 14.43	104.2 ± 14.79	104.5 ± 14.54	p= 0.0001
	Lean mass (kg)	75.2 ± 8.68	74.5 ± 8.35	75.3 ± 8.16	p<0.0001
	Fat mass (kg)	17.4 ± 6.46	19.1 ± 7.24	18.7 ± 7.06	p<0.0001
Forwards (n=26)	Body mass (kg)	111.2 ± 9.68	112.5 ± 10.12	112.6 ± 10.18	p= 0.0017
	Lean mass (kg)	79.3 ± 6.73	78.5 ± 6.22	79.4 ± 5.95	p= 0.0042
	Fat mass (kg)	20.9 ± 5.30	22.8 ± 6.13	22.23 ± 6.15	p= 0.0002
Backs (n=14)	Body mass (kg)	88.0 ± 8.10	88.8 ± 8.19	89.4 ± 7.55	p= 0.0344
	Lean mass (kg)	67.51 ± 6.49	66.96 ± 6.48	67.82 ± 6.18	p= 0.2427
	Fat mass (kg)	11.0 ± 1.54	12.2 ± 2.21	12.1 ± 2.30	p= 0.0086

There were some differences observed in body composition changes when the players were separated into positional groups of forwards (n=26), and backs (n=14) displayed in table 7.2.

Both groups increased body mass significantly over the course of the season. The forwards with an increase of 1.37± 2.27kg (p=0.0051) and backs with an increase of 1.41± 2.38kg (p=0.0106) from start-end. However, the backs increase from start-mid of 0.85± 1.33kg and mid-end of 0.56± 1.88kg were not significant changes, whereas the forwards experienced a larger 1.24± 1.68kg increase (p=0.0009) from start-mid and then experienced no significant change through the mid-end period of the season.

Both positional groups maintained lean mass throughout the season from start-end. However, whilst the backs experienced no significant variance across the three measures (p=0.2427), the forwards lost a significant (p=0.0062) 0.81± 1.38kg of lean mass from start-mid and then regained a significant (p=0.0030) 0.88± 1.37kg of lean mass from mid-end.

Changes in fat mass were similar in both groups with forwards gaining a significant (p=0.0045) 1.32± 1.59kg from start-end and backs gaining 1.14± 1.55kg (p=0.0169) through the whole season period. The pattern of change was mirrored also with both groups gaining most of the fat mass through the first period, start-mid; forwards 1.93± 1.63kg (p<0.0001) and backs 1.17± 0.90kg (p= 0.0003). Whereas the second period of the season mid-end saw no significant loss of body fat; forwards 0.61± 1.32kg and backs 0.04± 1.48kg.

7.4.3 Estimations of energy balance over the season

Mean energy balance in the whole group over the season from start to end was +44.0± 57.4 kcal·day^{-1}(range -41 to +188 kcal·day^{-1}). There was a significant difference between the periods of the season (p<0.0001) with a mean energy balance from start-mid of +104.8± 89.8 kcal·day^{-1} (range -79 to +331 kcal·day^{-1}) compared with mid-end of -36.5± 132.1 kcal·day^{-1} (range -302 to +275 kcal·day^{-1}). When comparing mean energy balance between positional groups there was no significant difference (p=0.3278) from start-end of the season, forwards +51.30± 60.66 kcal·day^{-1} and backs +34.40± 52.82 kcal·day^{-1}.

7.4.4 Correlations between body composition and energy balance

The whole in-season period (n=46) revealed significant correlations between energy balance and increases in body mass r^2= 0.38 (p<0.0001) and fat mass increases r^2= 0.98 (p<0.0001), but not with changes in lean mass r^2= 0.02 (p=0.37).

The interrelationships between changes in body mass, body composition and predicted energy balance are displayed in Fig 7.3 where the season has been divided into time periods of start-mid (left side) and mid-end (right side) including the three DXA scan timepoints (n=40). In both periods the changes in body mass were significantly correlated with energy

balance, start-mid r^2= 0.33 (p=0.0001), and mid-end r^2= 0.36 (p<0.0001). The reduction in lean mass start-mid was significantly inversely correlated r^2= 0.17 (p=0.0087) with energy balance, whilst the change in lean mass mid-end of season was not significantly correlated with energy balance r^2= 0.08 (p=0.0764). Changes in fat mass through both timepoints were also significantly correlated with energy balance, start-mid r^2= 0.96 (p<0.0001) and mid-end r^2= 0.96 (p<0.0001).

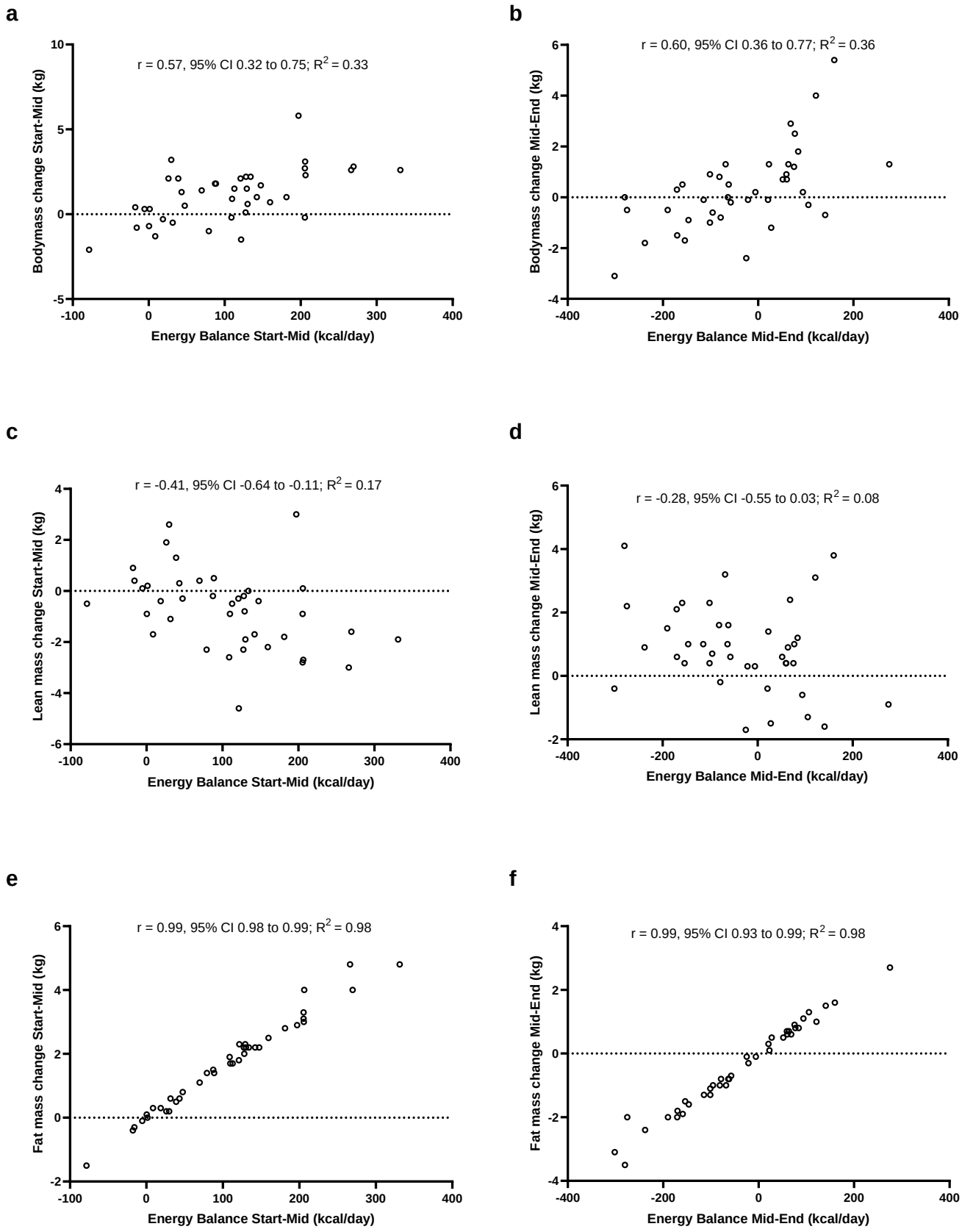


Figure 7.3 Correlations between body mass (a. – start to mid and b. – mid to end), lean mass changes (kg) (c. – start to mid and d. – mid to end), and fat mass (e. – start to mid and f. – mid to end), with predicted energy balance (kcal/day) in an elite rugby union squad (n=40).

7.4.5 Associations between training, match exposure and body composition

The correlations between match exposure measured as gametime in minutes, and changes in body composition and body mass (kg) are shown in Fig 7.4. The relationship between gametime and changes in body mass was not significant r^2= 0.0820 (p= 0.1853). The changes in lean mass from the start to end of the season were not significantly correlated with gametime r^2= 0.1333 (p= 0.0867). The increases in fat mass were significantly correlated with exposure to match play r= 0.6245 (p= 0.0014).

Body mass r^2= 0.0002 (p= 0.9486), lean mass r^2= 0.0005 (p= 0.9221), and fat mass r= 0.0366 (p= 0.8684) changes were not correlated with the amount of resistance training undertaken by the athletes throughout the season as measured by (AU) subjective units.

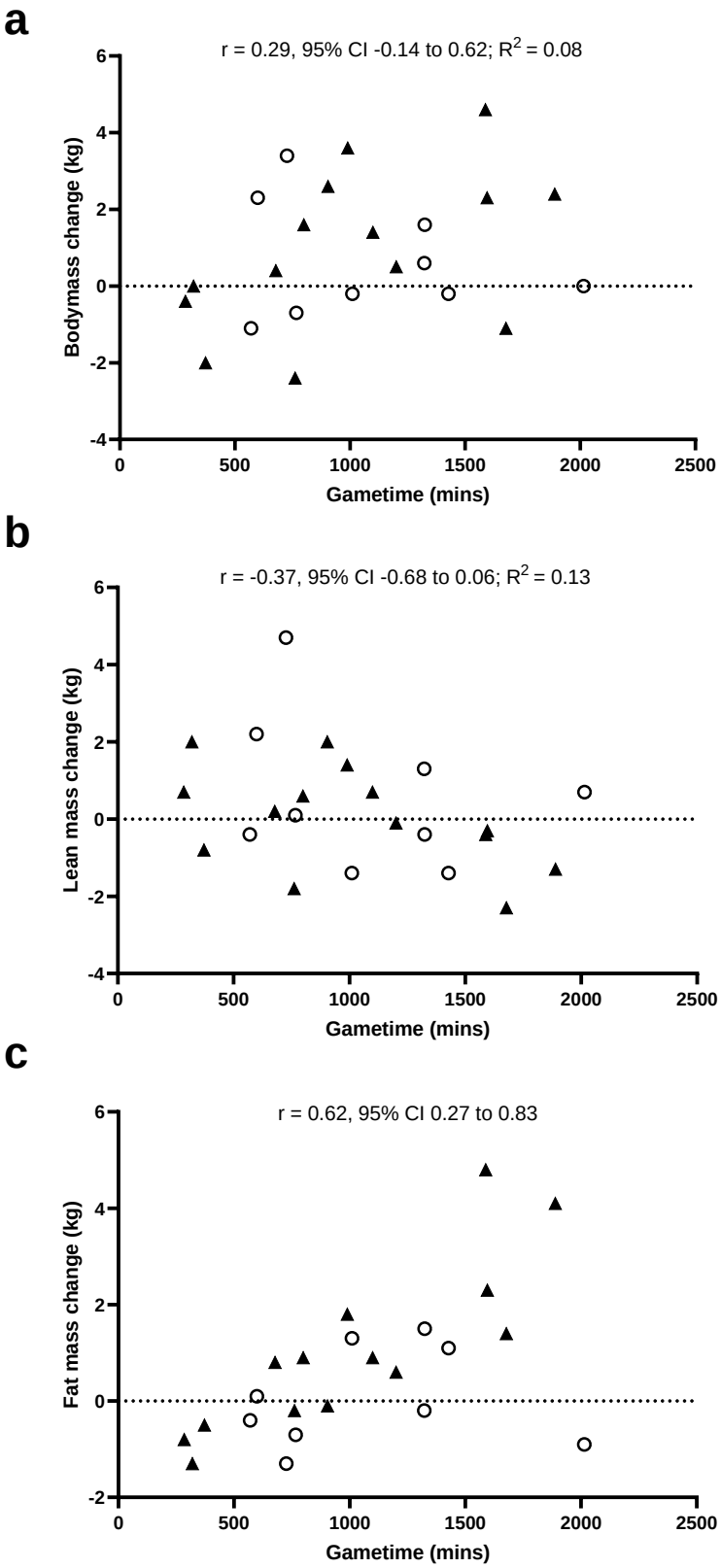


Figure 7.4 Relationship between match play exposure measured as gametime (mins) and the changes in a). body mass, b). lean mass, and c). fat mass over the in-season period (start-end) (n= 23). Forwards are represented by the filled black triangles (▲), and the backs by unfilled circles (○).

7.5 Discussion

The objectives of the current study were to investigate the body composition changes in elite rugby union players throughout an entire in-season period and to use these data to estimate energy balance over time. We also examined the relationships between changes in body composition with the training and match play demands. The findings in chapter 4 that resting metabolic rate increased significantly after match play (Hudson et al., 2020), coupled with the previously published increases in TEE associated with collisions in training (Costello et al., 2018), and the exceptionally high TEE of rugby league players (Morehen et al., 2016), led us to hypothesise that over the course of the season players may be in a mean energy deficit during periods of repeated match play. This may result in losses of body mass during the season which may be derived from the lean mass compartment.

Contrary to our hypothesis, we report that estimated mean energy balance across the whole season was in fact positive and this resulted in a significant increase in body mass, due to an increased fat mass compartment. This positive energy balance was reported in both the forwards and backs positional groups. Whilst the gain in fat mass reported here, as a result of a mean positive energy balance, may not be desirable to maintain physical performance throughout the season, sustained energy deficits are associated with undesirable ramifications (Thompson, Manore and Skinner, 1993; Deutz et al., 2000; Elliott-Sale et al., 2018), including impaired physical performance (Murphy et al., 2018). Although REDS is usually associated with aesthetics or sports where low overall body mass is desirable for performance, there is growing concern across all sports and in male athletes due to challenging body composition targets (Burke et al., 2018). Previous work using DLW in rugby athletes has reported a wide gap between TEI of 14.0MJ (3346kcal) and TEE OF 22.5MJ

(5378kcal) further exacerbating these concerns (Morehen et al., 2016). There has been a threshold of ±400 kcal·day^{-1} proposed to represent a limit for a desirable EB based upon predicted liver glycogen (Benardot, 2013). There were no players chronically below this limit in our research, or close to the extremely low levels reported in Australian rules football pre-season period of -935 kcal·day^{-1} (Bartlett et al., 2019), or in a combat sport athlete case study of -869 kcal·day^{-1} (Kasper et al., 2019). The research presented here provides an insight as to the chronic habits of these elite rugby union athletes and may allay fears of large consistent energy deficits, especially during periods of high TEE. Our results may also reinforce the need for nutrition support in rugby union performance teams, which is not the case in all professional environments (Sharples et al., 2021).

In a sport where high overall mass is desirable to generate momentum and win collision situations (Quarrie et al., 1995; Bevan et al., 2022) managing energy balance and limiting an increase in fat mass may be crucial for consistent physical performance over a season. Such increases in fat mass are not desirable as they may impact relative power and acceleration in backs (Posthumus et al., 2020) but also the ability of forwards, especially front five to meet the modern demands of speed and increased running distances (Bevan et al., 2022). The significant relationship between match play exposure and increases in fat mass over the season we present here, indicate that daily periodisation of energy warrants consideration. Rugby athletes are in pain and discomfort due to the collisions inherent with match play, throughout the entire season (Fletcher et al., 2016) and this may limit the volume of training they participate in, lowering TEE during parts of the training week, potentially leading to a surplus EI. A periodic surplus of energy during the training week could account for the mean positive EB reported here, causing the unfavourable increases in fat mass. Elite rugby union players therefore need to be mindful of their training intensity and load between matches,

and ensure they “fuel for the work required” (Impey et al., 2018) to prevent periods of energy surplus leading to the accrual of fat mass over a season.

There is evidence that elite rugby players periodises their dietary intake throughout a match week (Bradley et al., 2015a; Posthumus et al., 2021). The commonality between both published data sets and ours presented in chapter 5 of this thesis (Hudson et al., 2021), is the relatively low carbohydrate intake on GD+1. These observed intakes were below the mean for the week in both studies at ≈2.7 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Posthumus et al., 2021) and ≈3.1 & 3.2 $g \cdot kg^{-1} \cdot day^{-1}$ (Bradley et al., 2015a) for forwards & backs respectively and 2.93 ±0.64 $g \cdot kg^{-1} \cdot day^{-1}$ in our players (chapters 5 & 6). The findings of the preceding chapters of this thesis may explain how this could impact body composition over time. A significant increase in RMR due to the recovery from match play (chapter 4), coupled with the lower carbohydrate intakes reported, could create a significant temporary energy deficit. Previous work in elite female athletes reported a significant positive relationship between the number of hours spent in a deficit of >300kcal, and increases in body fat percentage as measured by DXA scanning (Deutz et al., 2000). The authors did not publish the body composition compartments though, so this increase in relative body fat percentage could be due to increased fat mass alone, reductions in lean mass, or both in tandem (Deutz et al., 2000). The metabolic perturbations following match play reported in chapters 5 and 6 of this thesis, indicated that recovery from match play requires carbohydrate to be available and that not meeting these needs may result in myofibrillar protein breakdown (Hudson et al., 2021). Therefore, periods of deficit in recovery from match play due to increased carbohydrate needs not being met, could explain why we have reported losses in lean mass, despite estimated mean energy surplus on a per day basis when viewed longitudinally. This

highlights the potential cumulative consequence of not meeting energy and carbohydrate needs in recovery from regular weekly match play.

To end the season with no overall losses of lean mass in the group was highly desirable especially as competitions are won at the end of the competitive calendar, and previous studies have reported significant reductions in lean mass at the season climax (Harley, Hind and O'Hara J, 2011; Lees et al., 2017). Due to the varied profiles of rugby union players (Duthie, Pyne and Hooper, 2003) and the differing demands (Cunniffe et al., 2009; Austin, Gabbett and Jenkins, 2011a), body composition profiles do vary between positions (Zemski, Slater and Broad, 2015), but the common theme for a collision-based sport is that high levels of lean mass are highly desirable especially at the elite level (Geeson-Brown et al., 2020). The importance is primarily due to the associations with standard of play (Smart, Hopkins and Gill, 2013; Bevan et al., 2022) and physical qualities of strength, power, and momentum (Smart et al., 2014; Posthumus et al., 2020) high levels of lean mass bring. There is evidence lean mass accrual can be achieved even whilst in an energy deficit as long as dietary protein intake is adequate (Carbone, McClung and Pasiakos, 2012) and whilst accompanied by an appropriate training stimulus. Indeed, the significant re-gain in lean mass during the second half of the season was achieved in an estimated mean daily energy deficit, as was reported in an Australian rules football pre-season period (Bartlett et al., 2019). Rugby populations typically report protein intakes above $2.0 g \cdot kg^{-1} \cdot day^{-1}$ (Black, Black and Baker, 2018) which is supported by observations in chapter 5 of this thesis and in other elite squads (Bradley et al., 2015a; Posthumus et al., 2021). However, neither the likely high protein intakes, a confirmed energy surplus, combined with the multiple resistance training sessions undertaken, prevented the significant losses in lean mass from start to mid-season reported here. Indeed, neither could the “re-gain” of lean mass in the second half of the

season be explained by simply undergoing more resistance training as there was no correlation between the internal loads of resistance training across the season and changes in lean mass.

The differences between forwards and backs positional groups may provide further insight into the reasons for the apparent loss in lean mass despite a mean positive energy balance during the period of intense match play. Forwards suffered significant reductions in lean mass during the first period whereas there was no significant change in lean mass when comparing all three scan timepoints in the backs. Forwards show greater strength and absolute power compared to backs with higher levels of both lean tissue and fat mass (Posthumus et al., 2020). These attributes are linked to the positional demands with forwards undergoing more high-level impacts than backs (Cunniffe et al., 2009). Given that our findings of increased RMR in chapter 4 were attributed to the collisions experienced in match play and, were significant in the forwards rather than the backs, we may have expected those positions involved in more collisions to be in an energy deficit. However, as we have presented, this was not the case. These athletes received both the stimulus and the necessary mean energy balances to maintain lean mass even during regular match play, which may drive us towards another potential explanation. The collisions inherent with rugby union match play cause ultrastructural damage to muscle tissue triggering a cascade of events involving an acute inflammatory response due to the actions of immune cells (Peake et al., 2017), increased inflammatory cytokines (Cunniffe et al., 2010; Cunniffe et al., 2011; Morehen et al., 2020) and accompanying oxidative stress (Lindsay et al., 2015; Lindsay et al., 2016a). The realisation of these events has been profiled in the metabolic perturbations presented in chapter 5 & 6 of this thesis, and in the increased metabolic rate and changes to substrate oxidation in recovery presented in chapter 4. This cascade of

events is akin to those seen in disease states where anabolic resistance resides, causing a desensitisation of the muscle to normal anabolic stimuli and reduced muscle protein synthesis (Paulussen et al., 2021). Therefore, the combination of increased muscle protein breakdown due to insufficient carbohydrate provision at GD+1 we proposed in chapter 6, with the potential anabolic resistance due to the prolonged inflammatory state associated with recovery from IIMD (Naughton, Miller and Slater, 2018a), may explain the losses of lean mass despite resistance training and an apparent mean daily positive energy balance in these athletes. Whist this suggestion seems plausible, it must be stressed this is speculative and future studies should now attempt to explore this through measurement of anabolic resistance using tracer techniques in the days following rugby match play.

When discussing the changes in body composition there must be an appreciation for the techniques used and to what degree the changes reported here are meaningful changes. There was a high degree of rigour imposed in the methodology to ensure that best possible practice was employed as this can more than double the deviations of change in lean mass especially between repeated scans (Nana et al., 2016). The granting of ethics to perform repeated DXA scans to ascertain a true coefficient of variance for the equipment under the operation of the researchers is not possible in the UK currently due to radiation exposure policy. In Australia for example, such permission is granted as arguably, the results of the research may not be interpreted to their fullest without the knowledge of what least significant change between scans may be. In similar research to this chapter, a CV generated with repeated scans of some participants, has allowed a further appreciation of results across a season (Bartlett et al., 2019). The measurement of skinfolds in tandem with DXA scans was employed here as they have been shown to predict the direction of change in fat mass and lean mass as measured by DXA (Zemski et al., 2018). The Hologic machine used

herein has been investigated in an older non-athletic population revealing a CV and least significant change (95% confidence) in lean mass of 0.51% and 1.41kg, with fat mass 0.89% and 2.46kg (Cheung, Roff and Grossmann, 2020). There is also data utilising another similar Hologic machine in athletic groups including elite rugby union athletes reporting a proposed technical error of measurement threshold of twice the TEM to determine real change in body composition compartments. These were reported to be 0.9% and 1.10kg lean mass, and, 3.9% and 0.72kg fat mass (Colyer et al., 2016). The significant correlation between changes in skinfolds and fat mass as measured by DXA reported herein, with these thresholds for significant change, supports the DXA derived changes in fat mass, especially in the first half of the season as real change. In the squad investigated here a CV of 0.51% would translate to 0.78kg lean mass (twice the TEM). However, when we consider that the mean changes in lean mass reported here between timepoints are less than 1.0kg, we cannot be certain a number of participants experienced real change in the lean mass compartment. Over a third of the playing group did experience changes between timepoints in lean mass greater than 1.0kg but further investigation into lean mass changes in these athletes is required, preferably with a measurement of least significant change generated as part of the research methodology and ethical permissions.

Overall, this research is the first to investigate body composition changes in an elite rugby union squad throughout an entire in-season period linking them with estimations of energy balance and interactions of match play exposure and training load. We report for the first time that it is possible to maintain lean body mass over the duration of the whole competitive season. However, despite nutrition support ensuring a positive mean daily energy balance was achieved, unfavourable changes in body composition with reductions in lean mass still occur during intense periods of match play. These observations highlight both

the need to meet the increased metabolic demands of recovery from match play 'fuelling for the damage induced', and also the management of players' gametime where possible. This periodisation of nutrition and management of match play exposure may support the maintenance of an athlete's optimal physical qualities throughout the competitive season. The summary of this chapter and major findings are displayed in infographic form in figure 7.5 below.

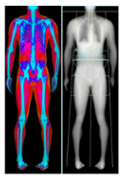
Rationale

Observational data on dietary intake from the literature and this thesis demonstrates rugby union athletes may not be meeting the metabolic requirements of recovery as reported in this thesis

Objective

Measure body composition employing DXA scanning technology at multiple occasions throughout the in-season period and use this data to estimate longitudinal energy balance. Investigate relationships between body composition, energy balance, and the training and match play demands of ellite rugby union

Results



	Body mass	Lean mass	Fat mass
Start-Mid	↑	↓	↑
Mid-End	↔	↑	↔
Start-End	↑	↔	↑

- Reductions in lean mass start-mid season are significantly correlated with a positive energy balance.
- Backs did not see any change in lean mass over the season whereas forwards experienced a significant reduction start-mid but re-gained this from mid-end.
- Losses in lean mass were associated with higher match exposure although not statistically significant.
- Increases in fat mass over the season are significantly correlated with higher match exposure.

Conclusion

The losses in lean mass were more apparent in forwards who undergo more collisions in match play and experience the significantly increased metabolic demands of recovery compared with the backs. However resistance training and a mean per day energy surplus did not prevent lean mass losses. Fat mass gain was also more apparent in those that played the most rugby.

Next Steps

We have proposed the muscle damage caused by collisions in rugby union match play had increased metabolic demands in recovery. The inflammation associated with this recovery period may be causing anabolic resistance challenging the ability of these athletes to maintain optimal body composition over the season.

Figure 7.5 Infographic overview of the research and major findings within chapter 7.

Chapter Eight

Synthesis of findings

The present chapter provides a summary of the findings from this thesis in relation to the original aims and objectives. A general discussion is then presented focussing upon how the findings developed throughout this PhD programme of work have furthered our understanding of the metabolic requirements of rugby union. Finally, the limitations, practical implications, and recommendations for future work are outlined.

8.1 Achievement of thesis aims and objectives

The overall aim of this thesis was to determine the metabolic requirements of recovery from match play in elite rugby union athletes. It was hypothesised that the findings derived from this thesis would formulate nutrition guidelines to best recover from the compounding physical movement and collision demands of rugby union. These underlying objectives to determine the metabolic requirements of recovery were achieved through a series of interlinked studies utilising laboratory and field-based techniques. An overview of each specific objective for each of Chapters 4, 5, 6, and 7 is provided below.

Objective 1 (Chapter 4) - Investigate whether exposure to elite rugby union training and match play alters resting metabolism on a day-to-day basis throughout a competitive match week.

Indirect calorimetry was used to measure resting metabolic rate (RMR), everyday across a 7-day competitive microcycle, apart from gameday itself. This was carried out utilising best practice RMR methodology whilst on-site at the training ground of the professional rugby club, which participated. The measures revealed a significant mean increase in RMR the morning after elite rugby union match play of approximately 10% or 231± 302 kcal.day^{-1}.

The forwards sub-group experienced this significant increase whilst the backs positional group did not. There were also a wide range of responses in RMR to match play with some outside backs experiencing no change, whilst there were individual examples of forwards and some backs (centres) displaying RMR increases of greater than 500 kcal.day^{-1}. In addition to the changes in RMR, there were also significant increases in RER at GD+2 and

GD+3. These increases corresponded with significant increases in carbohydrate oxidation at rest on both days and a significant reduction in fat oxidation at GD+3.

Monitoring the external and internal loads of the match week we were able to propose that the significant changes to metabolism following match play are due to the collisions and contact situations inherent with elite rugby union match play.

Objective 1 was successfully achieved and the results advocate that practitioners working withing elite rugby must consider these increased metabolic demands in the days following match play.

Objective 2 (Chapters 5 and 6) - Utilising a progressive, whole body, systems-based approach, investigate the metabolic perturbations associated with elite rugby union match play acutely and throughout recovery in serum (Chapter 5), urine, and saliva (Chapter 6).

To investigate metabolic perturbations across a week of match play, serum (chapter 5), urine and saliva (Chapter 6), were investigated utilising 1-NMR metabolomics and univariate and multivariate analyses techniques to identify key metabolites in both the acute period immediately post-match play and during the days after competition. Glycolysis, the TCA cycle, and glucose-alanine cycle were the predominant metabolic pathways accounting for energy production during match play. Due to the high intensity nature of this sport, at the elite level, the metabolism of carbohydrate for energy provision appeared to be the major contributor. The recovery period in the days following match play revealed a re-entry to gluconeogenesis coupled with markers of oxidative stress, structural protein degradation and altered fatty acid metabolism. There were also biomarkers associated with

adipogenesis, muscle protein and connective tissue degradation apparent in urine and saliva after competitive match play.

Objective two was successfully achieved in these chapters (5 & 6) and these data suggested that whilst carbohydrate metabolism during match play is the major energy source, the consequence of insufficient carbohydrate intake during recovery to meet the increased metabolic demands, may negatively affect the integrity of muscle and connective tissue protein.

Objective 3 (Chapter 7) - Measure body composition employing DXA scanning technology at multiple occasions throughout the in-season period in a population of elite rugby union players.

Having reported markers of muscle protein breakdown in the urine and saliva metabolomes (Chapter 6) with significant shifts in substrate oxidation (Chapter 4) during recovery from match play, the aim of this study was to investigate whether the repeated weekly bouts of match play and recovery cumulatively affected the body composition of these athletes. This research demonstrated that lean mass was maintained across the whole season, but that during periods of intense match play a significant reduction in lean mass was observed. The losses in lean mass were re-gained when the frequency of match play was reduced.

Both positional groups gained fat mass over the course of the season, with the significant gains observed in the first period of the season. Backs did not experience any changes in lean mass across the season. The forwards, however, lost a significant amount of lean mass during the period of intense match play but then regained this during the second half of the season.

Objective 4 (Chapter 7) - Utilise this body composition data to estimate longitudinal energy balance in this population and explore relationships between these and the training and match play these athletes are exposed to.

Due to the significant increases in RMR during recovery from match play detailed in chapter 4, coupled with the inability to measure TEE and TEI accurately for extended periods of time, this part of chapter 7 estimated energy balance derived from the changes to body composition. During the first half of the competitive season when match play occurred every week (21 matches over ≈148 days), the positive energy balance of the squad was significantly associated with gains in fat mass and overall body mass. Perhaps the most interesting finding from this section of the season was that the positive energy balance was significantly correlated with losses of lean mass. This lean mass was however regained despite small energy deficits, throughout the second half of the in-season period where match play was less frequent (12 matches over ≈103 days).

There was a trend that players with the most match play exposure lost more lean mass, however this was not statistically significant. By contrast, the gains in fat mass were significantly associated with those players playing the most minutes of competitive rugby. There was also no correlation between the resistance training loads (AU) players were exposed to through the season and changes to the lean mass compartment of body composition. These data demonstrate this squad of elite rugby union players were at least meeting their energy needs on a mean per day basis during intense periods of match play. Despite this however, unfavourable shifts in body composition with significant losses of lean mass and concomitant gains in fat mass were observed. Due to the differing positional changes in lean mass and the fact these occurred despite an apparent mean daily positive

energy balance with accompanying resistance training, we speculated the collisions inherent with elite rugby union may cause anabolic resistance in the muscle, challenging the ability to maintain lean mass during periods of intense match play.

8.2 General Discussion

8.2.1 Changes to metabolism in recovery

8.2.1.1 Resting metabolism measures

Measuring RMR under strict outpatient conditions we revealed a mean 231± 302 kcal or ≈10% increase on the morning after the game. Prior work demonstrating an increase in TEE of ≈5% during a training week involving collisions (Costello et al., 2018) supports these findings. We have furthered this insight demonstrating that the RMR compartment of TEE is increased significantly post-match play. We have also demonstrated that the collisions inherent with match play are likely responsible for this significant increase in the metabolic requirements in recovery. This is further supported in the positional differences observed. The backs subgroup did not see the significant increase in RMR nor significant changes to substrate oxidation compared to the forwards. Forwards experience more high-level collisions than backs (Cunniffe et al., 2009) which makes this separation between positions important but challenging as methods to quantify collisions and impacts still remains elusive in contact sports (Reardon et al., 2017a).

Lower intensity exercise activities such as jogging or cycling, even when energy matched, do not cause changes to RMR (Kolkhorst, Londeree and Thomas, 1994), whilst resistance training as opposed to aerobic exercise has previously been shown to cause an increase in RMR the following morning (Gillette, Bullough and Melby, 1994). The proposal from the authors (Gillette, Bullough and Melby, 1994) that the eccentric component of the resistance training caused this increase was supported by resistance work of high volume and an eccentric focus also increasing RMR in the days afterwards (Dolezal et al., 2000; Hackney, Engels and Gretebeck, 2008). This proposal that the muscle damage caused by the

resistance training is responsible for these RMR increases agrees with our conclusions that the metabolic requirements of recovery from match play are due to the damage caused by the collisions in rugby union. However, prior research into the changes to resting metabolism after exercise is limited as not all elements of gaseous exchange measures were published or recorded via indirect calorimetry. This means that reported increases in resting metabolism post exercise are likely due to extended periods of post exercise oxygen consumption (EPOC) rather than concomitant measures of carbon dioxide production and oxygen consumption (Fullmer et al., 2015). Our research differs in that all gaseous measures were recorded and analysed. These revealed significant increases in RER at GD+2 and GD+3 driven by significantly increased carbohydrate oxidation at the same timepoints. To our knowledge this has not been documented before in any research investigating changes in measures of indirect calorimetry after exercise interventions. These results are also corroborated in the investigations of the metabolome which followed.

8.2.1.2 Changes to the metabolome

The acute changes to the metabolome we reported, demonstrated high levels of carbohydrate metabolism via glycolysis and the activity of the TCA cycle which agrees with previous reports comparing HIT with moderate intensity energy matched work (Peake et al., 2014). Alanine increases also accompanied the post exercise changes after HIT exercise (Peake et al., 2014) implicating gluconeogenesis via the glucose-alanine cycle. Alanine was significantly increased at GD and GD+2 spectra in serum with both the serum and salivary metabolomes significantly ranking the glucose-alanine cycle and gluconeogenesis in recovery. When investigating differing phenotypes of athletes both endurance athletes and body builders demonstrated increases in the metabolites of these pathways after exhaustive exercise (Schranner et al., 2021).

Despite metabolites of carbohydrate metabolism and lactate formation being greater after HIT compared with moderate exercise, there were not differences in metabolites of fatty acid metabolism (Peake et al., 2014). Acute metabolome responses to rugby match play revealed serum acetate levels peaking at GD which the beta-oxidation of fatty acids could have contributed to. However, with no accumulation of acetoacetate and lower levels of 3-hydroxybutyrate compared to pre-match, the utilisation of fatty acids for energy provision in match play appears minimal. The accumulation of blood acetoacetate, 3-hydroxybutyrate, and acetone has been reported in NMR metabolomics after exhaustive endurance exercise indicating saturation of beta-oxidation (Kirwan et al., 2009; Stander et al., 2018; Bester et al., 2021). We report the levels of these metabolites in serum to remain below those at pre-match throughout recovery with support of a reduction in ketone body formation from the saliva metabolome also. The appearance of acylcarnitines associated with fatty acid metabolism reported in other exercise metabolomics studies may not be apparent here due to the technique of NMR being used rather than MS spectrometry (Khoramipour et al., 2022). As we have discussed though, this is not a limitation as the confidence we have around metabolite identification using NMR (Emwas, 2015; Emwas et al., 2019) together with these metabolites of fatty acid oxidation such as acetoacetate and 3-hydroxybutyrate being identified as key discriminators between sample timepoints, support our conclusions.

Reduction in blood amino acid levels have been observed across exercise metabolomics studies (Schranner et al., 2020) after resistance type training (Berton et al., 2017), and endurance exercise (Khoramipour et al., 2022). The extent of reductions in blood amino acids reported, acutely after exercise may be due to the form of training undertaken and to the high dietary intakes reported in a ‘bodybuilder’ phenotype (Schranner et al., 2021).

However, modern rugby union athletes undertake a combination of power, strength and high intensity training rather than purely muscle hypertrophy or endurance (Bevan et al., 2022). The reductions in serum amino acids in the extended recovery period have not been documented previously and were associated with the gluconeogenesis pathways identified in our research (Chapter 5). The saliva metabolome evidence complemented these findings demonstrating the potential of multi-biofluid analysis to build a complete picture of the metabolic perturbations (Do et al., 2015). The downstream metabolites of leucine, and connective tissue breakdown identified in urine, and the metabolites of muscle protein breakdown (3-MH) in both saliva and urine were reported in our research(Hudson et al., 2021). There are important differences in sample collection of saliva though as our conclusions were based upon fasted samples where this metabolite has been identified as a key discriminator and were cautious using the results of fed samples immediately post-game. For this reason, the appearance if 3-MH during the latter stages of basketball match play should be approached with caution (Khoramipour et al., 2020), whereas the accumulation of soccer match play causing increased 3-MH in saliva (Ra et al., 2014) supports our findings implicating increases in muscle protein breakdown during the recovery period.

The cumulative metabolic stress of rugby match play was signified via the key discriminatory metabolite 2-hydroxybutyrate which has also been reported following HIT exercise (Peake et al., 2014), and after intense high volume resistance training (Deminice et al., 2011). It is important to note that many of these metabolites signifying metabolic stress and fatigue do drop back to pre-exercise levels in the first few hours post exercise. This is true of 2-hydroxybutyrate after HIT (Peake et al., 2014) and in agreement with our observations in serum. However, the kynurenine pathway, upregulated and significantly ranked in urine

both acutely and in the recovery period in our research, both supported the high energy requirements of match play and were associated with the metabolic demands due to oxidative stress throughout the extended recovery period. This was also true of the purines identified as key discriminatory metabolites in saliva and urine. Levels of purines have previously returned to pre-exercise levels after exhaustive cycling within 24 hours (Arthur et al., 2018) and been associated with fatigue in soccer players (Prado et al., 2017; Pitti et al., 2019; Alzharani et al., 2020; Quintas et al., 2020), but our work demonstrated these changes lasted beyond this acute period into the following days signifying the elongated metabolic stress response in recovering from elite rugby union.

Discussing our findings beyond 24 hours post-match play is challenging as no prior metabolomic work has continued sampling in this timeline (Schranner et al., 2020; Khoramipour et al., 2022). Another novel aspect of our metabolomics work was the recording of dietary intake which both enabled the identification of metabolites due to specific foods in the analyses performed, but also to relate changes in the metabolome to energy and macronutrient intakes. We will therefore also discuss the novel changes to metabolic rate, substrate oxidation and the metabolome we have reported, with reference to the dietary intakes of this population.

8.2.2 Dietary intake

The dietary intake data we have presented in this thesis (Chapter 5 & 6) revealed no significant differences in daily fat (1.16± 0.31 $g \cdot kg^{-1}$) or protein (2.39± 0.53 $g \cdot kg^{-1}$) intakes across the days of the match week, with carbohydrate (3.17± 1.39 $g \cdot kg^{-1}$) being the macronutrient responsible for day-to-day fluctuations in total EI. A comparison with prior research in elite rugby union squads is included in table 8.1. The differences in energy and macronutrient intakes may be attributed to the far greater training loads of the southern hemisphere squad where they reported a player load of 4386 AU total for the week (Posthumus et al., 2021) compared to our groups 1922 AU (Chapter 4) and 1978 AU (Chapter 5 & 6). The loads in the work of Bradley et al. without gameday included were also greater than our group at ≈1776 AU for forwards and ≈1523 AU for backs compared to our ≈1300 AU (Chapter 4) and ≈1445 AU (Chapters 5 & 6) (Bradley et al., 2015a). The running distances are not comparable due to differences in thresholds used (Reardon, Tobin and Delahunt, 2015).

These far greater loads may explain the differences in fat intake between the northern (Bradley et al., 2015a) and southern hemisphere (Posthumus et al., 2021) groups whilst the carbohydrate and protein intakes are similar to previous observations (Black, Black and Baker, 2018). The carbohydrate has been consumed to 'eat to intensity' (Posthumus et al., 2021) or more akin to the 'fuel the work required' paradigm (Bradley et al., 2015a) with carbohydrate significantly increased in preparation for competition as it was in our research, as reported in Chapters 5 & 6.

Table 8.1 Dietary intakes from elite rugby union squads in prior research compared to our group reported in Chapters 5 & 6 of this thesis.

Study	Positional Group	Energy Intake		Carbohydrate	Protein	Fat
		kcal·day^{-1}	kcal·kg·day^{-1}	g·kg·day^{-1}	g·kg·day^{-1}	g·kg·day^{-1}
(Posthumus et al., 2021)	Forwards	4606 ± 719	40.5 ± 7.2	3.5 ± 0.8	2.5 ± 0.4	1.8 ± 0.4
	Backs	3761 ± 618	41.9 ± 7.2	3.7 ± 0.7	2.4 ± 0.5	1.8 ± 0.5
(Bradley et al., 2015a)	Forwards	≈3967	≈36.0	3.5 ± 0.8	2.7 ± 0.5	1.4 ± 0.2
	Backs	≈3394	≈36.3	3.4 ± 0.7	2.7 ± 0.3	1.4 ± 0.3
(Hudson et al., 2021)	Whole group	3323 ± 630	32.9 ± 7.1	3.2 ± 1.4	2.4 ± 0.5	1.2 ± 0.3

8.2.2.1 Periodisation of energy intake during the match week

The energy intake in our group of rugby players at GD+1 was 3272± 409kcal which is below the mean for the week at 3323± 637kcal, a common theme in the other elite group observations with the energy intakes at the mean for the week (Forwards ≈3976kcal and Backs ≈3394kcal) in northern hemisphere players (Bradley et al., 2015a) and below the mean for the week (Forwards ≈4606kcal and Backs ≈3761kcal) in southern hemisphere rugby union athletes (Posthumus et al., 2021). Our work in Chapter 4 demonstrating the increased metabolic demands of recovery at GD+1 means that energy intake as part of a periodised match week needs to be increased in rugby union athletes at GD+1 and it may be even more pertinent to promote this in those positions which undergo heavy, frequent collisions.

8.2.2.2 Carbohydrate intake in match preparation

Studies, which harvested muscle biopsies from rugby league match players demonstrated that a carbohydrate intake of 6g·kg^{-1} as opposed to 3g·kg^{-1} in the 36hr prior to match play may be beneficial in preventing glycogen levels dropping so low as to affect the athlete's ability to physically produce the repeated high intensity activities required to perform (Bradley et al., 2016). This intake has been observed in northern hemisphere rugby players (Bradley et al., 2015a) but southern hemisphere practices appear to not reach this total in the day before match play with intake at approximately ≈3.2 g·kg^{-1} on GD-1 but then on GD are able to consume 3.0± 0.9 g·kg^{-1} (Forwards) and 3.0± 0.5 g·kg^{-1} (Backs) prior to kick off. In preparation for match play the participants recruited in chapters 5 & 6 herein, consumed 4.32 g·kg^{-1} at GD-1 and carbohydrate rich meals prior to kick off contributing to the GD intake of 5.62 g·kg^{-1} meaning they would likely have achieved the 6 g·kg^{-1} target in the 36hr prior to kick off. The results of our metabolomics research indicate carbohydrate as the

predominant fuel source for match play with both serum and saliva metabolomes revealing that the glycolysis, gluconeogenesis, and the TCA cycles were upregulated in samples taken immediately post-match. Metabolites in serum and urine also demonstrated the acute metabolic stress and the kynurenine pathway upregulated to enable NAD+ production to facilitate the continued high energy needs. Previous work in academy-level professional rugby league players had identified lipid mobilisation via non-esterified fatty acids (NEFA) and Glycerol increases post-match (Bradley et al., 2016) as an important fuel source. This is in contrast to our data, where there were no indications that fatty acid metabolism increased and the reductions in blood ketones led to the conclusion that due to the intensity of senior elite rugby union match play the provision of carbohydrate is crucial for match day performance in the form of stored glycogen and exogenous sources.

These findings accentuate the need for carbohydrate intake in the 36hr prior to kick off, to comfortably meet this 6 $g \cdot kg^{-1}$ target in preparation for match play. This coupled with a light team run training session and low EE on GD-1 will allow glycogen stores to be prepared for competition.

8.2.2.3 Carbohydrate intake in recovery from match play

Prior investigations into glycogen replenishment after a rugby league simulation protocol implicated immediate high carbohydrate refeeding taking advantage of the exercise induced glucose transport into the muscle as preferable compared to delayed feeding (Bradley et al., 2017). Despite Bradley and colleagues not recording dietary intake on GD (Bradley et al., 2015a), the southern hemisphere elite group revealed intakes of 1.5± 0.7 $g \cdot kg^{-1}$ (Forwards) and 2.1± 0.8 $g \cdot kg^{-1}$ (Backs) carbohydrate post-match play of a total GD carbohydrate intake of ≈4.5 $g \cdot kg^{-1}$ (Forwards) and ≈5.1 $g \cdot kg^{-1}$ (Backs). Our group consumed 5.62 $g \cdot kg^{-1}$ on GD in total with over half of that being in the drinks and whole food meals consumed over the

three opportunities after the match concluded. This higher intake than previously reported and more in line with the aims of the replenishment study (Bradley et al., 2017), may explain why the metabolomics investigations herein, observed a normalisation of the amino acid levels and metabolites associated with the levels of gluconeogenesis at the morning after the match (GD+1) compared with immediately post-match. However, between the GD+1 timepoint and the samples at GD+2, we demonstrated a shift back to gluconeogenesis and the degradation of gluconeogenic amino acids together with pathways upregulated indicative of oxidative stress across all three biofluids. The significant changes to RER in Chapter 4 at GD+2 with increased carbohydrate oxidation at rest were corroborated by the pathways upregulated in our metabolomics research leading to the conclusion that greater carbohydrate intakes are required throughout GD+1 to meet the metabolic requirements of recovery from muscle damage caused by elite rugby collisions in match play.

We observed an intake of 2.9 $g \cdot kg^{-1}$ carbohydrate at GD+1 which was below the mean intake for the week. Prior work reported carbohydrate intakes below the mean for the week in each group, ≈2.7 & 3.1 $g \cdot kg^{-1} \cdot day^{-1}$ (Posthumus et al., 2021) and ≈3.1 & 3.2 $g \cdot kg^{-1} \cdot day^{-1}$ (Bradley et al., 2015a) for forwards & backs respectively. This indicated habitual intake at GD+1 is below levels required to 'fuel for the damage induced' and needed to support the processes of recovery caused by EIMD and IIMD after match play (Hudson et al., 2021). We suggest an increase in carbohydrate intake at GD+1 to 4.0-6.0 $g \cdot kg^{-1} \cdot day^{-1}$ would be more appropriate to facilitate recovery and meet the metabolic requirements.

8.2.3 Energy balance during the in-season period

Previous research using SenseWear armbands (EE) and dietary intake (EI) to estimate EB throughout an in-season week demonstrated a mean energy surplus of ≈167 kcal·day^{-1} in the forwards and ≈48 kcal·day^{-1} in the backs (Bradley et al., 2015a) compared to our longitudinal view over the season of forwards 51.30± 60.66 kcal·day^{-1} and backs 34.40± 52.82 kcal·day^{-1}. This is contrary to the view obtained in rugby league where a significant gap between TEI of 3346kcal·day^{-1} and TEE 5378kcal·day^{-1} was reported whilst using DLW (Morehen et al., 2016). Whilst the surplus reported in rugby union supports our findings, the limitations of SenseWear armbands not being worn for all activities and the observed in-season week not accounting for EI and EE of the gameday itself (Bradley et al., 2015a), means the insight provided by DLW which includes all activities and any increased metabolic demands after match play may be more applicable and represents the potential deficits which could occur in contact sports (Morehen et al., 2016). The results of our longitudinal estimation of energy balance across the season, rather than a short 7-14 day 'snapshot' may represent the chronic habits of rugby athletes. It is therefore positive that no chronic or extreme deficits were observed in our research as has previously been reported (Bartlett et al., 2019; Kasper et al., 2019) as those could cause declines in physical health and performance (Benardot, 2013; Elliott-Sale et al., 2018). We must also recognise though that these results are from one squad who do have nutrition support as part of their performance department and may not be representative of all rugby union clubs at the elite level.

Perhaps the most intriguing element to this insight to longitudinal energy balance is the loss of lean mass during heavily congested match play periods despite players being in a mean energy surplus. The undesirable gain in fat mass which accompanies this loss of lean mass in

the first half of the season was not surprising given the estimated mean energy surplus, but the loss in lean mass was not expected. This estimated energy balance is derived on a mean daily basis (kcal·day^{-1}) (de Jonge et al., 2007; Pieper et al., 2011). It cannot appreciate any potential intra-day variations in energy balance. It has been proposed that where extended periods of energy restriction or surplus occur, catabolism and anabolism of lean and fat mass occur (Benardot, 2013). Our findings that the metabolic requirements of recovery at GD+1 are increased in some individuals above the proposed 400kcal range (Benardot, 2013) and that dietary intake in our population of energy and carbohydrate was below the mean for the week, could create a period of deficit. This transient period of deficit may be masked by the mean positive energy balance estimated longitudinally therefore explaining both the increases in fat mass and losses of lean mass. If these periods of transient deficit are exacerbated by match play, this would further explain why we witnessed these unfavourable shifts in body composition during the first part of the season with continuous, intense fixtures. Moreover, given that the loss in lean mass was more apparent in the forwards positional group who undergo significantly more physical collisions than the backs (Cunniffe et al., 2009) supports this suggestion that if the increased metabolic demands due to damage caused by collisions is not met, a period of energy deficit at GD+1 may lead to the reported decrements in lean mass.

We also report that lean mass may be re-gained over the season when match frequency is lower even when a small mean energy deficit is observed in the group. This has been witnessed before in an Australian rules football pre-season period where lean mass gains were achieved despite a much larger energy deficit (Bartlett et al., 2019) and improvements in body composition were reported in a rugby union preseason again despite significant estimated energy deficits (Bradley et al., 2015b).

We have provided novel insight as to the metabolic requirements of the inflammation and immunoendocrine responses to match play (chapters 5 & 6), but there may also be effects of this recovery upon the maintenance of lean mass. Chronic inflammation is the hallmark of muscle wasting states or sarcopenic mechanisms observed in older humans (Dalle, Rossmeislova and Koppo, 2017). Despite a likely sufficient protein intake and regular resistance training exposure, these athletes still struggled to maintain lean mass during relentless periods of match play. We surmise a potential de-sensitisation of the muscle to anabolic stimulus in the recovery period (Paulussen et al., 2021), combined with the proposed muscle protein breakdown we reported in Chapter 6, lead to a loss of lean mass despite provision of stimulus, and a mean positive daily energy balance.

8.2.4 Body composition of Rugby union athletes

Given that high levels of lean mass are associated with success at the elite level in rugby (Geeson-Brown et al., 2020; Posthumus et al., 2020) it is positive we reported the maintenance of this lean mass across the season and is contrary to previous research in sub-elite rugby union (Lees et al., 2017) and elite rugby league (Harley, Hind and O'Hara J, 2011). It is critical that the elite squads do not suffer these losses, especially as modern competition formats reach their climax at the season end deciding an overall winner. Both our observations and those in elite rugby league agreed in that lean mass losses were suffered during the most intense periods of match play (Harley, Hind and O'Hara J, 2011).

Furthermore, there may be additional negative consequences for performance of the fat mass gains we observed in the first half of the season, which persisted to the end of the season. Higher levels of bodyfat have been related to decreased work-rates and reduced tackling ability, especially in the forward positional group (Smart et al., 2014). Increased bodyfat measures were also associated with decreased power, speed and aerobic fitness in

forwards (Posthumus et al., 2020). If bodyfat becomes excessive there may also be the negative consequence of decreased speed and acceleration (Duthie, Pyne and Hooper, 2003) which specifically relates to the ability to make line breaks and score tries in the backs positions (Smart et al., 2014). The unfavourable shifts previously reported in rugby athletes did not result in an increase in body mass though, rather a significant increase in fat mass concurrently with the lean mass decreases we have discussed (Harley, Hind and O'Hara J, 2011; Lees et al., 2017). Due to the retention in lean mass, our group's gains in fat mass were apparent as increases in body mass over the whole season. The novel aspect to our work which advanced previous observations was that the unfavourable shifts in body composition were associated with match play exposure. Gains in fat mass were significantly associated with gametime over the season and there was a trend for the lean mass losses observed to also be more prevalent in those playing more competitive rugby.

We would therefore propose rugby coaches in tandem with members of the performance team need to manage player's gametime and periodise training loads, where possible throughout the season. This may enable players to maintain lean mass consistently and limit gains in fat mass therefore maintaining their optimal physical qualities.

8.3 Practical implications

8.3.1 Measurement of resting metabolism in athletes

When strict outpatient protocols are adhered to, there is no difference in resting measures of energy expenditure compared to inpatient procedures in athletes (Bone and Burke, 2018b). In the work of Bone and Burke (2018) SenseWear monitors were worn to ensure training activities were complied with prior to measures (Bone and Burke, 2018b), and our work herein supports the need to carefully schedule measurements of RMR in athletes. Otherwise, a likely inflation of RMR will be reported due to either; EPOC from prior training (Kolkhorst, Londeree and Thomas, 1994), a high eccentric focussed resistance training load (Dolezal et al., 2000; Hackney, Engels and Gretebeck, 2008), or contacts within collision sports (Hudson et al., 2020). At least 48hrs rest from training may be prudent to ensure these are not confounding factors. When we consider the variety of inaccuracies in deriving RMR from equations, especially in athletes with large amounts of lean mass (Carlsohn et al., 2011; Morehen et al., 2016; Jagim et al., 2018; Smith et al., 2018) accurate measures of RMR are still crucial for practitioners to accurately predict energy needs in specific situations.

8.3.2 The periodisation of nutrition in rugby union

The periodisation of nutrition across the competitive microcycle should be considered with the primary outcome of maintaining physical qualities of collision sport athletes to allow a high level of consistent physical performance throughout a long in-season period. In endurance sports the periodisation of carbohydrate under the “Fuel for the work required” paradigm seeks to acutely manipulate carbohydrate availability to augment cell signalling related to mitochondrial biogenesis (Impey et al., 2018). Improvements in body composition often accompany these day-by-day, meal-by-meal changes to carbohydrate intake with endurance performance markers (Marquet et al., 2016) and there is evidence a rugby preseason period may benefit from similar improvements (Bradley et al., 2015b). Here, we

suggest that the novel findings from the research presented allow a weekly competitive microcycle to be periodised to meet the increased energy requirements of recovery (Chapter 4), together with the proposal that this needs to be derived from carbohydrate intake at GD+1 (Chapter 5 & 6). The aim being to allay gluconeogenesis and the potential degradation of myofibrillar protein and prevent the loss of lean mass observed during intense match play periods of the season (Chapter 7). This change in mindset away from carbohydrate solely reloading glycogen for the next competitive event, but towards providing the necessary fuel to recover from the damage induced, includes an increase in energy intake primarily from carbohydrate on GD+1 to meet the increased metabolic demands. It is important to note this suggested framework is using the scheduled training intensity and volume as per the squad researched in this thesis and may need to be adapted to a specific schedule. There may also need be individual considerations with differing training and body composition goals at times in season.

Table 8.2 Training content of the weekly competitive microcycle commencing with the first day return to training post-match at GD+2 moving towards the next fixture. This provides the session content and some considerations for why carbohydrate content may be periodised as below (Table 8.3) around training and recovery days to create energy balance.

Time Point	GD+2	GD+3	GD+4	GD−2	GD−1	GD	GD+1
Daily purpose	Resistance train, Installation & Tactical learning	Resistance train, Unit skills under fatigue, Overload run volume	Rest & Recovery	Intensity Execute tactical specifics at high intensity	Team Run Low intensity rehearsal of game plan	Match Play Maximal physical performance	Rest & Recovery
Resistance training content	Lower Limb Strength (45 min)	Upper Limb Strength (45 min)	None	Upper Limb Strength (45 min)	None	None	None
Physical rugby content	Low intensity attack shapes and defensive system installation. (45 min)	High Intensity throughout rugby specific drills. (75-90 min)	None	Specific Game Prep (45 min) Unit Split (30 min)	Execution of specific game plan at a low intensity (30 min)	Individual & Team prep. Rugby Match Play (80 min).	None
Rationale for feeding strategy	Intensity very low in rugby with no high-speed efforts and short duration. Resistance training volume not low-mod strength.	Time high carbohydrate meals around the training sessions especially rugby specific training on this day to support high intensity and longest session duration of the training week.	Meet protein & energy needs, focus on nutrient quality. Low overall carbohydrate intake in total.	Provide carbohydrate early in the day to facilitate high intensity of power, speed, & rugby session. Carbohydrate may be lower through the rest of the day.	High carbohydrate intake at all meals to meet ≈600g total. A combination of high & low G.I. foods may promote reaching this target and palatability.	High but comfortable carbohydrate fuelling into KO with gels/drinks in match and emphasis on immediate refuelling at every meal post game.	High carbohydrate meals to fuel the response to muscle damage and inflammation. Forwards & high collision backs may have greater requirements than 9's, 10's and back 3 (11/14/15).

Table 8.3 Suggested practical model integrating carbohydrate periodisation into rugby union players as a one game per week and full 7-day turnaround into the next competitive fixture. CHO – carbohydrate, Fwds – forwards positions, Backs – backs positions.

Day	Typical Loads	Breakfast	Inter-session	Lunch	Snack	Dinner	Evening Snack
Monday (GD+2)	Duration = 45min RD = 0.5m-1km HSR ≈50m	Medium CHO 0.75-1 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}	Medium CHO 1 g·kg^{-1}	None	Medium CHO 0.75-1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}
Tuesday (GD+3)	Duration = 75-90min RD = 1.5-2km HSR = 300-450m	Medium CHO 1 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}	High CHO 1.2-1.5 g·kg^{-1}	None	Medium CHO 1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}
Wednesday (GD+4)	Rest & Recovery	Medium CHO 0.5-1 g·kg^{-1}	None	Medium CHO 0.5-1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}	Medium CHO 0.5-1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}
Thursday (GD-2)	Duration = 75min RD = 1-1.5km HSR = 200-350m	Medium CHO 1 g·kg^{-1}	None	Medium CHO 1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}	Medium CHO 0.5-1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}
Friday (GD-1)	Duration = 30min RD ≈ 1km HSR <50m	High CHO 1.2-1.5 g·kg^{-1}	None	High CHO 1.2-1.5 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}	High CHO 1.2-1.5 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}
		Breakfast	Pre-match	During Game	Post-Game		
Saturday (GD)	Duration = 80 mins RD = 1.5-3km (Fwds) 2-3.5km (Backs) HSR = 150-350m (Fwds) 350-500m (Backs)	High CHO 1.2-1.5 g·kg^{-1}	High CHO 1-1.5 g·kg^{-1}	High CHO 60 g·hr^{-1}	High CHO 1-1.2 g·kg^{-1} for 3 hourly opportunities; Changing rooms, Meal 1 & Meal 2		
		Breakfast	Inter-session	Lunch	Snack	Dinner	Evening Snack
Sunday (GD+1) 1-8 & 12/13	Active & Passive Recovery with Rest	High CHO 1.2-1.5 g·kg^{-1}	None	High CHO 1.2-1.5 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}	High CHO 1.2-1.5 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}
Sunday (GD+1) 9/10, Back 3		High CHO 1.2-1.5 g·kg^{-1}	None	High CHO 1.2-1.5 g·kg^{-1}	Medium CHO 0.5-1 g·kg^{-1}	Medium CHO 0.75-1 g·kg^{-1}	Low CHO 0.5 g·kg^{-1}

8.4 Limitations

Despite being collected in the applied field all the studies reported within the thesis were conducted with rigour and planning to ensure the controlled laboratory environment was taken into the field rather than sacrifices being made in data quality to facilitate research within elite populations. The equipment utilised and replication of laboratory conditions were facilitated in large part due to the privilege of space afforded by the participating rugby club and generous loans of equipment from university facilities. It is however vital we acknowledge limitations in this research that remain, and how the conclusions and hypotheses generated within, can be contextualised in their application.

8.4.1 The population

It is important to highlight that the findings from all four chapters are from a single professional rugby team competing in the English Premiership competition. Study 3 (Chapter 7) involved nearly every member of the full-time professional squad with only a few omitted due to long term injury which reduced their continuous training load and availability for selection. The participants for study 1 (Chapter 4), and study 2 (Chapters 5 & 6) are skewed towards the forwards positional group due to the individuals who gave consent to take part. As further work in this area is developed and potentially less invasive techniques are utilised, greater sample sizes will be engaged, with the ability to stratify the playing positions with a balanced view of the simplistic forwards and backs subgroups.

We also acknowledge that this squad may not be representative of all groups within elite professional rugby union, as the sports science and nutrition support does vary between organisations and countries. The lead researcher's role as performance nutritionist afforded

them three days per week contact with the squad throughout the season and this had been in place throughout the period of research conducted within this thesis. This level of contact time per week and the length of time allowed to embed a culture around performance food together with consistent education and strategies change behaviours is more than many clubs in the English Premiership would have. The support English Premiership players receive is wide ranging from full-time to as little as one day per week at the time of writing. It is therefore likely the participant players in this research may be better informed and educated in all aspects performance nutrition than many of their peers. This may have been advantageous in this research as the appreciation for how the research being conducted could positively influence the support that could then be provided may have achieved greater buy-in and conformity to research protocols.

8.4.2 GPS

Early work in rugby union utilising GPS technology employed absolute speed zones to classify for example, jogging, running, and high speed running (Roberts et al., 2008; Austin, Gabbett and Jenkins, 2011a). However, there is evidence that relative speed zones may provide us with more information on positional running demands in field sport athletes (Dwyer and Gabbett, 2012) and rugby union (Reardon, Tobin and Delahunt, 2015), therefore, this was employed throughout the thesis studies. This is important to understand the context of how training and match demands were analysed when referencing prior research in this sport to give context. It is a limitation with regards to the comparison with prior research, but is now best practice when analysing GPS training and match demands in this population. Crucially we were also aiming to compare the output of the participants in chapters 5, 6, and 7 across the days of the microcycle to validate the view that these athletes are regularly exposed to running demands similar, if not greater than that of match

play but without the full collisions of a game, rather than comparing previously reported workloads or GPS running demands.

8.4.3 Data collection timepoints

The collection of resting metabolism data via indirect calorimetry occurred everyday across the competitive match week, apart from the morning of the match itself in Chapter 4. Ideally RMR would have been measured on the morning of the match day also, as would a sample of blood, urine and saliva during sample collection for Chapters 5 & 6. However, due to the population being elite professional athletes, and the matches included in this research being fully competitive fixtures, this was not consented. The participating athletes felt this disrupted their individual preparation and presented logistical issues with the timing of waking and meal consumption. The challenge of maintaining rigorous standards of data collection for indirect calorimetry make this challenging to obtain a measure of RMR on the day of competition. However, if future work can utilise less invasive biofluids such as urine or saliva for targeted metabolite research, then this collection timepoint may be permissible in this population.

8.4.4 Body composition changes and performance

We have discussed the potential ramifications of the unfavourable changes to body composition that were reported in chapter 7 during the competitive season. However, we do not know whether these changes were meaningful in terms of the physical capacities of the players. Although the gains in fat mass across the season were statistically significant it would have added further application of the results had measures of strength, power or speed been incorporated into the testing at the timepoints.

8.5 Future work

Building upon the findings from this thesis, further research is required to advance our understanding of how best to fulfil the metabolic requirements of recovery from rugby union match play, from a sport science and a nutrition perspective. The research questions that we believe will further our knowledge may be addressed in the following recommendations:

1. **Repeat measures of indirect calorimetry on GD-1, GD+1, GD+2 and GD+3 under outpatient conditions and incorporate new technology to quantify the frequency and intensity of collision activities.** The limitations of GPS technology for measurement and quantification of collision activities have been discussed within this thesis. However, wearable mouthguard technology to measure collisions via the inclusion of accelerometers and gyroscopes within a traditional gumshield worn by rugby athletes is evolving. There are systems now validated with the HitlQ (Hit IQ Pty. Ltd., Melbourne Australia(Stitt et al., 2021) and work being undertaken on the 'Protecht' solution within rugby union and rugby league in England currently. This could both challenge and/or validate our conclusion that the collisions in match play were responsible for the increased metabolic demand in recovery but also begin to determine whether increases in RMR at rest on GD+1 can be correlated to the number and severity of collision experienced by the players, potentially influencing recovery strategies on an individual basis.
2. **Prescribed dietary intake at GD+1 to investigate the effect of 6 $g \cdot kg^{-1}$ vs. 3 $g \cdot kg^{-1}$ carbohydrate upon recovery from elite rugby union match play.** Providing the dietary intake for the period prior to and in the days after the match, keeping

protein and fat intake consistent between groups but manipulating carbohydrate intake would allow investigation as to whether the gluconeogenesis and amino acid oxidation reported at GD+2 could be allayed. The markers of myofibrillar and connective tissue degradation may also be reduced. Due to the congruency we observed between pathways of saliva and serum, a less invasive method for sample collection could be used rather than venepuncture. The use of a targeted metabolomics investigation may mean a mass spectrometry technique could be employed. Potentially easier sample collection and cost-effective targeted techniques could allow more samples to be tested across playing positions and potentially involve more than one team.

3. **Determine whether the energy balance data reported here is representative of the wider population of elite rugby union players.** Repeat measures of body composition throughout the in-season period via DXA scanning and investigate whether the mean per day energy surplus in this elite population is representative in other squads not only in Europe but globally.
4. **Investigate whether the muscle of elite rugby union players demonstrates anabolic resistance in the recovery period from match play.** To determine why losses of lean mass are apparent even when resistance training is undertaken and feeding results in a mean per day energy surplus, methods to assess anabolic resistance could be employed. The use of clamp studies with stable isotope tracers (Millward and Smith, 2019), whilst appropriate in willing participants from the general population, it may not be possible in athletes. The use of these infusions of essential amino acids also not be representative of feeding in our population. These deficits in anabolic signalling are associated with a decreased responsiveness of muscle protein

synthesis (MPS) to essential amino acid (EAA) provision, which is independent of increased insulin, but changes to insulin sensitivity are also present (Cuthbertson et al., 2005). We propose a mixed meal be ingested containing sufficient protein to stimulate MPS maximally, alongside carbohydrate after a resistance training session a GD+2. Control participants could have undergone the same training but without the involvement of the match play component to the days prior. Blood samples would then be drawn at intervals to examine the amino acid profile, glucose, and insulin via mass spectrometry.

Biopsy techniques have been implemented with rugby union players previously (Bradley et al., 2016) and although their use in elite athletes may not be possible, it may be permissible in a group of highly trained players. The 'Combined Oral Stable Isotope Assessment of Muscle' or 'COSIAM' is far more applicable and less invasive than previous clamp techniques (Cegielski et al., 2021). Myofibrillar MPS, whole body muscle protein breakdown and muscle mass can be measured simultaneously using urine, saliva, blood, and muscle samples. However, with only two blood samples and a single ≈30mg muscle biopsy required during the 72hour period of investigation, the reduced burden upon participants may be consented to by athletes.

8.6 Summary

In summary the research undertaken in this thesis provides novel data on resting metabolism, multi biofluid metabolomics, changes to body composition throughout competition, and longitudinal energy balance in English Premiership rugby union players. A summary infographic of the research rationale, major findings, and practical messages is below in figure 8.1.

The significant increases in resting metabolic rate in recovery from match play together with shifts in substrate oxidation were attributed to the collisions inherent with the sport of rugby union. These metabolic requirements of the recovery period, when investigated utilising a whole body, systems-based approach revealed upregulation of pathways pertaining to gluconeogenesis, amino acid degradation, and oxidative stress. The ramifications of which may compromise the integrity of muscle protein and connective tissues. The accumulation of these bouts of match play and recovery, repeated throughout a season, challenge the maintenance of optimal body compositions even when energy needs appear to be met. Nutrition interventions to shift the status quo and increase carbohydrate intakes in the day after competition may provide the necessary substrate for these metabolic requirements to be met. Elite rugby union athletes must 'fuel the work' but also now consider 'fuelling for the damage induced'.

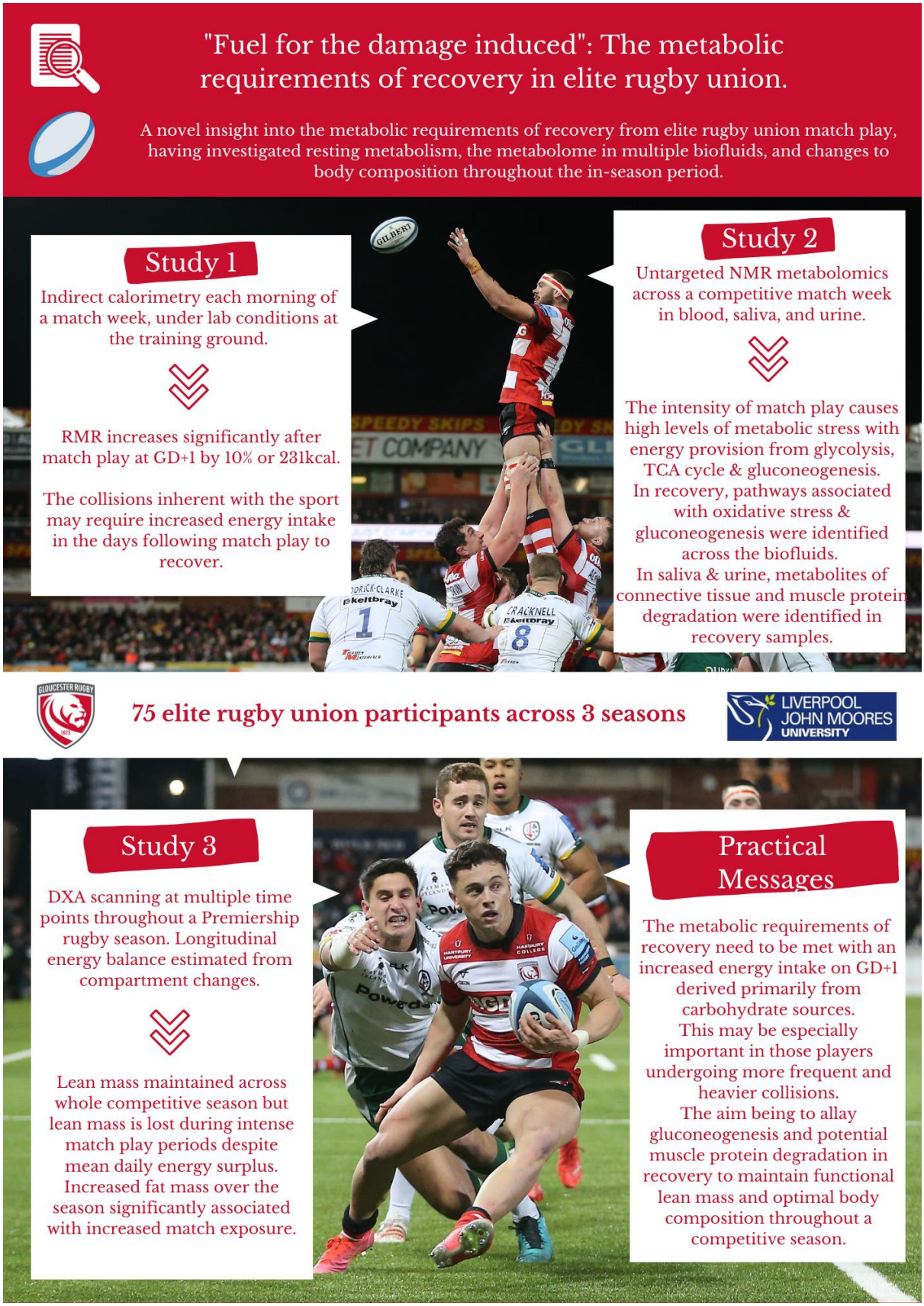


Figure 8.1 Summary infographic of this body of work and the practical messages.

Chapter Nine

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