Can exercise mitigate the negative metabolic effects associated with sleep loss?

This thesis is submitted in fulfilment of the requirements for the degree

Doctor of Philosophy

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Abstract

The increased time-demands imposed on modern society means that many people don't meet nightly sleep recommendations. However, despite the obvious importance of sleep for the maintenance of good health, the effects of sleep loss are remarkably understudied. The consequences of sleep loss on aspects of metabolic health are becoming more apparent, with detrimental changes to glucose tolerance, insulin sensitivity, and an increased risk of numerous metabolic conditions being reported. Comparatively, the underlying mechanisms that lead to these changes are not well characterised, but may include circadian misalignment, changes in mitochondrial function, and inhibition of the molecular signalling pathways that govern protein synthesis. Due to the emergence of these detrimental metabolic changes, interventions that are capable of mitigating these effects (which remain following bouts of 'recovery sleep') should be investigated. Exercise improves glucose tolerance, improves mitochondrial function and is also thought to be able to shift circadian rhythms, making it an ideal candidate to mitigate some of the detrimental effects of sleep loss. Accordingly, the overall aim of this thesis was to investigate the metabolic consequences of sleep loss, and the underlying cellular mechanisms, and to determine the effectiveness of exercise on aspects of metabolic health during a period of sleep loss.

To investigate these aims, 24 healthy young males underwent a sleep study protocol of eight nights in duration (consisting of two nights of controlled baseline sleep, a five-night intervention period, and one night of *ad libitum* recovery sleep). Participants were matched in 3 parallel groups (based on $\dot{V}O_{2peak}$ and mitochondrial respiratory function), with each group undergoing a different intervention. A normal sleep (NS) group spent 8 h time in bed (TIB) each night, a sleep restriction (SR) group spent 4 h TIB each night, and a sleep restriction and exercise group (SR+EX) also spent 4 h TIB but performed three sessions of exercise on the middle three days (at 10:00 am) of the intervention period. High-intensity interval exercise (HIIE) consisting of 10 x 60-s intervals at 90% $\dot{W}_{peak}$ on a cycle ergometer, interspersed with 75 s of active recovery, was used as the exercise intervention. Muscle biopsies were obtained, and oral glucose tolerance tests (OGTT) were performed pre- and post-intervention. Peripheral skin temperature was recorded throughout the study and sleep was assessed via sleep watch actigraphy and polysomnography (PSG) measures.

The first study investigated the effect of sleep restriction and exercise on the markers and regulators of peripheral tissue circadian rhythms. Changes in peripheral skin temperature amplitude and stability, and skeletal muscle clock gene mRNA expression and protein content, were examined. The mRNA expression of the key molecular clock gene, *Bmal1*, was reduced from pre- to post-intervention in the SR group (-29 ± 33%, CI [-6, 64%], $P = 0.031$). This change

was not seen in either the NS group (2 ± 22%, CI [-40, 35%], $P > 0.999$) or the SR+EX group (7 ± 51%, CI [-29, 43%], $P > 0.999$). Analysis of peripheral wrist skin temperature amplitude showed a significant decrease in the amplitude of both the SR (mean ± SD °C, *P* value, 0.85 ± 0.72°C, CI [0.20, 1.45°C], $P = 0.007$) and SR+EX groups (0.80 ± 0.70°C, CI [0.20, 1.54°C], $P = 0.018$), but not the NS group (0.04 ± 0.67°C, CI [-0.59, 0.66°C], $P > 0.999$). Together, these data demonstrate that sleep restriction causes alterations to circadian rhythms as assessed via skeletal muscle clock gene expression and peripheral skin temperature analysis. Further, it suggests that HIIE prevented the changes observed in skeletal muscle *Bmal1* mRNA expression, but not wrist skin temperature amplitude. These results provide evidence linking sleep loss to circadian disruption and elucidates the potential of HIIE in counteracting this. This is of importance given the emergence of circadian misalignment as a contributing factor in the development of different metabolic diseases.

Study 2 investigated potential mechanisms behind sleep-loss-induced reductions in glucose tolerance and the effect of exercise on these changes. Glucose tolerance was assessed via an OGTT pre- and post-intervention and there was an increase in glucose area under the curve (AUC) (mean change, A.U.) in the SR group (149 ± 115 A.U., CI [54, 243 A.U.], $P = 0.002$), which was mitigated by performing HIIE (67 ± 57, CI [-161, 27.7 A.U.], $P = 0.239$). Analysis of skeletal muscle biopsies indicated reductions in mitochondrial respiratory function from pre- to post-intervention ($(ETF+CI+CII)_P$, mean change ± SD pmol $O_2.s^{-1}.mg^{-1}$, 95% CI, *P* value) in the SR group (-15.86 ± 12.41 pmol $O_2.s^{-1}.mg^{-1}$, CI [-26, -6 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.001$), but not the SR+EX group (0.67 ± 11.82 pmol $O_2.s^{-1}.mg^{-1}$, CI [-9, 10 pmol $O_2.s^{-1}.mg^{-1}$], $P = 0.997$). Furthermore, sarcoplasmic fractional synthetic rate (FSR) (FSR %/day ± SD) was lower in the SR group, compared to the NS and SR+EX groups (NS, 1.72 ± 0.18; SR, 1.10 ± 0.25; SR+EX, 1.77 ± 0.22, $P < 0.05$). Differences between the SR and SR+EX groups were also observed for the expression of *Glut4* and *Mfn2* mRNA (both decreased in the SR group), and in p-p38 MAPK protein content (increased in the SR+EX group) ($P < 0.05$). Together, these data demonstrate for the first time a concomitant reduction in aspects of mitochondrial biology (respiratory function, sarcoplasmic protein synthesis, and mitochondrial signalling pathways) and glucose tolerance, while the same decrements were not observed in the SR+EX group. This suggests that performing exercise during a period of sleep restriction can mitigate the detrimental effects on glucose tolerance and mitochondrial function.

The aim of study 3 was to investigate the effect of sleep restriction, with or without HIIE, on the potential mechanisms underpinning previously reported sleep-loss-induced changes to muscle mass. Myofibrillar protein FSR and the molecular signalling pathways that underpin skeletal muscle protein synthesis and degradation were also assessed. Myofibrillar protein FSR (FSR

%/day ± SD) was lower in the SR group, compared to the NS and SR+EX groups (NS, 1.53 ± 0.09; SR, 1.24 ± 0.21; SR+EX, 1.61 ± 0.14, $P < 0.05$). However, no change in the purported regulators of protein synthesis, from skeletal muscle mRNA expression (*Foxo1/3, Mafbx, Murf1* and *myostatin*) or protein content (p-mTOR, p-p70S6K and Caspase-3) were observed from pre- to post-intervention in any of the groups. This data demonstrates that myofibrillar protein synthesis is reduced by sleep restriction, but this is mitigated by performing HIIE. However, there was a dissociation between the molecular markers of protein synthesis and myofibrillar protein synthesis. Given the previously reported reductions in muscle mass following sleep loss, this data provides new insight into the functional mechanisms underpinning this and suggests HIIE may be of therapeutic value to mitigate this loss of muscle mass.

In summary, the data presented in this thesis provides novel information relating to the detrimental metabolic effects caused by a period of sleep restriction. Furthermore, it examines the potential role of HIIE as a therapeutic intervention to counteract these detrimental changes. This data also expands on previous literature by examining the potential mechanisms that may underpin sleep restriction induced alterations to circadian rhythms, glucose tolerance and protein synthesis. Specifically, these data suggest that sleep restriction alters circadian gene expression and that HIIE may help to realign this. Furthermore, a concomitant reduction in glucose tolerance, mitochondrial respiratory function and both myofibrillar and sarcoplasmic protein FSR, is reported following sleep restriction, all of which were mitigated by performing HIIE. This body of work will help to establish evidence-based guidelines to support behavioural changes in those not attaining adequate sleep. Further, there is potential economic and social benefits that can be gained by improving our understanding of the mechanisms causing the detrimental effects of sleep loss and the best methods to counteract them. The findings of this thesis should be further expanded with additional research that investigates how translatable these data are in the context of sleep loss commonly experienced by the general population (i.e., sleep time of ~6 h per night).

Student Declaration

I, Mr Nicholas Saner, declare that the PhD thesis entitled "Can exercise mitigate the negative metabolic effects associated with sleep loss?" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 11/06/2019

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List of Publications, Conferences, Awards, and Grants

Below is a list of publications, conference presentations, awards, and grants that were obtained during the PhD. These result from research conducted both in direct relation to this thesis and also from contributions to additional projects during this time.

Publications

- **Saner N.J**, Bishop, D.J., Bartlett, J.D, "Is exercise a viable therapeutic intervention to mitigate mitochondrial dysfunction and insulin resistance induced by sleep loss?" **Sleep Med Rev**. 2018, 37: 60-68. (IF = 10.60).

- Grgic J., Lazinica B., Garofolini G., Schoenfeld B., **Saner N.J**, Mikulic P., "The effects of time of day-specific resistance training on adaptations in skeletal muscle hypertrophy and muscle strength: a systematic review and meta-analysis", **Chronobiol Int**, 2019, 36(4):449-460, (IF = 3.54).

- Bishop D., Botella J., Genders A., Lee M., **Saner N.J,** Kuang J., Yan X., Granata C. "High-intensity exercise and mitocondrial biogenesis: current controversies and future research directions", **Physiology**, 2019, 34(1):56-70, (IF = 4.86).

- Andrade-Souza, V., Ghiarone, T., Sansonio, A., Silva, K., Tomazini, F., Arcoverde, L., Fyfe, J., **Saner, N**.J, Kuang, J., Bertuzzi, R., Leandro C., Bishop, D., and Lima-Silva, A., "Exercise twice-a-day potentiates skeletal muscle signaling responses associated with mitochondrial biogenesis in humans, which are independent of lowered muscle glycogen content", **bioRxiv doi**: 10.1101/547489 and submitted to FASEB J, April 2019 (IF = 5.5)

- Botella J, **Saner N.J**, Granata C, "Guardian of mitochondrial function: an expanded role of Parkin in skeletal muscle", **J Physiol**, 2018, 596(24): 6139-6140, (IF = 4.74).

- Tan, B., Kwek, J., Wong, C., **Saner, N**.J, Yap, C., Felquer, F., Morris, M., Gardener, D., Rathjen, P., and Rathjen, J., 2016 – "Src family kinases and p38 mitogen-activated protein kinases regulate pluripotent cell differentiation in culture", **PLoS One**. 2016; 11(10): e0163244, (IF = 3.54).

Conference presentations

- **Saner, N**.**J**, Bishop, D.J**.,** Bartlett, J.D. (2018). *Can exercise prevent the negative metabolic effects of sleep loss?* Presented at the 8th ESSA conference, Brisbane, Australia, March 2018 (10-minute oral presentation). **Winner of Young Investigator Award for Exercise and Health.**

- **Saner, N.J**, Bishop, D.J., Bartlett, J.D. (2018). *Exercise as an intervention to mitigate mitochondrial dysfunction and impaired glucose tolerance induced by sleep loss*. Presented at the 65th Annual ACSM conference, Minneapolis, U.S.A, May 2018 (10-minute oral presentation).

- **Saner, N**.**J**, Bishop, D.J**.,** Bartlett, J.D. (2018). *Can exercise prevent the negative metabolic effects of sleep loss?* Presented at the 23rd Annual Congress of the ECSS,

Dublin, Ireland, July 2018. ESSA-ECSS exchange program (Invited Presentation – 10-minute oral).

- Bishop, D.J., **Saner, N.J.**, Bartlett, J.D. (2019). *Sounding the Alarm: Sleep, Clock Genes, and Mitochondria*. Presented by Prof. David Bishop (invited speaker) at the World Congress on the Basic Science of Exercise, Circadian Rhythms and Sleep - ACSM conference 2019, Orlando, U.S.A.

Awards and grants

- Sports Medicine Australia (SMA) Research Grant, 2016 - $2,000.
- Exercise and Sport Science Australia (ESSA) Exercise and Health Young Investigator Award, ESSA 2018. Awarded $4000 and a conference position as an invited presenter to the European College of Sport Science Conference, Dublin, 2018.
- Victoria University, Institute of Sport Exercise and Active Living HDR conference, 2015 Best poster presentation.
- Victoria University, Institute of Sport Exercise and Active Living HDR conference, 2017 1st place student conference presentation award.

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List of Abbreviations

18S	18S ribosomal 5
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ACSM	American College of Sports Medicine
ACTB	Actin β
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
APE	Atomic percentage excess
AS160	Akt substrate of 160 kDa
ATF2	Activating transcription factor 2
AUC	Area under the curve
B2M	β-2-microglobulin
Bmal1	Brain and muscle Arnt-like protein-1
BMI	Body mass index
CAMK	Calmodulin-dependent protein kinase
CCG	Clock controlled genes
Clock	Circadian locomotor output cycles kaput
COX-1	Cytochrome c oxidase subunit 1
CREB	Cyclic-AMP response element binding protein
Cry 1/2	Cryptochrome 1 and 2
CS	Citrate synthase
CSA	Cross-sectional area
CV	Coefficient of variation
D_2O	Deuterium oxide
DEXA	Dual-energy x-ray absorptiometry
Drp1	Dynamin related protein 1
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
EMG	Electromyogram
EOG	Electrooculogram
ETS	Electron transport system
FOXO1/3	Forkhead box-O 1/3
FSR	Fractional synthetic rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-IRMS	Gas chromatography isotope ratio mass spectrometry

GLUT4	glucose transporter 4
GXT	Graded exercise test
HbA1c	glycated hemoglobin
HEC	hyperinsulinaemic-euglycaemic clamp
HIIE	High-intensity interval exercise
HIIT	High-intensity interval training
IL-6	Interleukin-6
IPAQ	International Physical Activity Questionnaire
IRS1	Insulin receptor substrate-1
LC3B	Microtubule-associated protein 1A/1B-light chain 3
LKB-1	Liver kinase B1
MAFBx	Muscle-specific atrophy F box
MEF2	Myocyte enhancer factor 2
MEQ	Morning-Eveningness Questionnaire
MFN1	Mitofusin-1
MFN2	Mitofusin-2
MICT	Moderate-intensity continuous training
MitoPS	Mitochondrial protein synthesis
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
MuRF1	Muscle RING finger-1
MyoPS	Myofibrillar protein synthesis
NAD_p	Nicotinamide adenine dinucleotide
nREM	non-rapid eye movement
NRF1/2	Nuclear respiratory factor 1/2
NS	Normal sleep
OGTT	Oral glucose tolerance test
OSA	Obstructive sleep apnea
p38MAPK	p38 mitogen-activated protein kinase
p62	Ubiquitin binding protein p62
p70S6K	70 kilodalton ribosomal S6 protein kinase
PARK2	Parkin RBR E3 ubiquitin protein ligase
PDK4	Phosphoinositol-dependent kinase-1
Per 1/2/3	Period 1,2, and 3

PGC-1α	Peroxisome proliferator-activated receptor coactivator 1α
Pink1	PTEN-induced kinase 1
PPAR	Peroxisome proliferator-activated receptor
PSG	Polysomnography
REM	Rapid eye movement
Rev-erbα (NR1D1)	Nuclear receptor subfamily 1, group D, member 1
ROR-α	RAR-related orphan receptor a
RPE	Rating of perceived exertion
RT-PCR	Real-time polymerase chain reaction
SarcPS	Sarcoplasmic protein synthesis
SCN	Suprachiasmatic nucleus
SIRT1/3	Sirtuin 1/3
SOL	Sleep onset latency
SR	Sleep restriction
SR+EX	Sleep restriction and exercise
SUIT	Substrate–uncoupler–inhibitor-titration
SWS	Slow wave sleep
T2DM	Type 2 diabetes mellitus
TBP	TATA-box binding protein
TEM	Transmission electron microscopy
Tfam	Mitochondrial transcription factor A
TIB	Time in bed
TNF-α	Tumour necrosis factor alpha
TSC1	Tuberous sclerosis complex 1
TST	Total sleep time
WASO	Wake after sleep onset
$\dot{W}_{Peak}$	Peak power output
β-HAD	β-Hydroxacyl-CoA dehydrogenase
$\dot{V}O_{2peak}$	Peak rate of oxygen uptake

Chapter 1. Thesis Introduction

1.1 Background

The U.S. National Sleep Foundation recommends that adults sleep between seven and nine hours each night (Hirshkowitz, Whiton et al. 2015). Despite this, a large proportion of the population in developed nations do not meet these guidelines. Estimates within Australia suggest that approximately 40% of the population experience inadequate sleep (defined as < 7 h sleep per night) (Sleep Health Foundation 2017), while recent statistics from the U.S. suggest that up to 70 million adults are sleeping less than six hours a night (Ford, Cunningham et al. 2015). This is a concerning trend, as sleep loss has emerged as a risk factor for a number of chronic diseases including obesity, stroke, depression, and cardiovascular disease (Sleep Health Foundation 2017). This research has particularly highlighted the metabolic consequences of sleep loss, with strong links to the development of insulin resistance and Type 2 Diabetes Mellitus (T2DM). As such, reduced sleep duration has now been reported as a comparable risk factor to that of physical inactivity for the development of insulin resistance and Type 2 Diabetes Mellitus (T2DM) (Anothaisintawee, Reutrakul et al. 2015). It is likely that the full extent of the detrimental metabolic effects caused by sleep loss are still to be uncovered.

Despite the clear association between sleep loss and the development of a range of chronic diseases, information regarding the underlying physiological mechanisms that cause these detrimental effects is remarkably limited. In particular, the reduction in glucose tolerance and the subsequent increased risk for developing T2DM with reduced sleep duration is now well-documented in both laboratory and epidemiological studies (Spiegel, Leproult et al. 1999, Anothaisintawee, Reutrakul et al. 2015). However, the mechanisms that underpin these changes are yet to be elucidated. Amongst the proposed mechanisms linked to sleep loss induced reductions in glucose tolerance, is a disruption to the body's regular circadian rhythms (the ~24 h endogenous oscillations of biological processes, driven by the suprachiasmatic nucleus in the hypothalamus and adjusted by the molecular clocks in peripheral tissues, such as skeletal muscle). It's been suggested that disruption of the sleep/wake cycle may cause the misalignment of circadian rhythms, which has been linked to a multitude of detrimental metabolic effects, including reduced glucose tolerance (Wefers, van Moorsel et al. 2018). How a period of sleep restriction influences markers of circadian rhythms in humans requires further investigation.

Mitochondria are dynamic cellular organelles responsible for the production of ATP via oxidative phosphorylation (Bishop, Botella et al. 2019). The development of insulin resistance and reduced glucose tolerance has often been associated with reductions in mitochondrial content and function

(Kelley, He et al. 2002, Lowell and Shulman 2005, Mogensen, Sahlin et al. 2007, Fazakerley, Minard et al. 2018). However, the effect of recurrent sleep restriction on mitochondrial content and function has not been investigated previously and may be a contributing factor for the reported development of sleep loss induced glucose intolerance. Improving our understanding of the mechanisms that regulate these changes will help to inform decisions regarding potential therapeutic interventions that may be able to mitigate or counteract these detrimental effects.

Sleep has also been implicated in the maintenance of muscle mass. The loss of muscle mass can have considerable detrimental effects due to its important roles for mobility and strength (which is highlighted in disease states such as sarcopenia and cachexia), as well as its key metabolic functions (i.e., glucose metabolism). Recent studies have demonstrated that sleep deprivation and sleep restriction may promote the increased loss of muscle mass (Nedeltcheva, Kilkus et al. 2010, Dattilo, Antunes et al. 2012, Monico-Neto, Giampa et al. 2015, de Sa Souza, Antunes et al. 2016). However, the mechanisms underpinning these effects are not well understood and to date, have not been extensively investigated. As the balance between protein synthesis and protein degradation, regulates changes in muscle mass, sleep loss may influence these processes and the molecular signalling pathways that regulate them. Indeed, previous studies investigating the effect of 96 h of sleep deprivation in rats report a decrease in muscle fibre cross sectional area (CSA), with a concomitant increase in molecular markers that are involved in protein degradation signalling pathways (Dattilo, Antunes et al. 2012, Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016). Additionally, recent evidence suggests that this may also be true in humans, with a study reporting increased losses of muscle mass in people sleeping 5.5 hours a night, compared to 8.5 hours a night (for 14 nights) (Nedeltcheva, Kilkus et al. 2010). However, how these results translate in human participants and actually influence the rates of protein synthesis can only be speculated.

Despite the recognition that people commonly experience inadequate sleep, initiatives to improve sleep and to 'sleep more' are not working. By improving the understanding of the underlying mechanisms involved in the detrimental metabolic effects that are associated with sleep loss, targeted therapeutic interventions can be explored as a means to mitigate the effects. Studies investigating the benefits of 'weekend recovery sleep' remain equivocal (Broussard, Wroblewski et al. 2016, Depner, Melanson et al. 2019) and therefore suitable time-efficient interventions that can be incorporated into daily routines may be of importance for modern society. One potential intervention is exercise, which has previously been demonstrated to be highly effective for the treatment and prevention of a range of chronic diseases, supported by the phrase, 'exercise as medicine' (Pedersen and Saltin 2015). In relation to T2DM, it's well documented that even single bouts of exercise are able to improve glucose tolerance and insulin sensitivity (Richter and

Hargreaves 2013), while longer-term exercise interventions and habits have overwhelmingly been shown to lower the risk of developing T2DM and reduce rates of all cause morbidity (Pedersen and Saltin 2015). However, whether exercise is able to mitigate the detrimental effects induced by sleep loss remains to be elucidated. The molecular pathways that are known to regulate the beneficial effects of exercise, may overlap with the mechanisms that are thought to underpin the detrimental effects of sleep loss. Further, exercise has been shown to 'shift' or 'realign' circadian rhythms, and induce increases in mitochondrial content, function, and muscle protein synthesis (Wolff and Esser 2012, Bell, Seguin et al. 2015, Granata, Jamnick et al. 2018). Therefore, exercise may be a potential therapeutic intervention, which can be targeted at the mechanisms that cause the detrimental effects of sleep loss.

1.2 Aims and objectives

The overall aim of this thesis was to investigate the potential mechanisms underlying some of the detrimental metabolic effects that have been associated with sleep loss and accordingly to determine the potential role of exercise as a therapeutic intervention to mitigate these effects. As such, it is hoped that the knowledge generated from this thesis will contribute to the development of evidence-based guidelines and recommendations for the many people who commonly experience inadequate sleep and to potentially support the use of exercise as a targeted therapeutic intervention to mitigate the detrimental effects of sleep loss.

The aims were achieved by investigating the following questions:

1. Does sleep restriction disrupt circadian rhythms and can exercise mitigate this effect? (Chapter 4).

2. What are some of the potential mechanisms underlying the reductions in glucose tolerance that are induced by sleep restriction? Further, is exercise a potential therapeutic intervention to prevent these changes? (Chapter 5).

3. Can changes in myofibrillar protein synthesis explain, in part, the mechanisms underpinning the previously demonstrated loss of muscle mass following sleep restriction? Further, can three sessions of HIIE prevent any potential changes? (Chapter 6)

Chapter 2. Literature Review

The information within this chapter has been published in manuscript form in Sleep Medicine Reviews (Appendix Error! Reference source not found.). Given the emergence of new data since publication of this review, specific content has been updated and adapted for the presentation and thesis:

Saner N. J, Bishop D. J, Bartlett, J. D. Is exercise a viable therapeutic intervention to mitigate mitochondrial dysfunction and insulin resistance induced by sleep-loss? Sleep Med Rev, 37: 60-68, 2018

2.1 Summary

Sleep loss has emerged as a risk factor comparable to that of physical inactivity for the development of insulin resistance, impaired glucose tolerance and Type 2 Diabetes Mellitus. This is a concern as it was estimated in 2012 that approximately 70 million adults in the United States are sleeping less than 6 hours each night, and the average nightly sleep duration of a representative sample of the U.S adult population is reported to be significantly less than in previous decades. The underlying mechanisms responsible for chronic sleep loss induced insulin resistance include modifications in the regulation of hormone secretion, peripheral clock gene regulation, and the cellular signalling processes associated with regulating mitochondrial respiratory function. Emerging evidence shows these mechanisms share similar biochemical signalling pathways to those underpinning exercise-induced adaptations, which together suggest exercise might be a viable, suitable, and potent treatment alternative to alleviate sleep loss induced insulin resistance and glucose intolerance. In this review, a summary of the impact of reduced sleep duration and quality on mitochondrial function and insulin resistance is provided, before detailing the possible underlying mechanisms. Finally, it is proposed how and why regular exercise may be a therapeutic intervention to mitigate sleep loss induced mitochondrial dysfunction and insulin resistance.

2.2 Introduction

The prevalence of type 2 diabetes mellitus (T2DM) continues to rise in modern society. The World Health Organization (WHO) estimates that by the year 2025, 300 million people across the world will be diagnosed with diabetes (World Health Organization 1998). To give this additional context, T2DM was the 7th leading cause of death in the United States in 2012 and costs their economy an estimated $245 billion each year (Centers for Disease Control and Prevention 2014). Typically, the onset of T2DM coincides with impaired glucose tolerance (characterized by a fasting blood glucose level >7 mmol/L) and increased insulin resistance (reduced efficiency of insulin-stimulated glucose uptake) (National Collaborating Centre for Chronic Conditions 2008). Furthermore, the health consequences arising from diabetic complications (such as heart disease, stroke and kidney damage) add to the already extraordinary burden of this metabolic disease on health services (Centers for Disease Control and Prevention 2014).

The most common risk factors associated with the onset of insulin resistance and glucose intolerance include genetic predisposition and lifestyle factors such as diet and physical inactivity (Anothaisintawee, Reutrakul et al. 2015). However, in the last 15 years sleep has emerged as a prominent factor influencing the development of T2DM. In fact, sleep loss and reduced sleep quality are now reported to be comparable to physical inactivity (Anothaisintawee, Reutrakul et al. 2015) in terms of their relative contribution to the risk of developing T2DM. The percentage of sleep deficient people who develop insulin resistance and T2DM is difficult to determine with the available data. Nonetheless, studies investigating shift workers (a cohort often associated with insufficient sleep and reduced sleep quality, (Akerstedt 2003)) indicate a significantly increased risk (40%) of developing diabetes compared to those performing day work (Anothaisintawee, Reutrakul et al. 2015). Considering the significant reduction in average nightly sleep duration compared to previous decades (Ford, Cunningham et al. 2015), and that it was recently estimated via self-reported measures that ~40% of the population are sleeping less than the recommended 7 hours per night (National Sleep Foundation 2013), the role of sleep loss in contributing to the already high prevalence of T2DM is an important area for further investigation.

Potential mechanisms by which sleep loss may contribute to insulin resistance and glucose intolerance have been identified, and include modifications to the regulation of hormone secretion (Spiegel, Leproult et al. 1999), misalignment of the molecular clock (i.e., circadian misalignment) (Cedernaes, Osler et al. 2015), and disruptions to cellular signalling processes (such as those involved in the insulin signalling pathway and the pathways associated with mitochondrial respiratory function) (Vondra, Brodan et al. 1981, Broussard, Ehrmann et al. 2012, Sweeney, Jeromson et al. 2017). If sleep loss affects signalling pathways related to mitochondrial

respiratory function, then this has important implications as mitochondrial dysfunction has been associated with increased insulin resistance and T2DM (Lowell and Shulman 2005, Mogensen, Sahlin et al. 2007). The gold standard for the assessment of mitochondrial respiratory function is the measurement of oxygen utilization in isolated mitochondria or permeabilised tissue, and indicates the ability of mitochondria to generate ATP via oxidative phosphorylation (Bishop, Granata et al. 2014). Using this method, it has been reported that mitochondrial respiratory function is significantly reduced in the skeletal muscle of T2DM patients compared with obese non-diabetic subjects (Mogensen, Sahlin et al. 2007). Furthermore, this decrease in mitochondrial respiratory function was negatively associated with HbA1c results (glycated haemoglobin - a test that reflects blood glucose levels of the previous 3 months) (Mogensen, Sahlin et al. 2007). However, the effects of sleep loss on mitochondrial function are not well characterized.

Many of the aforementioned mechanisms that are potentially disrupted with sleep loss, particularly insulin signalling and mitochondrial function, share similar biochemical signalling pathways to those connected with exercise (an area of research that is comparably well studied). This suggests exercise as a potential intervention to counteract some of the negative effects associated with sleep loss. For example, single (Bartlett, Hwa Joo et al. 2012) and multiple (Perry, Lally et al. 2010) bouts of exercise induce a cascade of cellular signalling events that lead to improvements in mitochondrial function and insulin sensitivity (Meex, Schrauwen-Hinderling et al. 2010). Exercise has long been considered the cornerstone for the treatment and prevention of T2DM, with reports that those who regularly engage in moderate physical activity (even brisk walking) have approximately a 30% lower risk of developing T2DM compared to sedentary individuals (Jeon, Lokken et al. 2007). However, limited research has investigated the ability of exercise to counteract the negative effects of sleep loss on glucose tolerance and insulin resistance (VanHelder, Symons et al. 1993). Nevertheless, it is plausible that by increasing basal levels of exercise some of the detrimental effects associated with sleep loss might be mitigated.

The aim of this review is to draw on the available literature and propose exercise as a viable strategy to alleviate the negative effects of sleep loss. In doing so this review contextualises the effect of reduced sleep duration and quality on insulin resistance, glucose tolerance, and T2DM, along with the potential underlying mechanisms, while demonstrating how and why engaging in regular exercise may act as a therapeutic intervention to mitigate sleep loss induced insulin resistance and T2DM.

2.3 Sleep loss and health

The U.S-based National Sleep Foundation recommends adults between the age of 18 and 64 sleep 7 to 9 hours each night (National Sleep Foundation 2013). Despite this approximately 70 million

Americans (30% of the population) sleep less than 6 h per night - significantly less per night than that reported in 1985 (22%), based on a large representative sample of the U.S. adult population. This reduction in sleep has been attributed to a range of lifestyle factors and work pressures, including shift work, increased work demands, changing social pressures and roles, increased travel and the consequent jet lag, sleep disorders, and stress (Sleep Health Foundation 2011). Changes in sleep habits have important consequences and have been linked to increased absenteeism, disability, industrial and motor vehicle accidents, increased alcohol consumption, risk of cardiovascular disease, and possibly increased mental health issues (Banks and Dinges 2007, Perlis, Grandner et al. 2016).

Recent epidemiological and laboratory-based studies have also associated sleep loss with a range of chronic health concerns, particularly in relation to insulin resistance and T2DM (Banks and Dinges 2007, Cappuccio, D'Elia et al. 2010, Anothaisintawee, Reutrakul et al. 2015). In humans, a 40% reduction in glucose tolerance and a 30% reduction in the acute insulin response following a glucose tolerance test (measurement of glucose and insulin levels following oral glucose administration) has been reported after 6 nights of 4 h of time in bed (TIB) per night (Spiegel, Leproult et al. 1999). More recently, it was reported that just one night of 4 h of sleep time reduces insulin sensitivity in healthy human populations (Donga, van Dijk et al. 2010, Cedernaes, Lampola et al. 2015, van den Berg, Mook-Kanamori et al. 2016), with many other studies consistently reporting sleep loss induced insulin resistance (Donga, van Dijk et al. 2010, Broussard, Ehrmann et al. 2012, Robertson, Russell-Jones et al. 2013, Cedernaes, Lampola et al. 2015, Rao, Neylan et al. 2015, van den Berg, Mook-Kanamori et al. 2016). Thus, evidence from both epidemiological studies and intervention studies support a key role for sleep in the regulation of glucose tolerance and insulin resistance.

2.4 Sleep loss, circadian misalignment, and mitochondrial function

One proposed mechanism contributing to sleep loss induced changes in glucose tolerance and insulin resistance is circadian misalignment, a condition in which the exogenous behavioural sleep/wake schedule and feeding schedule are not aligned with endogenously generated circadian rhythms (Leproult, Holmback et al. 2014). These circadian rhythms include changes in hormone secretion, body temperature, heart rate, muscle tone, and substrate utilization/metabolism, which persist throughout a 24-h period under constant conditions (i.e., without environmental/exogenous time cues) (Dunlap 2004, Colten 2006). Circadian rhythms are regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, and the molecular clocks of peripheral tissues (such as skeletal muscle) acting synchronously as biological timekeepers. Altering the sleep/wake cycle (i.e., reduced sleep duration) may also independently alter the central and peripheral molecular clocks of human skeletal muscle (Cedernaes, Osler et al. 2015)

such that they become desynchronized with exogenous stimuli – a notion supported by rodent studies (Antle and Mistlberger 2000, Yamazaki, Numano et al. 2000). As over 800 genes are regulated in a circadian manner within skeletal muscle of mice (McCarthy, Andrews et al. 2007), sleep loss induced disruption of the molecular clocks that regulate the expression of these genes may have far-reaching effects, particularly in regard to normal cellular function and health. As such, the misalignment of both the central and peripheral clocks by exogenous stimuli has been proposed as another potential mechanism contributing to sleep loss induced insulin resistance and impaired glucose tolerance (Andrews, Zhang et al. 2010, Dyar, Ciciliot et al. 2014, Leproult, Holmback et al. 2014, Cedernaes, Osler et al. 2015).

Shift workers who undertake variable rotating day and night shifts have an increased risk of circadian misalignment (Nagano, Adachi et al. 2003). These workers experience reduced sleep durations, a constantly changing light/dark cycle, altered sleep/wake patterns, and mixed feeding patterns, which together can contribute to circadian misalignment (Nagano, Adachi et al. 2003, Wolff and Esser 2012). Of significance, these changes in daily behaviours are also associated with a 40% increase in the risk of developing diabetes (Anothaisintawee, Reutrakul et al. 2015). Indeed, a longitudinal study (conducted between 1991 – 2001) of Japanese alternating shift workers concluded that, compared to day shift workers, alternating shift work was an independent risk factor for the onset of T2DM (Suwazono, Sakata et al. 2006). Importantly, in both human and mouse models, exercise has been shown to be capable of causing a phase shift (i.e., advance or delay the circadian phase depending on the time of day that the exercise occurs) (Wolff and Esser 2012, Richardson, Gradisar et al. 2016), and has thus been suggested as a potential intervention to help realign circadian and diurnal rhythms in those with sleep issues (Richardson, Gradisar et al. 2016). Linked to phase shifts of endogenously-regulated circadian rhythms, exercise has also been shown to cause shifts of the peripheral molecular clock in skeletal muscle (both rodent and human), thus highlighting the potential role for exercise to realign disrupted metabolic rhythms to their optimal state (Zambon, McDearmon et al. 2003, Wolff and Esser 2012).

Emerging evidence demonstrates that skeletal muscle function, and specifically that of mitochondrial respiration, also fluctuates rhythmically throughout the day. When mitochondrial respiratory function was measured in human skeletal muscle across a 24-h period, ADP-stimulated mitochondrial respiration oscillated in a “robust day/night rhythm”, with a difference of approximately 20% between the highest (11 PM) and lowest (1 PM) values (van Moorsel, Hansen et al. 2016). In mice, the expression of key rate-limiting mitochondrial enzymes (i.e., pyruvate dehydrogenase and carnitine palymitoyl transferase) and ~38% of the mitochondrial proteome, which are linked to the regulation of glucose tolerance and insulin resistance, also

display a diurnal rhythm (Neufeld-Cohen, Robles et al. 2016). Similar to circadian rhythms, a diurnal rhythm follows a distinct 24-h cycle, but is synchronized to exogenous stimuli such as day and night and other factors such as the timing of meals (Scheer 2011). Therefore, it is possible that misalignment of these diurnal rhythms is detrimental to mitochondrial function, which would have further implications for glucose tolerance and insulin resistance.

As mitochondrial function is regulated in a diurnal manner, and sleep loss can lead to circadian misalignment, it is plausible that sleep loss may alter mitochondrial function - even if this remains to be fully elucidated in well-controlled laboratory studies. It has been reported that 120 h of sustained wakefulness (i.e., sleep deprivation) reduces the activity of citrate synthase (CS) (24%), malate dehydrogenase (35%), and glycerol-3-phosphate dehydrogenase (17%) in human skeletal muscle (Vondra, Brodan et al. 1981), collectively suggesting a decreased functional capacity of the mitochondria. More recently, 72-h of sleep deprivation was associated with reduced mitochondrial respiratory function in the hypothalamus of rats (Andreazza, Andersen et al. 2010). Whilst these studies indicate that mitochondrial function may be directly reduced as a consequence of severe sleep loss, these initial findings have not been characterized in models that replicate the sleep loss encountered by humans. Nonetheless, the implications are significant considering the proposed contribution of mitochondrial dysfunction to the development of insulin resistance and T2DM (Lowell and Shulman 2005, Mogensen, Sahlin et al. 2007). Although the relationship between mitochondrial dysfunction and insulin resistance is regularly debated, and some suggest insulin resistance may itself cause mitochondrial dysfunction, there is undoubtedly a relationship between the two factors (Mogensen, Sahlin et al. 2007, Fazakerley, Minard et al. 2018). It may therefore be hypothesized that chronic sleep loss, such as that experienced by at least 30% of the American population, leads to defects in mitochondrial function and a consequent increase in the risk of developing insulin resistance and T2DM (Koliaki and Roden 2016). Strategies to improve mitochondrial function, such as exercise (Bishop, Granata et al. 2014), might be useful to combat some of the negative consequences of sleep loss.

2.5 Mechanistic pathways connecting sleep loss, misalignment of circadian rhythms, and the potential benefits of exercise

At a molecular level, circadian rhythms are controlled by a number of genes - collectively known as clock genes. A transcriptional: translational feedback loop, which includes the core clock genes *Bmal1*, *Clock* and *Ror-α* (often referred to as the activators of the feedback loop), and *Per 1-3*, *Cry 1/2* and *Rev-erb-α* (considered the repressors of the feedback loop), together coordinate circadian rhythmicity and metabolism at a cellular level via their ability to regulate the transcriptional activity of a host of other genes (clock controlled genes – CCGs) (see (Harfmann,

Schroder et al. 2015) for review). Of significant note, the mRNA expression in human skeletal muscle of two of these clock genes (*Bmal1* and *Cry1*) is decreased following a night of simulated shift work (one night of wakefulness) (Cedernaes, Osler et al. 2015).

Central to metabolic health and circadian regulation is the energy sensor, AMP-activated protein kinase (AMPK). AMPK is activated upon changes in the AMP:ATP ratio, is important in the regulation of mitochondrial biogenesis, and interacts closely with clock genes. Activation of AMPK causes the phosphorylation and destabilization of *Cry1*, leading to de-repression of the BMAL1:CLOCK complex (Lamia, Sachdeva et al. 2009). Moreover, REV-ERB-α (a regulator of the clock gene transcriptional feedback loop and mitochondrial biogenesis) (Woldt, Sebti et al. 2013) regulates AMPK (Duez and Staels 2009) via LKB1 - a protein responsible for upstream regulation of AMPK. It has also been suggested that clock genes within skeletal muscle (i.e., *Per1*, *Cry2*) are regulated by AMPKγ3 (Vieira, Nilsson et al. 2008) - an AMPK isoform specific to skeletal muscle and essential for the regulation of glucose tolerance and insulin sensitivity (Barnes, Marklund et al. 2004). Exercise also activates and phosphorylates AMPK (Bartlett, Hwa Joo et al. 2012), which subsequently plays a role in GLUT4 (glucose transporter 4) mediated skeletal muscle glucose uptake (Hayashi, Hirshman et al. 1998). Furthermore, muscle-specific *Bmal1* knockout mice have decreased skeletal muscle glucose uptake through decreased GLUT4 translocation (Dyar, Ciciliot et al. 2014), which may be due to reduced expression of *Tbc1d1* - a Rab-GTPase member involved in the GLUT4 translocation process and a potential target of the CLOCK:BMAL1 complex (Kumaki, Ukai-Tadenuma et al. 2008). Together, this suggests exercise-induced AMPK activation might help counteract impairments in insulin resistance and glucose tolerance that may be associated with abnormalities in clock gene expression.

Downstream of AMPK is the transcriptional co-activator peroxisome proliferator-activated receptor γ (PGC-1α) (Jager, Handschin et al. 2007), which is also upregulated in response to exercise (Bartlett, Hwa Joo et al. 2012). PGC-1α is often referred to as the 'master regulator' of mitochondrial biogenesis, due to its role in the transcriptional co-activation of a number of transcription factors (NRF 1/2, TFAM and PPARs) involved in the regulation of mitochondrial respiratory function and content (Perry, Lally et al. 2010). Importantly, PGC-1α also regulates the expression of, and is in itself regulated by interactions with, members of the clock gene family (as demonstrated in a number of rodent studies) (Liu, Li et al. 2007, Andrews, Zhang et al. 2010, Pastore and Hood 2013). For example, PGC-1α knockout mice models demonstrate reduced clock gene expression, while *Bmal1* knockout mice models and mice with a mutated CLOCK protein (truncated $CLOCK^{\Delta 19}$ protein) display reduced PGC-1α protein content (Liu, Li et al. 2007, Andrews, Zhang et al. 2010, Pastore and Hood 2013). Decreases in PGC-1α protein content may

contribute to the reductions in mitochondrial content and respiration that are observed in *Bmal1* knockout mice and CLOCK$^{\Delta 19}$ mutant mice (Andrews, Zhang et al. 2010, Pastore and Hood 2013). These same clock gene mutant mice have also been shown to have reduced insulin sensitivity, and the early development of T2DM (Rudic, McNamara et al. 2004, Turek, Joshu et al. 2005, Lefta, Wolff et al. 2011, Harfmann, Schroder et al. 2015), which may be explained in part by the close association between mitochondrial dysfunction and the development of insulin resistance and T2DM (Lowell and Shulman 2005, Mogensen, Sahlin et al. 2007, Montgomery and Turner 2015, Koliaki and Roden 2016). These findings suggest that reductions in the protein content and expression of clock genes and PGC-1α may help to explain the reported effects of sleep loss on insulin sensitivity and T2DM. Importantly, both single and multiple bouts of exercise in humans increase PGC-1α expression (Bartlett, Hwa Joo et al. 2012, Edge, Mundel et al. 2015) and mitochondrial function (Bishop, Thomas et al. 2010, Granata, Oliveira et al. 2016), with subsequent improvements in insulin sensitivity (Meex, Schrauwen-Hinderling et al. 2010). Thus, there is emerging evidence suggesting exercise might be a viable strategy to counteract the effects of sleep loss on clock gene expression, PGC-1α expression, reduced mitochondrial function, and subsequently increased insulin resistance.

While the role of PGC-1α as a key metabolic regulator is well established, it is also important to note its role may be determined by factors such as tissue specificity and its level of expression (Benton, Wright et al. 2008, Liang, Balas et al. 2009). Hepatic overexpression of PGC-1α in mice induces hepatic insulin resistance, potentially via increased stimulation of gluconeogenesis, leading to hyperglycaemia (Liang, Balas et al. 2009). However, increased expression of PGC-1α within physiological levels (as seen with exercise), improves mitochondrial content, GLUT4 expression, and insulin sensitivity in skeletal muscle (Benton, Wright et al. 2008).

Another key regulator of mitochondrial function, which can also be classified as a clock-controlled protein, is SIRT3 (a member of the sirtuin family) (Peek, Affinati et al. 2013). SIRT3 is a mitochondrial protein that controls the acetylation levels of key functional oxidative metabolism and fatty acid oxidation proteins (i.e., long-chain acyl dehydrogenase), and thus can influence the overall respiratory function of the mitochondria (Peek, Affinati et al. 2013). Knockout of SIRT3 in mice causes a reduction in the expression of mitochondria related genes that are induced by PGC-1α (Kong, Wang et al. 2010). Using liver-specific *Bmal1* knockout mice, it was reported that mitochondrial respiration in the liver could be influenced in a circadian manner via clock gene dependent regulation of nicotinamide adenine dinucleotide (NAD^+ - a mitochondrial co-enzyme), which in turn controls the deacetylase activity of SIRT3 (Peek, Affinati et al. 2013). Another member of the sirtuin family, SIRT1 – a regulator of insulin

sensitivity in mouse skeletal muscle, also appears to be activated by CLOCK and BMAL1, with mutation and knockout of these clock proteins, respectively, leading to the induction of insulin resistance (Liu, Zhou et al. 2016). This indicates the dysregulation of clock genes, suggested to occur in response to one night of wakefulness (simulated shift work) (Cedernaes, Osler et al. 2015), may also lead to increased insulin resistance via a reduced content of SIRT proteins.

Of note, and similar to both AMPK and PGC-1α, it has been shown that SIRT1 and SIRT3 can be upregulated by exercise in the skeletal muscle of both animals (Suwa, Nakano et al. 2008, Palacios, Carmona et al. 2009) and humans (Gurd, Perry et al. 2010). Elevated SIRT1 activity has been shown to be important in the subsequent deacetylation and induction of PGC-1α transcriptional activity (Gurd 2011). Together, this demonstrates that both clock genes and exercise influence many regulators of mitochondrial biogenesis. This might help to explain why there is a diurnal rhythm to mitochondrial function and enzyme activity, why these may be affected by sleep loss, and equally why exercise may play a role in mitigating sleep loss induced insulin resistance. The connection between molecular clock proteins (BMAL1, CLOCK, PER1/2/3, and CRY1/2), AMPK, PGC-1α, SIRT1 and SIRT3 is summarized in **Figure 2.1**.

2.6 Exercise increases mitochondrial signalling and clock gene expression

Exercise induced increases in the activation of AMPK, the protein content of PGC-1α, and activity of SIRT1 and SIRT3, are important signalling events that coordinate the expression of mitochondria-related genes (leading to increased mitochondrial content and function) as well as improvements in insulin sensitivity (Puigserver, Wu et al. 1998, Leick, Fentz et al. 2010). Among the genes that PGC-1α increases the expression of, are the mitochondrial enzymes that have been reported to have diminished activity following sleep loss in humans. For example, CS activity (a key enzyme of the citric acid cycle and a marker of mitochondrial content) is significantly reduced in human skeletal muscle following 120 h of sustained wakefulness (Vondra, Brodan et al. 1981). Importantly, skeletal muscle CS mRNA and PGC-1α mRNA is increased following a single bout of high-intensity interval exercise (HIIE), with subsequent increases in CS activity and PGC-1α content (Perry, Lally et al. 2010). Moreover, activity of the same complexes in the electron transport system that are reduced following sleep deprivation in rats (Andreazza, Andersen et al. 2010) can be improved via exercise training (Granata, Oliveira et al. 2016). Therefore, exercise can promote increased activity and content of mitochondrial enzymes that are reduced following sleep loss and which have been linked to insulin resistance and T2DM.

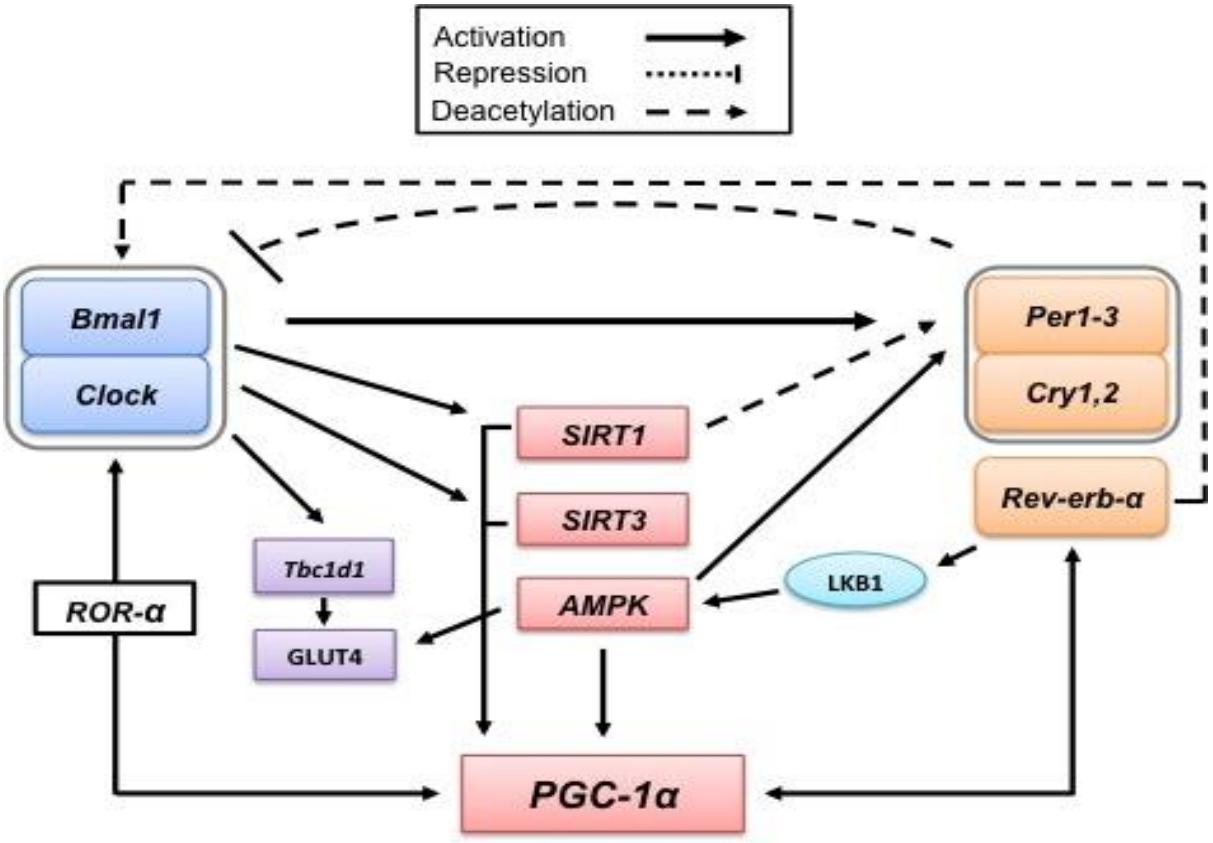

Figure 2.1 - Interplay between peripheral molecular clock proteins, AMPK, SIRT1, SIRT3 and PGC-1α.
BMAL1 and CLOCK activate PER1-3 and CRY1,2. PER1-3 and CRY1,2 transcriptionally repress the action of BMAL1 and CLOCK. AMPK phosphorylates CRY1,2 leading to less repression of BMAL1 and CLOCK, whilst PGC-1α transcriptionally regulates and is itself regulated by REV-ERB-α. Rev-erb-α also represses the effects of BMAL1. PGC-1α regulates Clock through a ROR-α dependent pathway and is regulated also by the CLOCK:BMAL1 complex. CLOCK:BMAL1 can activate both SIRT1 and SIRT3 whereby SIRT1 deacetylates Per2, whilst SIRT1 and SIRT3 upregulates transcription of PGC-1α. Furthermore, BMAL1:CLOCK directly exerts its influence on Tbc1d1 which is required for GLUT4 translocation. AMPK also regulates GLUT4 translocation to the plasma membrane. Rev-erb-α activates AMPK via LKB-1, which directly upregulates transcription of PGC-1α.

Despite our increased understanding of mitochondrial signalling, and models of sleep disruption and circadian misalignment, there are a number of additional exercise-induced signalling pathways associated with improved mitochondrial function that have yet to be investigated in response to sleep loss. In humans muscle contraction leads to the activation of kinases such as calmodulin-dependent protein kinase (CAMK) and p38 mitogen activated protein kinase (p38MAPK) (in addition to AMPK), via changes in calcium and reactive oxygen species, respectively, which converge on myocyte enhancer factor 2 (MEF-2), activating transcription factor 2 (ATF2) and cyclic-AMP response element binding protein (CREB), and exert their influence on enhancing PGC-1α promoter activity (Egan, Carson et al. 2010). In addition, Perry et al. (16) demonstrated that in humans, exercise training modulates mitochondrial fission and fusion proteins that are important for regulating mitochondrial integrity and turnover (via a process known as mitochondrial dynamics). Together, the fission and fusion proteins, along with

mitophagy proteins are involved in a process that defines the overall mitochondrial network, morphology and subsequent function of mitochondria. The dysregulation of these regulatory processes has previously been linked to the development of T2DM (Holstrom et al 2012, Liu et al. 2014). Exercise training has been shown to decrease the activity of the key fission proteins dynamin-related protein 1 (DRP1) and mitochondrial fission 1 (FIS1), with corresponding improvements in insulin sensitivity in insulin-resistant adult skeletal muscle (Fealy et al. 2014 and Axelrod et al. 2018). Moreover, we have reported in human skeletal muscle that single (Bartlett, Hwa Joo et al. 2012) and multiple (Edge, Mundel et al. 2015) bouts of exercise increase phosphorylation and protein levels of the tumour suppressor protein, p53. Ablation of p53 content in mice results in reduced mitochondrial respiration, lowered PGC-1α content and decreased exercise capacity (Saleem, Adhihetty et al. 2009), whilst p53 has also been shown to regulate insulin resistance (Kung and Murphy 2016). Collectively, these pathways represent varying avenues for future research in the context of sleep loss, exercise, mitochondrial function and insulin resistance.

The detrimental mitochondrial characteristics previously demonstrated in clock gene knockout mice can also be somewhat ameliorated by exercise (Pastore and Hood 2013). Following an 8-week exercise training program, mice with mutated $CLOCK^{\Delta 19}$ proteins were able to restore PGC-1α protein content to that of trained wild-type mice (Pastore and Hood 2013). This was accompanied by similar increases in COX-I activity (another marker of mitochondrial content (Larsen, Nielsen et al. 2012)). Moreover, a single bout of resistance exercise is associated with increased expression of *Per2*, *Cry1* and *Bmal1* in human skeletal muscle, compared with resting muscle (Zambon, McDearmon et al. 2003). Together, these associations between clock controlled genes, PGC-1α, and mitochondrial dysfunction (Liu, Li et al. 2007, Andrews, Zhang et al. 2010) provide a theoretical mechanism by which exercise-induced contractile activity may play a role in ameliorating sleep loss induced disruptions of clock genes following a night of wakefulness (simulated shift work) ((Cedernaes, Osler et al. 2015) and hence mitigate consequent impairments in mitochondrial function and insulin sensitivity. That said, future research should focus on uncovering the specific relationships and underpinning mechanisms between sleep loss, changes in skeletal muscle mitochondrial function, and exercise. A schematic overview summarizing the hypothesized connection between sleep, exercise and insulin resistance is shown in **Figure 2.2**.

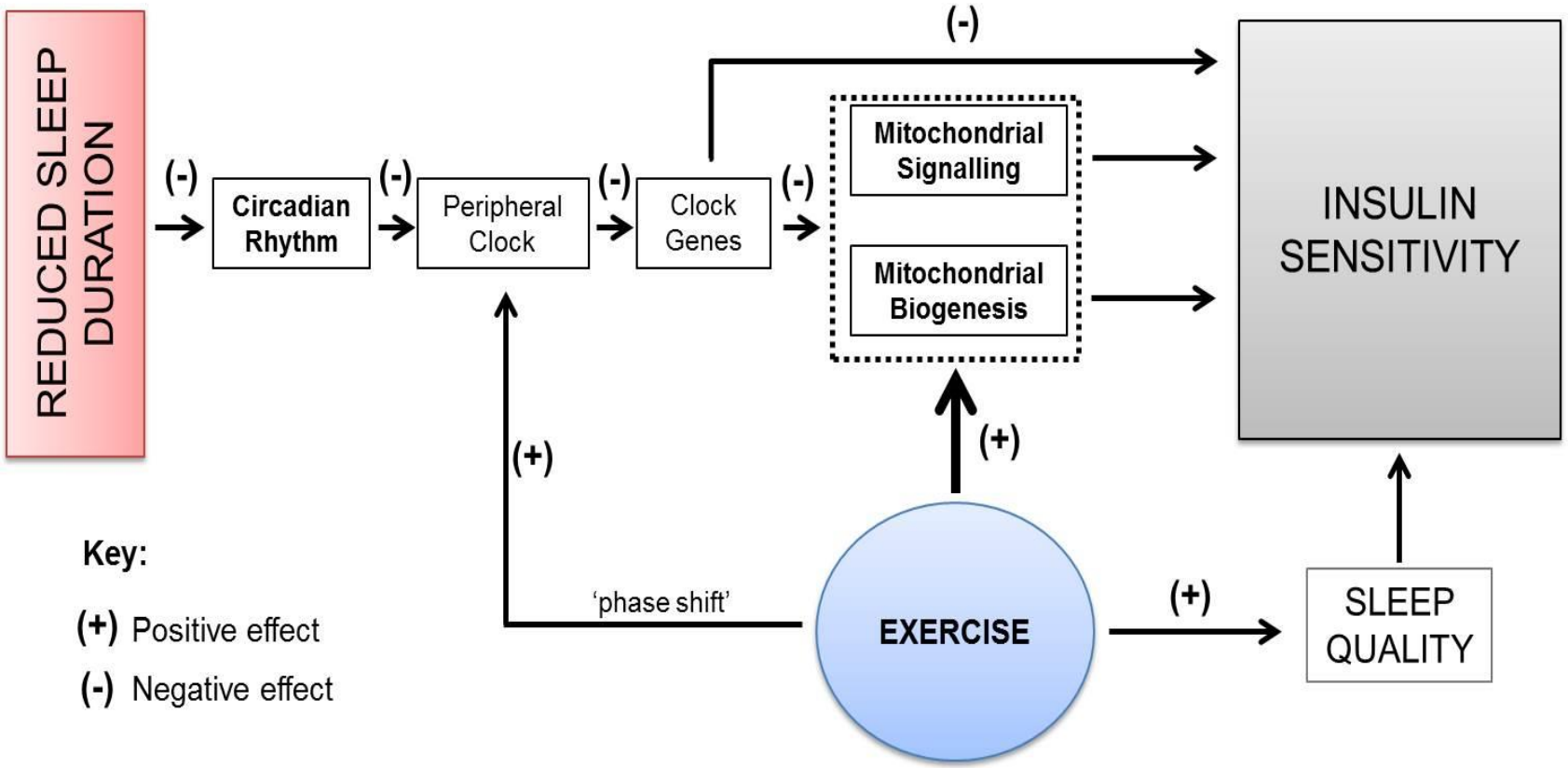

Figure 2.2 - One of the main proposed models, based on current literature, of the impact of sleep loss with or without exercise on mitochondrial signalling, mitochondrial biogenesis, and insulin sensitivity.
Reduced sleep duration disrupts circadian rhythm and alters molecular clock gene expression, which subsequently impacts on mitochondrial signalling events associated with mitochondrial biogenesis and results in a reduction in insulin sensitivity. Conversely, exercise activates the mitochondrial signalling events associated with mitochondrial biogenesis and, therefore, may be a viable strategy to mitigate sleep loss induced reductions in insulin sensitivity. Furthermore, exercise may induce a phase shift and thus a 'resyncing' of the peripheral clock. While reduced sleep quality also induces reductions in insulin sensitivity, exercise induced improvements in sleep quality may mitigate these response.

2.7 The role of sleep on muscle mass and insulin resistance

As skeletal muscle is the major site for glucose disposal, it is conceivable that maintenance of muscle mass and function is also important for maintaining glucose tolerance and insulin sensitivity. In this context, it has been reported that sleep loss can lead to a decrease in muscle mass – likely due to an increased catabolic and reduced anabolic hormone profile (Dattilo, Antunes et al. 2012, Bell, Kilkus et al. 2013, Monico-Neto, Antunes et al. 2015). Moreover, while on a calorie restricted diet, curtailing sleep opportunities (i.e., sleep restriction) to 5.5 h a night for 14 consecutive nights increased the fraction of muscle mass lost compared to participants who had an 8.5 h sleep opportunity each night (Nedeltcheva, Kilkus et al. 2010). It was speculated that an increased conversion of protein to glucose to support the prolonged metabolic requirements in the sleep restricted state may explain the results. Further, 96 h of sleep deprivation in rats resulted in reduced muscle fibre cross-sectional area (CSA), which was attenuated in rats that had performed resistance-based exercise prior to sleep deprivation (Monico-Neto et al. 2015). Increases in catabolic hormones (such as cortisol) and a decrease in anabolic hormones (such as testosterone and IGF-1) were suggested as possible mechanisms to explain these changes. Furthermore, these changes in skeletal muscle CSA coincided with an increase in autophagy and protein degradation related proteins, LC3-1, p62/SQSTM1 and FOXO3 in the sleep-deprived rats.

Muscle mass is regulated via the net balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Rennie, Edwards et al. 1982). In previously reported reductions of muscle mass (i.e., step reduction and limb immobilisation models) a decrease in MPS, rather than an increase in MPB is acknowledged as the primary mechanism underlying reductions in muscle mass (Rennie 1985, de Boer, Selby et al. 2007), however the effect of sleep restriction on this has not been examined. At the molecular level, protein synthesis is regulated via the activation of the AKT-mTOR-p70S6K signalling pathway, which subsequently leads to increases in strength and muscle mass over time (Bodine, Stitt et al. 2001). However, little is known as to how sleep restriction may influence these pathways. Following 96 h of sleep deprivation, either no change in p-mTOR^{ser2448} and p-p70S6K^{thr389} (Monico-Neto, Antunes et al. 2015) or an increase in p-mTOR^{ser2448} and p-4EBP1^{ser65} (de Sa Souza, Antunes et al. 2016) were observed in rodent skeletal muscle. How these signalling pathways may be influenced in humans experiencing sleep restriction is not known at this stage (**Figure 2.3**). Although resistance exercise is regarded as the most potent stimulus to induce protein synthesis, high-intensity interval exercise (HIIE) is also capable of activating protein synthesis signalling pathways and muscle protein synthesis (Miller, Olesen et al. 2005, Di Donato, West et al. 2014, Bell, Seguin et al. 2015). These signalling pathways and effects on protein synthesis and the maintenance of muscle mass mark another possible convergence point by which sleep *and* exercise may interact.

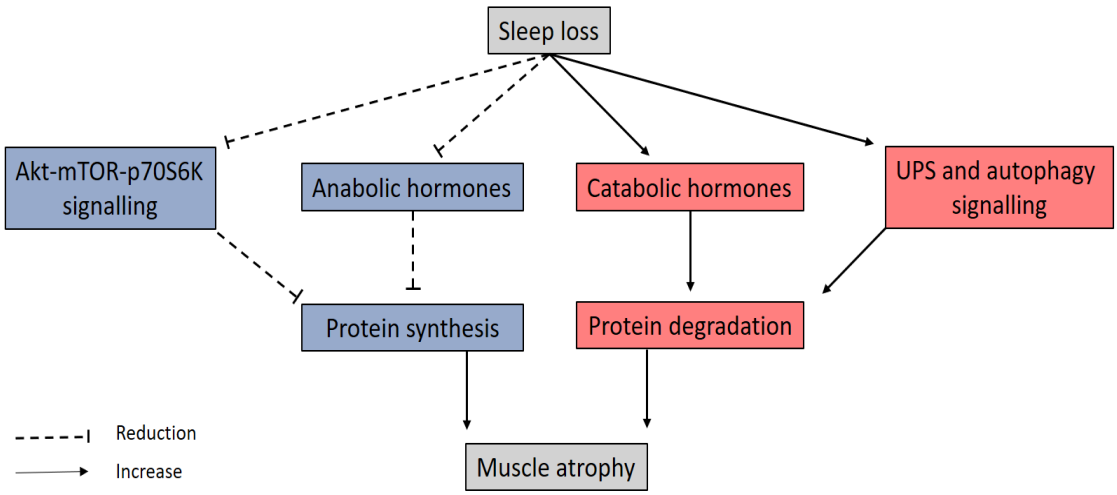

Figure 2.3 - Schematic representation of the potential mechanisms leading to sleep-loss-induced reductions in muscle mass.
This schematic has been adapted from (Dattilo, Antunes et al. 2011) and demonstrates the potential detrimental effect of sleep loss on anabolic hormone profiles and the protein synthesis signalling pathway, which subsequently leads to reduction in protein synthesis and increase in muscle atrophy. Furthermore, a concomitant increase in catabolic hormones and the UPS/autophagy signalling pathways leads to an increase in protein degradation, potentially leading to muscle atrophy. UPS – ubiquitin proteasome system.

While inconclusive thus far, evidence from animal models used to investigate muscle atrophy and sarcopenia (a progressive loss of muscle mass, quality and function, associated with aging (Mitchell, Williams et al. 2012)) point towards the disruption of clock genes as a contributing mechanism. For example, *Bmal1* knockout mice display features associated with advanced aging and sarcopenia, including significantly reduced strength and altered myofilament structure (Andrews, Zhang et al. 2010, Schroder, Harfmann et al. 2015). This suggests that the molecular clock is necessary for the maintenance of skeletal muscle function and phenotype. Another mechanism thought important in the regulation of muscle mass is inflammation. Indeed, lifestyle factors such as sedentary behaviours and sleep loss, as well as obesity and T2DM, induce dramatic increases in pro-inflammatory signalling (Mullington, Simpson et al. 2010), which may also be implicated in muscle atrophy (Haddad, Zaldivar et al. 2005).

Strategies that can regulate the molecular clock, such as resistance-based exercise interventions, have also been shown to increase muscle mass and to protect against sarcopenia (Taylor, Cable et al. 2004). Furthermore, exercise has been shown to have anti-inflammatory properties (Mathur and Pedersen 2008), providing a potential additional mechanism to counteract sleep loss induced muscle loss. Importantly, it has been shown in rats that resistance exercise training performed prior to sleep deprivation attenuates muscle atrophy (Monico-Neto, Antunes et al. 2015), providing preliminary evidence of the role contractile activity can play in protecting against sleep loss induced muscle mass loss. Whether it also helps reduce the development of insulin resistance,

by maintaining skeletal muscle functionality, remains to be determined. Nonetheless, considering the increased prevalence of sarcopenia in T2DM patients (Lim, Kim et al. 2010, Kim, Park et al. 2013), this appears an important area of emerging research.

2.8 Sleep quality, insulin sensitivity and exercise

In addition to sleep loss, sleep quality may also be a critical factor influencing the regulation of insulin sensitivity (Tasali, Leproult et al. 2008). It was recently reported that, like sleep loss, reduced sleep quality is associated with a similar increase in the risk of T2DM as physical inactivity (~40%) (Anothaisintawee, Reutrakul et al. 2015). Additionally, patients with sleep disorders, such as obstructive sleep apnea (OSA), in which sleep quality is significantly reduced, have an increased risk of insulin resistance (Anothaisintawee, Reutrakul et al. 2015). Sleep quality can be determined via polysomnography, which analyses multiple physiological parameters relevant to sleep. Specifically, the time spent in different stages of sleep can be quantified (i.e., non-rapid eye movement (nREM, stages 1-3) and rapid eye movement (REM)) (Moser, Anderer et al. 2009). A reduction in the time spent in slow wave sleep (SWS, nREM stage 3) (i.e., restorative sleep) is also associated with increased insulin resistance (Tasali, Leproult et al. 2008, Herzog, Jauch-Chara et al. 2013). Indeed, when SWS is disturbed, but the overall time spent asleep is not reduced, there are increases in insulin resistance of between 20-25%. However, a night of REM sleep disturbance did not produce similar reductions in insulin sensitivity, suggesting that SWS rather than REM sleep may play a role in the regulation of insulin sensitivity (Herzog, Jauch-Chara et al. 2013). In considering there were no associations between sleep stages and fasting insulin resistance following 1 night of restricted sleep (Cedernaes, Lampola et al. 2015), when taken together, this suggests more research is warranted to determine the role of REM and SWS on the regulation of glucose tolerance and insulin resistance.

Given that sleep quality is an important determinant of glucose tolerance and insulin sensitivity (Tasali, Leproult et al. 2008, Cappuccio, D'Elia et al. 2010), improving sleep quality by means of regular engagement in exercise could also prove beneficial in the treatment and prevention of insulin resistance and T2DM. There are a number of strategies for improving sleep quality (e.g., sleep hygiene practices such as caffeine restriction and reducing the use of electronic devices prior to bed). However, engaging in physical activity and exercise (both acutely and chronically), as alternative or complementary approaches, may be beneficial (Yang, Ho et al. 2012, Kredlow, Capozzoli et al. 2015). In a comparison of older adults who were assigned to either a moderate-intensity exercise program or a health education control program over a 12-month period, an exercise program was shown to improve aspects of sleep quality (King, Oman et al. 1997). Indeed, decreases in nREM stage 1 sleep (considered a transitional state from wake to sleep), increases in

nREM stage 2 sleep (considered more stable sleep than stage 1) and reduced sleep disturbances (as assessed via polysomnography) were reported, which coincided with improved self-reported parameters of sleep quality following the exercise program. Moreover, exercise is currently prescribed, and has been shown to be effective in improving sleep quality, for a variety of clinical sleep disorders, including insomnia and obstructive sleep apnea, in which sleep quality is typically reduced (Kubitz, Landers et al. 1996, Baron, Reid et al. 2013). However, further research is required to determine whether, in addition to metabolic function, exercise-induced improvements in sleep quality (regardless of duration) can help to maintain glucose tolerance and insulin resistance.

2.9 Conclusion

To date, sleep *and* exercise in the context of human health have remained relatively independent lines of research. However, with the emergence of sleep loss as a significant risk factor for the development of insulin resistance and T2DM, engagement in exercise to mitigate these risks may offer an alternative solution. Indeed, this review presents and uncovers a framework and mechanistic underpinning of how exercise can be of significant benefit to counteract the mechanisms by which sleep loss might increase the risk of impaired mitochondrial function and the subsequent development of insulin resistance and T2DM.

Chapter 3. General Methodology

The overarching aim of this thesis was to investigate the potential mechanisms underlying the detrimental effects associated with sleep loss, and to examine the effect of exercise as a therapeutic intervention to mitigate these effects. To achieve this, a single study protocol was designed that allowed for the investigation of each of the specific objectives outlined within section **1.2**, using the same cohort of participants. The following chapter contains extensive detail regarding the design of the study protocol, and all experimental procedures, and analysis encapsulated within it. The subsequent experimental chapters contain a brief summary of the specific procedures used to investigate their particular objectives and refer back to this chapter where necessary.

3.1 Participants

Twenty-four healthy men, who were recreationally active, and aged between 18 and 40 years of age, volunteered to take part in this study. After medical screening to rule out any conditions that may have precluded their participation (e.g., cardiovascular, metabolic, or musculoskeletal problems), the participants were informed of the study requirements, benefits, and risks, before giving written informed consent. Only participants who 1) were free of medications before and during the study, 2) were not currently (or within the previous 3 months) performing shift work, 3) had regular sleeping habits and no previously diagnosed sleep disorders, 4) had not travelled overseas in the previous two months, and 5) had a body mass index between 19 and 30, were included in the study. Following an initial screening session to assess the suitability of volunteers for the study (completion of risk factor questionnaires and consent forms (**Appendix A**), one week of habitual sleep monitoring was conducted prior to the commencement of the study. Habitual sleep monitoring was assessed via sleep watch actigraphy (Actiwatch 2, Philips Respironics, Murrysville, PA, USA) and confirmed via sleep diary entries (**Appendix B**). Participants who slept less than 6 and more than 9 hours per night on average during the week of monitoring were also excluded from the study. Participants were considered 'recreationally active' and underwent one week of habitual physical activity and exercise monitoring prior to inclusion in the study. 'Recreationally active' was defined as engaging in moderate physical activity two to five times per week and assessed via the International Physical Activity Questionnaire (IPAQ) (**Appendix B**), and via self-reporting of physical activity conducted during the pre-study physical activity assessment. Daily step counts were recorded via accelerometers, so that they could be replicated throughout the study to avoid the detrimental effect of sedentary behaviour on glucose tolerance and muscle protein synthesis (Shad, Thompson et al. 2019) (**Table 3.1**). Due to issues with participant compliance and equipment reliability, step counts for some participants are not reported.

Table 3.1 - Habitual and sleep study step counts for participants in each group

Step Count	NS	SR	SR+EX
Habitual	12260 ± 3964	10965 ± 2136	11831 ± 919
Study	10652 ± 2476	10033 ± 1839	10953 ± 2316

Values are mean ± SD. NS – Normal sleep, SR – Sleep restriction and SR+EX – Sleep Restriction and Exercise. n = 4, 5 and 4 for NS, SR, and SR+EX respectively. There were no significant differences between step counts, habitually and within the study protocol, in any of the groups.

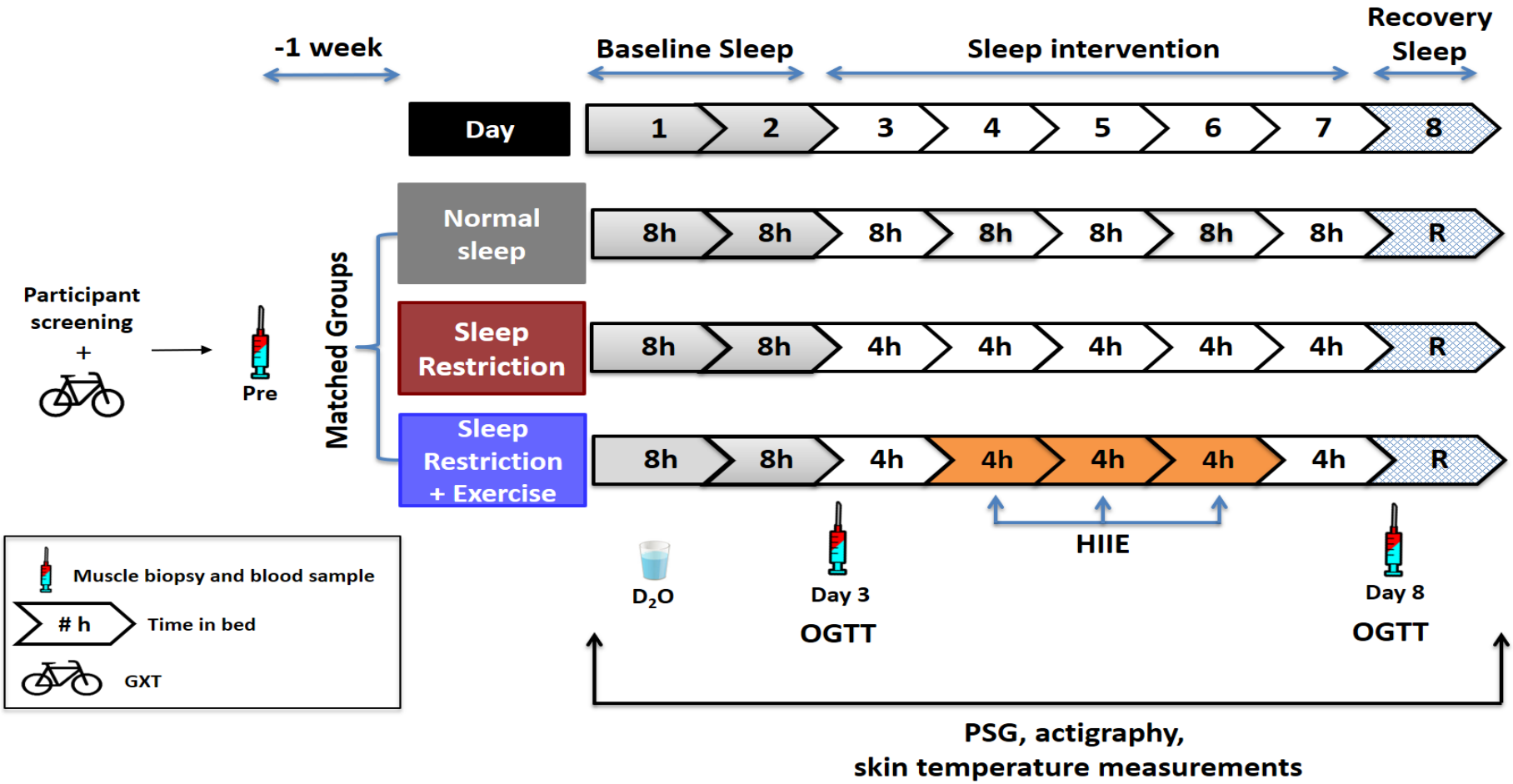

Figure 3.1 - Schematic representation of the study protocol
GXT – Graded exercise test, D2O – deuterium oxide ingestion, OGTT – oral glucose tolerance test, PSG – polysomnography sleep analysis, HIIE – high-intensity interval exercise, R – *ad libitum* recovery sleep, participant screening refers to medical questionnaires, exclusion criteria and, habitual sleep and physical activity monitoring. OGTT's were performed at 08:00 am, muscle biopsies were performed at 10:00 am, and HIIE sessions were performed at 10:00 am.

3.1 Study overview

For an overview of the study protocol see **Figure 3.1**. Following the initial screening procedures and having met all inclusion criteria, participants were asked to attend the exercise physiology laboratory to provide baseline physical measurements (i.e., age, height, and body mass) before performing a graded exercise test (GXT) to assess aerobic fitness ($\dot{V}O_{2peak}$). Participants were also asked to provide a pre-study blood sample (see section **3.3.4**), for assessment of fasting blood glucose levels, and a skeletal muscle biopsy (see section **3.3.3**) to determine baseline mitochondrial respiration values. Based on these preliminary assessments, participants were then assigned to one of three experimental groups (in a counterbalanced order, matched for age, BMI, $\dot{V}O_{2peak}$, and mitochondrial respiratory function): Normal Sleep (NS), Sleep Restriction (SR) or Sleep Restriction and Exercise (SR+EX). Baseline participant characteristics for the three groups are shown in **Table 3.2**.

Table 3.2 - Baseline characteristics of participants

	Group characteristics		
	NS (n=8)	**SR** (n=8)	**SR+EX** (n=8)
Age (y)	24 ± 4	25 ± 5	24 ± 4
Height (cm)	177 ± 8	179 ± 6	179 ± 7
Mass (kg)	78.7 ± 13.3	74.5 ± 11.7	80.2 ± 9.5
BMI	25.2 ± 3.6	23.3 ± 3.0	24.6 ± 2.5
$\dot{V}O_{2peak}$ (mL.kg^{-1}.min^{-1})	43.7± 9.7	47.2 ± 6.7	48.0 ± 5.0
$\dot{V}O_{2peak}$ (mL.min^{-1})	3370 ± 514.7	3633 ± 475.5	3868 ± 593.2
W_{peak} (W)	319.0 ± 59.0	330.0 ± 44.0	362.0 ± 48.0
Fasting plasma glucose (mmol/L^{-1})	5.1 ± 0.5	5.2 ± 0.4	5.2 ± 0.2
Mitochondrial respiration (ETF+CI+CII)p (pmol.s^{-1}.mg tissue^{-1})	80.8 ± 17.7	88.3 ± 25.6	81.2 ± 19.7
Habitual sleep duration (min)	457 ± 45	428 ± 44	437 ± 39
Mean habitual wake time (h:min)	07:41 ± 01:03	07:24 ± 00:36	06:48 ± 00:45
Mean habitual bed time (h:min)	23:45 ± 00:57	23:44 ± 00:44	23:22 ± 00:26

Values are mean ± SD. There were no statistically significant differences between the 3 groups for any of the baseline characteristics. NS – Normal sleep, SR – Sleep restriction and SR+EX – Sleep Restriction and Exercise, BMI – body mass index, W_{peak} – peak power output (W), (ETF+CI+CII)p – maximal oxidative phosphorylation through electron transferring flavoprotein, complex 1 and complex 2 of the electron transport system.

The experimental component of the study consisted of an eight-night stay within the sleep laboratory. The protocol for each of the three experimental groups consisted of an initial two

nights of baseline sleep, followed by a five-night intervention period and then a final night of recovery sleep. During the baseline sleep period all participants spent 8 h TIB, between 23:00 h and 07:00 h. For the remaining 5 nights, the NS group spent 8 h TIB (between 23:00 h and 07:00 h), while both the SR and SR+EX groups spent 4 h TIB (between 03:00 and 07:00) each night. The SR and SR+EX group TIB (03:00 h – 07:00 h) was standardised to ensure all participants woke at the same time each morning and that the subsequent testing procedures were performed following the same time duration post waking. Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux to reduce the effect of lighting on circadian rhythms (Duffy and Wright 2005).

Two experimental testing sessions were conducted during the participant's stay. At 08:00 h on the morning of Day 3 (following 2 nights of baseline sleep), the pre-intervention testing procedures were performed, this included the collection of a fasting venous blood sample and oral glucose tolerance test (OGTT), followed by a resting skeletal muscle biopsy. This same experimental protocol was performed again at 08:00 h on the morning of Day 8 (following the final night of the sleep intervention). For the duration of the study, each participant was provided with a standardised diet relative to body mass consisting of fixed proportions of carbohydrates (4.5 $g.kg^{-1}.d^{-1}$), protein (1.5 $g.kg^{-1}.d^{-1}$) and fat (1 $g.kg^{-1}.d^{-1}$). All meal times (there were six throughout the day – of varying caloric value) were kept constant throughout the study and participants were asked to eat all food provided. Meal timings were kept the same for all groups. An identical menu was provided on the days prior to both experimental sessions (an example of the daily diet is provided in **Appendix B**. Participants remained within the laboratory and were monitored throughout each study. To match the activity count prior to the commencement of the study, participants were instructed to walk outside the facility at designated periods throughout the day, while being accompanied by a member of the research staff. Waking hours were spent watching television, reading, working on a computer, or talking to staff. Approval of the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee (HRE15-294).

3.2 Study setting

The study was conducted in the exercise physiology and sleep laboratories of the Victoria University, Footscray Park campus. The sleep laboratory is sound-attenuated and all bedrooms have blacked out windows to limit the influence of outside noise and light. Temperature within the facility was kept constant throughout the study (21°C ± 1°C). The laboratory had the capacity for three participants to complete the study at any one time. Each participant had their own

bedroom, and shared a common living room, kitchen, and bathroom facility (**Figure 3.2**). Participants were monitored by a member of the research team at all times throughout the study.

a) b)

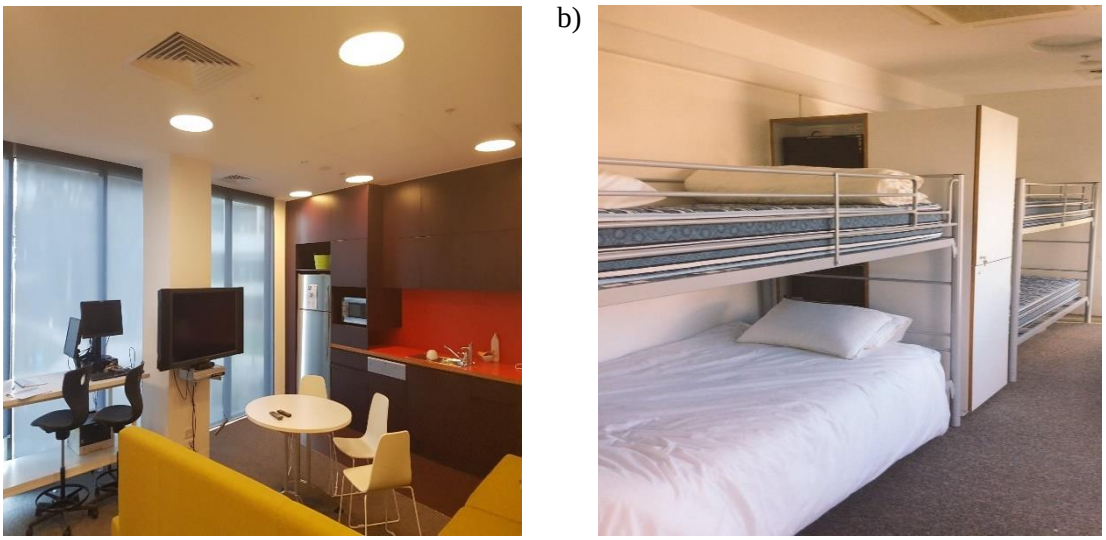

Figure 3.2 - Images of the sleep laboratory at Victoria University
a) Kitchen and communal area, b) Individual participant's bedroom.

3.3 Experimental procedures

3.3.1 Sleep monitoring

Participants wore sleep actigraphy watches (Actiwatch 2, Philips Respironics, Murrysville, PA, USA) throughout the study to objectively measure total sleep time (TST) and indicators of sleep quality such as sleep onset latency (SOL), wake after sleep onset (WASO), and sleep efficiency. These measures were determined using the Actiwatch 2 software, Philips. Sleep data was recorded each night of the study period via polysomnography (PSG) (Compumedics, AUS). Electrode placement for PSG monitoring was determined using the 10-20 electrode placement system (Berry, Gamaldo et al. 2015). An example of the electrode placement can be seen in

Figure 3.3. This was used to assess electrooculogram (EOG) activity, chin electromyogram activity (EMG), and electroencephalogram activity (EEG) and subsequently to score the PSG recordings into specific sleep stages. Analysis of PSG data (n=4 for each group) was conducted on recordings from night 6 of the study protocol (the night following the final exercise session for the SR+EX group) as an objective measure of sleep quality. PSG data from all nights of the protocol were collected; however, due to financial limitations and limited availability of the sleep scorer this has not been assessed at this point. Sleep stages were scored in accordance with standard criteria and guidelines (Rechtschaffen 1968, Iber 2007) by an experienced scorer blinded to the experimental conditions. The participant's time in bed (TIB) was assigned into either wake, rapid eye movement (REM) or non-rapid eye movement (nREM) sleep, with nREM being further defined as either stage 1 (N1), stage 2 (N2) or stage 3 (N3) (**Figure 3.4**). A member

of the research team recorded the exact time that bedroom lights were switched on and off, allowing participants the designated sleep opportunity each night.

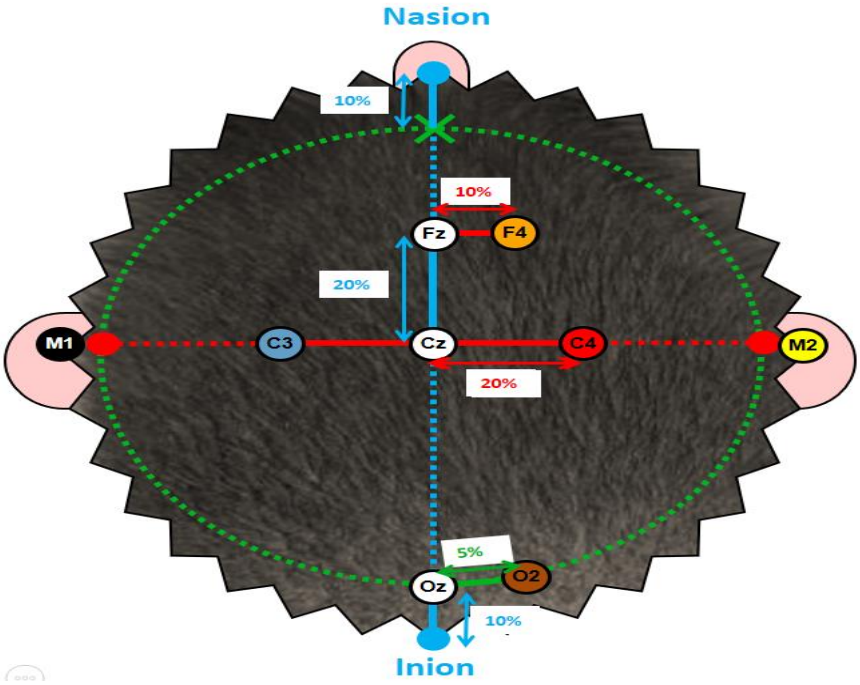

Figure 3.3 - Schematic representation of the PSG electroencephalogram (EEG) lead placement that was determined using the 10-20 electrode placement system.

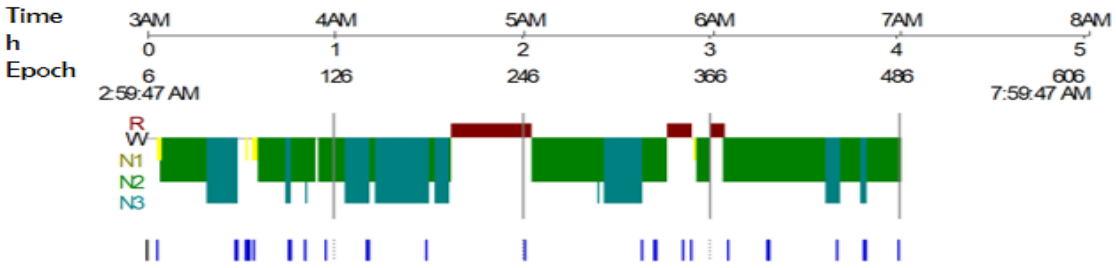

Figure 3.4 - Schematic representation of PSG sleep scoring from a night of sleep for a participant in the sleep restriction group.
The sleep stages are designated as either R – rapid eye movement, W – wake, N1 – non-rapid eye movement stage 1, N2 – non-rapid eye movement stage 2 and N3 – non-rapid eye movement stage 3, grey lines represent awakenings, blue lines represent arousals.

3.3.2 Graded exercise test

Upon meeting the inclusion criteria, participants underwent a pre-trial assessment of aerobic fitness (i.e., peak oxygen uptake ($\dot{V}O_{2peak}$) and peak power output ($\dot{W}_{Peak}$)) in the form of a graded exercise test performed on an electronically-braked cycle ergometer (Excalibur, V2.0; Lode, Groningen, the Netherlands). The protocol began with a 5-minute warm up at 30 Watts (W), followed by an incremental ramp protocol whereby the ergometer increased by 1 W every 2 seconds (30 W/minute) and continued until exhaustion (i.e., cadence fell below 60 rpm). $\dot{V}O_{2peak}$

was used to match the participants in each group, while W_{Peak} was used to determine exercise session intensity for the SR+EX group. During the GXT, expired air was continuously analysed for O_2 and CO_2 concentrations via a gas analyser (Moxus 2010; AEI Technologies, Pittsburgh, PA, USA), which was calibrated immediately before each test. The ventilometer was calibrated with a 3-L syringe (Hans Rudolph, Shawnee, KS, USA). VO_2 was recorded every 15 s and the average of the 2 highest, consecutive, 15-s readings was recorded as a participant's $\dot{V}O_{2peak}$.

3.3.3 Muscle biopsies

Three resting muscle biopsies were taken from the *vastus lateralis* muscle of the same leg using a biopsy needle with suction, under local anaesthesia of the skin and fascia (1% xylocaine). Muscle biopsies were collected one week prior to the commencement of the study, and then again during the experimental sessions of the study (Day 3 and Day 8). Samples collected on Day 3 and Day 8 were obtained at 10:00 h, following an overnight fast and then a 2 h oral glucose tolerance test (OGTT, see section **3.3.8**). The Day 8 biopsy was collected 48 h post the final exercise session to avoid the acute residual effects exercise has on glucose tolerance (Richter and Hargreaves 2013) and mitochondrial respiration (Trewin, Parker et al. 2018). The muscle biopsy obtained prior to the study (also at 10:00 h) was collected following an overnight fast and was used to assess background enrichment of deuterium oxide (D_2O) (see section **3.4.3**) (prior to the ingestion of the D_2O bolus) for the subsequent myofibrillar and sarcoplasmic protein synthesis analysis, as well as for matching the experimental groups based on pre-study mitochondrial respiratory function (see **Table 3.2**). The collected muscle samples were cleaned of excess blood, fat, and connective tissue; 1 portion (10 to 20 mg) was immediately immersed in 2 mL of ice-cold biopsy preservation solution (BioPS; see section **3.4.4**) for *in situ* measurement of mitochondrial respiratory function. The remaining portion was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses. All participants refrained from exercise for 48 h prior to the muscle biopsies.

3.3.4 Venous blood sample collection

Venous blood samples were obtained from an antecubital vein using standard venepuncture and intravenous cannulation techniques. The collection of samples occurred one week prior to commencement of the study, and on the morning of Day 3 and Day 8 of the study protocol following an overnight fast. The same cannulas were used for the oral glucose tolerance testing (OGTT) (see OGTT section below) that followed on Day 3 and Day 8. These samples were stored in ethylenediaminetetraacetic acid (EDTA), prior to being separated into plasma by centrifugation (10 min at 2200 RCF, 4 °C) and stored at -80 °C until subsequent analysis.

3.3.5 High-intensity interval exercise (HIIE)

Participants in the SR+EX group performed three sessions of HIIE on days 4, 5 and 6 at 10:00 h. The HIIE protocol consisted of 10 x 60-second intervals of exercise on a cycle ergometer (Velotron; RacerMate) at 90% of the participant's peak power (W_{peak} – as determined in the pre-trial graded exercise test). Each interval was interspersed with 75 seconds of active recovery (at 60 W) (**Figure 3.5**). All HIIE sessions started with a 3-minute warm up at 60 W.

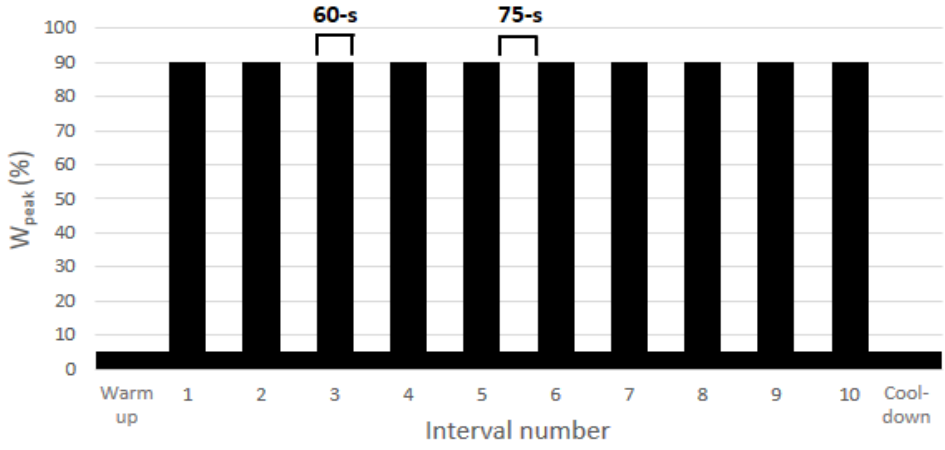

Figure 3.5 - Schematic representation of high-intensity interval exercise protocol.
10 x 60-s intervals at 90% $\dot{W}_{peak}$ on a cycle ergometer, interspersed by 75 s of active recovery. $\dot{W}_{peak}$ – peak power output ($\dot{W}$).

Heart rate was measured (Polar FT1, Kempele, Finland) at rest and during the last 15 seconds of each interval and rest period. Ratings of perceived exertion (RPE) were collected at the end of each interval (Borg 1970). If participants were unable to complete the exercise protocol at the designated intensity (as occurred with two of the participants, in their initial session only), the intensity was reduced to 85% $\dot{W}_{peak}$ and then again to 80% $\dot{W}_{peak}$ during the session (**Error! Reference source not found.**). High-intensity interval exercise has been shown to induce similar, if not greater, adaptations to cardio-metabolic health compared to traditional continuous endurance exercise (Tjonna, Lee et al. 2008, Milanovic, Sporis et al. 2015). The protocol used in this study was adapted from previous work (Little, Safdar et al. 2010) that lead to improvements in endurance performance testing, markers of mitochondrial content (i.e., citrate synthase activity), and total GLUT4, protein content following 6 sessions of the protocol. Similar low-volume, high-intensity protocols have been used to assess their effectiveness in diabetic populations, with reductions in hyperglycaemia and improvements in citrate synthase (CS) activity having been reported (Little, Gillen et al. 2011). Importantly, such protocols were well tolerated by these clinical populations.

Table 3.3 - Characteristics of the high-intensity interval exercise sessions

HIIE session characteristics	
Average power (W)	181 ± 35
Average interval power (W)	318 ± 53
Average heart rate (bpm)	156 ± 13
Peak heart rate (bpm)	182 ± 12
Average interval RPE	15 ± 2
Session RPE	9 ± 1

Values are mean $\pm$ SD, RPE – rating of perceived exertion, Session RPE (1 to 10 scale), interval RPE (6 to 20 scale).

3.3.6 Wrist skin temperature measurements

Wrist skin temperature was measured every 10 min for the duration of the study (8 days), using non-invasive temperature recording devices (iButtons) (Thermochron iButton; Embedded Data Systems, Lawrenceburg, KY). An example of the skin temperature data is shown in **Figure 3.6**.

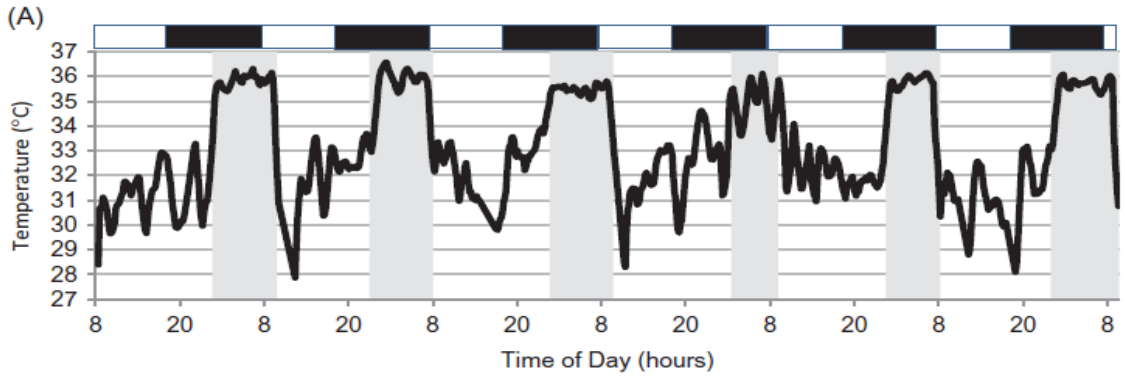

Figure 3.6 - Example of wrist skin temperature data collected across 6 days.
At the top of the graph, white bars represent daylight and the black bars represent night time. The grey vertical bars represent reported sleep time. Solid dark line represents a 30-min moving average of skin temperature data. This diagram has been adapted from (Tranel, Schroder et al. 2015).

The iButton was programmed to record temperature every 10 minutes (at a sensitivity of 0.0625°C) for the duration of the study. The use of peripheral skin temperature recorded from iButtons has been shown to be a reliable and valid method for evaluating circadian rhythmicity, with peripheral wrist skin temperature reported to have an inverse relationship to core body temperature (Sarabia, Rol et al. 2008, Tranel, Schroder et al. 2015). iButtons were securely attached to the inside of the participant's wrist (non-dominant hand) using strips of breathable latex-free dressing tape. Removal of iButtons only occurred prior to participants bathing or coming into direct contact with water. Information from the iButtons was transferred via a USB adapter (SK-IB-R-iButton, Connectivity Kit; Embedded Data Systems, Lawrenceburg, KY) and stored in customised excel spreadsheets. The temperature data was analysed for variations in temperature amplitude and stability for each participant, using a modified version of the software

program JTK_CYCLE (Hughes, Hogenesch et al. 2010). Analysis of wrist skin temperature data was performed by Professor Karyn Esser and Assistant Professor Elizabeth Schroder as previously described (Tranel, Schroder et al. 2015). Temperature amplitude is defined as the difference between the maximum (or minimum) value of the trace and the mesor (mean value of the data after smoothing with a cosine function) across a 24-h period (Corbalan-Tutau, Madrid et al. 2011), with changes in amplitude having previously been proposed to provide an indication of circadian alignment (Sarabia, Rol et al. 2008, Harfmann, Schroder et al. 2017) The degree of phase homogeneity of a rhythm during the period of data collection is considered a description of the 'stability' of the rhythm (i.e., where the oscillatory pattern of the rhythm is nearly identical from one day to the next stability is considered high and when there are large discrepancies between oscillatory patterns from day to day stability would be considered low) (Sarabia, Rol et al. 2008, Harfmann, Schroder et al. 2017). These principles are illustrated further in **Figure 3.7** below. Data from 8 participants were included in both the NS and SR groups; however, due to participant non-compliance, the SR+EX group includes data from seven participants.

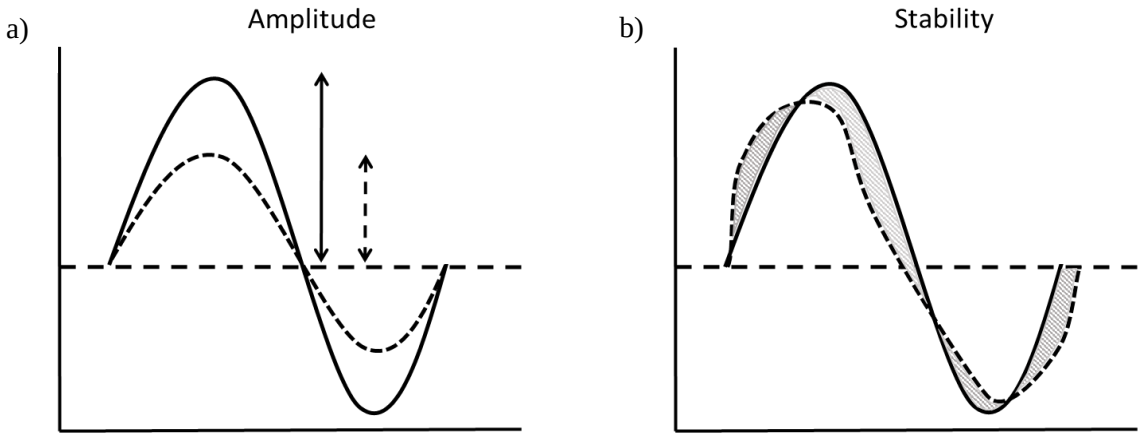

Figure 3.7 - Illustrative representation of the assessment of skin temperature amplitude and stability.
a) Temperature amplitude is defined as the difference between the maximum (or minimum) value of the trace and the mesor (mean value of the data after smoothing with a cosine function) across a 24-h period (Corbalan-Tutau, Madrid et al. 2011). b) The degree of phase homogeneity of a rhythm during the period of data collection is considered a description of the 'stability' of the rhythm. (Sarabia, Rol et al. 2008, Corbalan-Tutau, Madrid et al. 2011). The shaded area in b) represents the change in stability between the solid line and the broken line.

3.3.7 Morning-eveningness Questionnaire (MEQ)

The MEQ has 19 multiple-choice questions designed for the self-assessment of chronotype (Horne and Ostberg 1976) (**Appendix B**). Each answer is assigned a number of points that are used to calculate a final score, which is associated with a category for 'definite evening', 'moderate evening', 'intermediate', 'moderate morning' or 'definite morning' chronotype (**Table 3.4**).

Table 3.4 - Chronotype distribution of each group based on scores from the Horne-Ostberg Morningness-Eveningness Questionnaire.

	Chronotype				
Group	**Definite Evening**	**Moderate Evening**	**Intermediate**	**Moderate Morning**	**Definite Morning**
NS (n=8)	0	0	6	1	1
SR (n=8)	0	2	5	1	0
SR+EX (n=8)	0	0	3	5	0

NS – Normal Sleep, SR – Sleep Restriction and SR+EX – Sleep Restriction and Exercise.

3.3.8 Oral glucose tolerance testing (OGTT)

OGTT tests were performed on the mornings of Day 3 and Day 8 (following the initial 2 nights of baseline sleep and again following the final night of the sleep intervention), to assess glucose metabolism. At 08:00 h, following an overnight fast, participants consumed a 300 mL solution containing 75 g of glucose (Point of Care Diagnostics-Scientific, NSW, Australia). Blood samples (10 mL) were drawn via an intravenous cannula over the course of 120 minutes and stored in EDTA vacutainers, before being centrifuged at 2205 RCF for 15 minutes at 4°C to separate the blood components. Plasma samples were then aliquoted into 1.5 mL Eppendorf tubes and assessed for plasma glucose concentrations on a glucose/lactate analyser (YSI, 2300 STAT plus, Yellow Spring, OH, USA), before being stored at -80°C for subsequent analysis. Blood samples were collected at 0, 10, 20, 30, 60, 90 and 120 minute time points. Total area under the curve (AUC) was then calculated using the trapezoidal rule.

3.4 Analysis

3.4.1 Plasma insulin analysis

Plasma insulin concentrations were determined by analysis of stored plasma samples from the Day 3 and Day 8 OGTTs, using an Insulin ELISA kit (ALPCO. 80-INSHU-E01.1, E10.1, Salem, NH, USA). The ELISA kit was run as per the manufacturer's instructions. In brief, a 96-well microplate was loaded with insulin standards (25 µL), known manufacturer's insulin controls (25 µL), and plasma samples (25 µL), and then incubated on a plate shaker (700 rpm) with the detection antibody (100 µL) for 1 hour. All samples were run in duplicate. The plate was manually washed 6 times with wash buffer and blotted dry, before TMB substrate (100 µL) was added prior to incubation on the plate shaker (700 rpm) for a further 15 minutes at room temperature. A stop solution (100 µL) was then added and the optical density was measured by spectrophotometry, with the intensity of the colour generated being directly proportional to the amount of insulin in the sample. Results were obtained via calculation of a standard curve from the insulin standards and then compared to the optical density of each sample to give an insulin concentration (µIU/mL). Total area under the curve was calculated using the trapezoidal rule.

3.4.2 Plasma interleukin-6 analysis

Plasma interleukin-6 (IL-6) concentrations were determined by analysis of stored plasma samples from the Day 3 and Day 8 OGTTs, using an IL-6 ELISA kit (abcam IL-6 ELISA kit High Sensitivity, ab46042). The ELISA kit was run as per the manufacturer's instructions. . In brief, a 96-well microplate was loaded with IL-6 standards (100 µL), known manufacturers insulin controls (100 µL), and plasma samples (100 µL), and then incubated (covered) at room temperature with the detection antibody (1 X Biotinylated anti-IL-6) (50 µL) for 3 hours. All samples were run in duplicate. The plate was manually washed 6 times with wash buffer and blotted dry, before 1 X Streptavidin-HRP substrate (100 µL) was added prior to further incubation (covered and at room temperature) for a further 30 minutes. Previous wash steps were repeated using the wash buffer. Chromogen TMB substrate solution (100 µL) was added to each well and incubated for 15 minutes in the dark. A stop solution (100 µL) was then added and the optical density was measured by spectrophotometry, with the intensity of the colour generated being directly proportional to the amount of insulin in the sample. Results were obtained via calculation of a standard curve from the IL-6 standards and then compared to the optical density of each sample to give an insulin concentration (µIU/mL).

3.4.3 Assessment of myofibrillar and sarcoplasmic protein synthesis

The preparation of myofibrillar, sarcoplasmic, and saliva samples were performed in Professor Stuart Phillips' laboratory at McMaster University, Hamilton, Canada. Analysis of muscle protein synthesis was performed by Metabolic Solutions, Inc. (U.S.A).

3.4.3.1 Deuterium Oxide (D_2O) ingestion and saliva sample collection

The fractional synthetic rate (FSR) was assessed via the Deuterium Oxide (D_2O) tracer technique as an indicator of myofibrillar and sarcoplasmic protein synthesis. At 18:00 h on Day 1, each participant ingested 150 mL of D_2O (70 atom %, Cambridge Isotope Laboratories). Saliva samples were collected prior to D_2O ingestion and then on Days 3, 5, 7 and 8 to determine body water enrichment. Total body water ^2H enrichment was used as a surrogate for plasma alanine ^2H labelling (Wilkinson, Franchi et al. 2014).

3.4.3.2 Saliva sample collection and analysis

Saliva samples were obtained by asking participants to chew on cotton buds provided in the salivettes, which were then centrifuged at 1500 g for 3 minutes at 4°C. Saliva was then transferred to 1.5 mL Eppendorf tubes and centrifuged at 10,000 g for 10 minutes to remove any contaminants. Saliva was transferred to 2 mL Eppendorf tubes and stored at -80°C until the analysis was performed. Saliva samples were collected prior to D_2O ingestion on the day of the

pre-study muscle biopsy and upon arrival on Day 1 of the intervention. Samples were then collected on Day 3, 5, 7 and 8 as well.

Saliva samples were prepared for ^{2}H enrichment analysis by centrifuging at 18,400 RCF for 10 minutes at 4°C to remove any remaining contaminants. A 1 in 35 dilution of the saliva in distilled water was prepared in screw top auto-sampler vials (Thermo Scientific, Cat #0339114) and vortexed thoroughly. ^{2}H enrichment of saliva was determined by a cavity ring-down spectroscopy with the use of a liquid isotope analyser (Picarro L2130-I analyser, Picarro, Santa Clara, CA) with an automated injection system. Saliva samples were injected 6 times and the average of the last 3 measurements used for data analysis (coefficient of variation was <0.5%). Known low, intermediate, and high standard samples were run before and after each participant's samples were run. The ^{2}H isotopic enrichments for both muscle and saliva initially expressed as $\delta^{2}H\%$ were converted to atomic percentage excess (APE) using standard equations (Wilkinson, Franchi et al. 2014). This saliva sample preparation and analysis was performed in Professor Stuart Phillips' laboratory at McMaster University, Hamilton, Canada.

3.4.3.3 Muscle preparation to determine myofibrillar and sarcoplasmic fractional synthesis rate

Frozen muscle samples (approximately 40 to 60 mg) were homogenised in 500 µL of ice-cold Tris homogenisation buffer (25 mM Tris-HCl, TritonX-100 (0.5% final volume), protease (Roche, Cat # 4693116001) and phosphatase (Roche, Cat #4906845001) inhibitors). Metal beads were added to all samples and run at 20 Hz for 40 s in a tissue homogeniser (Tissue Lyser II, Qiagen). This was repeated if the entire sample was not sufficiently homogenised. The muscle homogenate was then centrifuged at 1,900 RCF for 10 minutes at 4°C. The supernatant was removed to prepare the sarcoplasmic fraction and the pellet was used to prepare the myofibrillar fraction. The sarcoplasmic fraction was used as a proxy for the mitochondrial fraction, as the tissue requirements needed to obtain a mitochondrial fraction were far in excess of the tissue samples we obtained. Justification for using the sarcoplasmic fraction as a representation of mitochondrial protein synthesis is provided in section **3.4.3.8**.

3.4.3.4 Myofibrillar fraction preparation

The myofibrillar pellet was washed and resuspended in 500 µL of distilled water, before undergoing centrifugation at 1500 rpm for 10 minutes at 4°C. The supernatant was discarded and 1 mL of 0.3M sodium hydroxide (NaOH) was added to the pellet to assist with the removal of collagen from the sample. The sample was heated at 50°C for 30 minutes, and vortexed every 10 minutes, prior to centrifugation at 9,400 RCF for 5 minutes at 4°C. The supernatant (now

containing the myofibrillar fraction) was placed into a 4 mL glass vial and NaOH was again added to the collagen pellet, before undertaking a repeat of the centrifugation process. Again, the supernatant was removed and added to the glass vial. 1 mL of 1M perchloric acid (PCA) was added to the myofibrillar fraction and spun at 580 RCF for 10 minutes at 4°C. The supernatant was discarded and precipitation of the large white pellet was encouraged with the addition 1 mL of 70% ethanol and centrifuged at 580 RCF for 10 minutes at 4°C. Ethanol was removed, prior to the addition of 1 mL of 1M HCl and 1 mL of Dowex (Dowex 50wx8-200 ion exchange resin, Sigma Aldrich) to assist in the release of protein-bound amino acids (via acid hydrolysis). Samples were placed in an oven at 110°C for 72 h, with thorough mixing via vortex occurring every 24 h (**Figure 3.8**).

3.4.3.5 Sarcoplasmic fraction preparation

1 mL of PCA was added to sarcoplasmic samples and centrifuged at 9,400 RCF for 10 minutes at 4°C. The supernatant was discarded and the sarcoplasmic pellet was washed in 70% ethanol twice, with repeat centrifugation steps in between. Sarcoplasmic pellets were lyophilised overnight and placed into a 4 mL glass vial. 1 mL of 6M HCl and 1 mL of Dowex were added to each sample to assist in the release of protein-bound amino acids (via acid hydrolysis), prior to being placed in an oven at 110°C for 72 h, with thorough mixing occurring every 24 h (**Figure 3.8**).

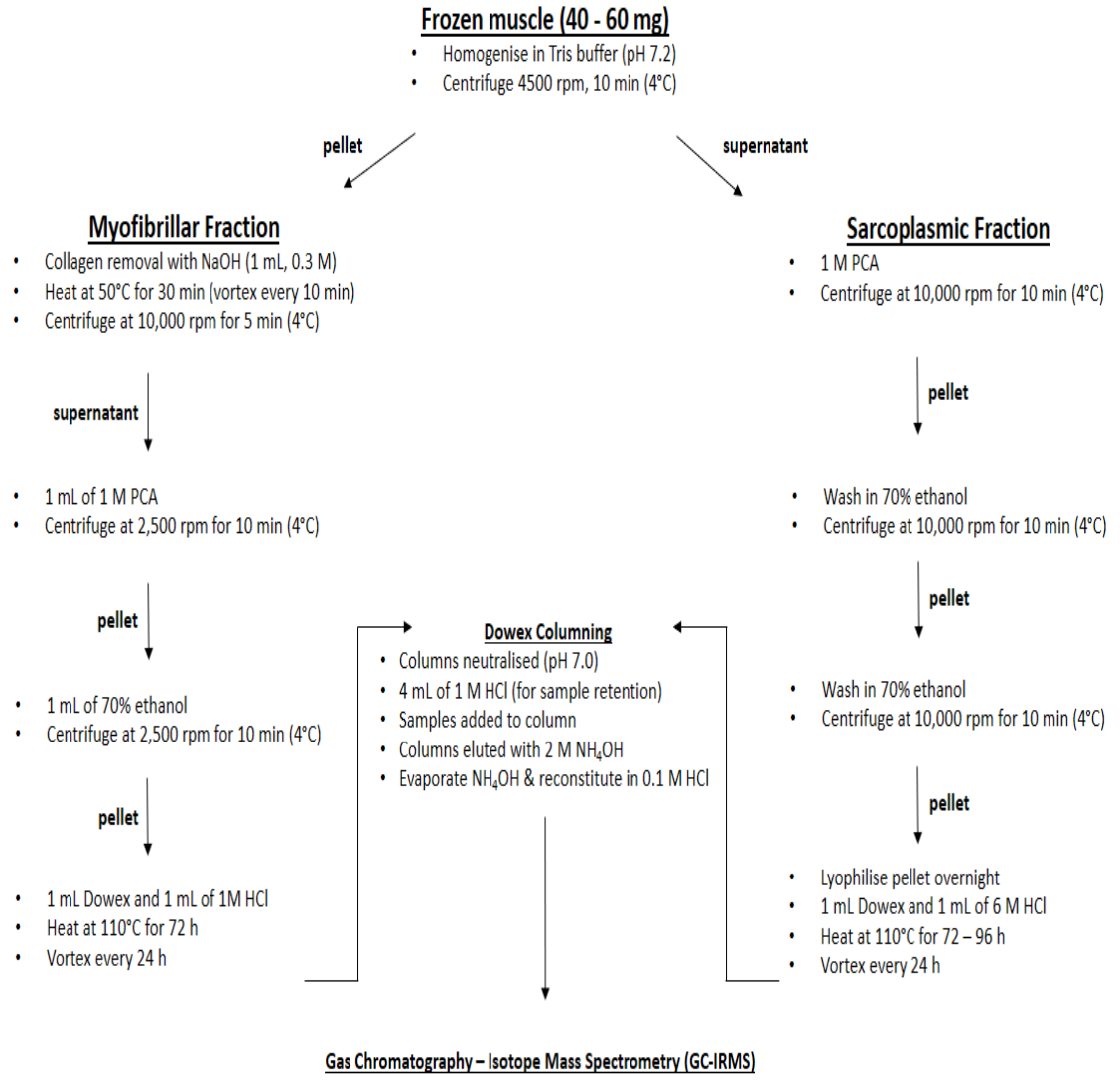

Figure 3.8 - Schematic representation of the myofibrillar and sarcoplasmic fractionation process. NaOH – sodium hydroxide, PCA – perchloric acid, NH4OH – ammonium hydroxide, HCl – hydrochloric acid.

3.4.3.6 Dowex column clean-up and reconstitution

Cation exchange chromatography was performed using columns containing Dowex resin (Dowex 50wx8-200 ion exchange resin, Sigma Aldrich) to extract free amino acids from the myofibrillar and sarcoplasmic fractions (as described previously) (Burd, West et al. 2010). In brief, samples were eluted through Dowex columns with ammonium hydroxide (2M, NH_4OH) into culture tubes, with the columns having previously undergone a series of wash steps to remove contaminants (2M, NH_4OH), to neutralise, and then acidify (1M HCl) the column (pH 1). Culture tubes containing the amino acids in NH_4OH solution were then dried under nitrogen at 60°C. Samples were then reconstituted by adding 500 µL of 0.1M HCl. The amino acid samples were then derivatised as their n-methoxycarbonyl methyl esters (MCME), as per previous protocols (Husek and Liebich 1994) (**Figure 3.9**).

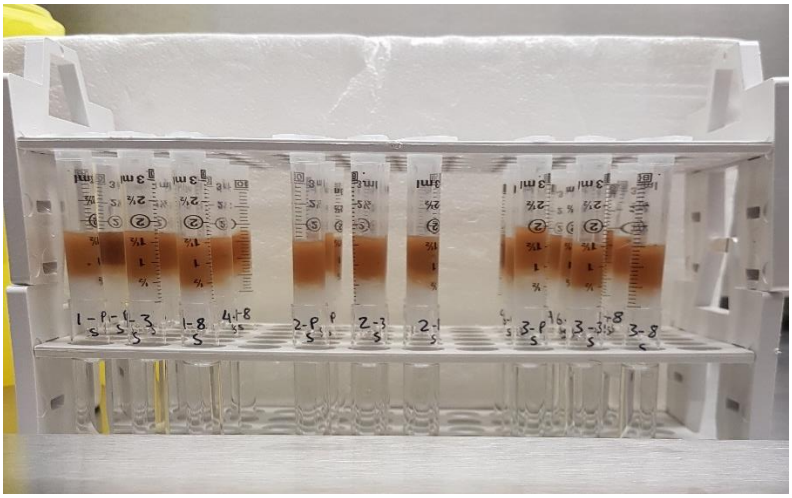

Figure 3.9 - Dowex column set up used in the fractionation process.
Columns are stacked on top of a glass culture tube, following preparation of the column to the appropriate pH level (pH = 1) and addition of either the myofibrillar or sarcoplasmic samples to the columns. The amino acids from the samples are retained in the column (due to pH level). The columns are then neutralised with deionised water, before the addition of ammonium hydroxide causes the amino acids to elute into the culture tubes.

3.4.3.7 Fractional synthetic rate (FSR)

The $^{2}H/^{1}H$ ratio of the myofibrillar and sarcoplasmic samples were determined using gas chromatography isotope mass spectrometry (GC-IRMS), which assessed the incorporation of deuterium into protein-bound alanine. This analysis was performed by Metabolic Solutions, Inc. (U.S.A). This was used to assess the FSR of myofibrillar and sarcoplasmic proteins with the use of the enrichment of body water, corrected for the mean number of deuterium moieties incorporated per alanine (as previously described (Wilkinson, Franchi et al. 2014), as the surrogate precursor labelling between subsequent biopsies.

The following standard equation (Wilkinson, Franchi et al. 2014) was used to determine FSR:

$$FSR\ (\%/day) = ((E_{t1}-E_{t0}) / (E_p \times time)) \times 100$$

Where; FSR = fractional synthetic rate, E_{t1} = APE day 8, E_{t0} = APE day 3, E_p = average saliva atom percent excess (APE), time = time between biopsies, in days.

3.4.3.8 Citrate synthase activity of fractionated muscle

Citrate Synthase (CS) activity was determined (see CS methodology below) on whole-muscle lysate, as well as the myofibrillar and sarcoplasmic fractions that were obtained from the same muscle sample (see **Figure 3.10**). As CS activity has been reported to be a valid biomarker for mitochondrial content, this analysis was conducted to provide an indication of the mitochondrial

content present in each fraction and an indication that the sarcoplasmic fraction is enriched in mitochondria, in comparison to the myofibrillar and whole-muscle lysate, as has previously been suggested (Larsen, Nielsen et al. 2012, Bell, Seguin et al. 2015). Homogenisation of muscle samples (approximately 60 mg of frozen muscle) was performed as per the myofibrillar and sarcoplasmic fractionation process previously described (**Figure 3.10**). Approximately 100 μL of lysate was removed as the whole-muscle lysate sample, with approximately 400 μL of lysate kept to perform the fractionation process. Myofibrillar pellets were then resuspended in the homogenisation buffer, with the same volume of buffer added to match the volume of sarcoplasmic supernatant collected (i.e. both myofibrillar and sarcoplasmic fractions were the same volume). Each CS sample was diluted (with homogenisation buffer) to contain 6 μg/μL of total protein (as determined via protein concentration assay following fractionation).

The whole-muscle CS activity was significantly higher than the myofibrillar fraction (mean difference CS activity (mol/h/kg protein) ± SD, 1.30 ± 0.48, CI [0.64, 1.97], P=0.0004). The sarcoplasmic fraction CS activity was also significantly higher than the myofibrillar fraction (2.05 ± 0.35, CI [-2.71, -1.39], P<0.0001). The sarcoplasmic fraction had significantly higher citrate synthase activity compared to the whole-muscle fraction (0.74 ± 0.44, CI [-1.40, -0.07], P=0.0276). These results demonstrate that the sarcoplasmic fractions are enriched with mitochondria, compared to both the myofibrillar fraction and whole-muscle sample, and, therefore, may provide insight into rates of mitochondrial protein synthesis.

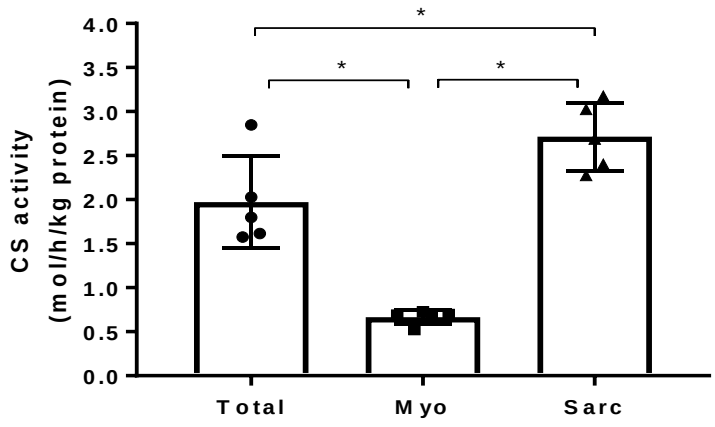

Figure 3.10 - Citrate synthase activity from fractionated skeletal muscle samples.
Whole lysate (Total), myofibrillar (Myo) and sarcoplasmic (Sarc) fractions were assessed from the same muscle samples (n=5). * denotes statistical significance between fractions (P<0.05).

3.4.4 High-resolution respirometry

3.4.4.1 Muscle fibre preparation and analysis

Immediately after the muscle biopsies, muscle fibres were placed in ice-cold biopsy preserving solution (BioPS): (containing 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 50 mM 2 (Nmorpholino)ethanesulfonic acid (MES), 15 mM Na_2-phosphocreatine, 20 mM imidazole, and 0.5 mM DTT adjusted to pH 7.1). Muscle fibres were then mechanically separated with pointed forceps in BioPS solution under a dissecting microscope to maximise fibre surface area. The plasma membrane was then permeabilised by gentle agitation for 30 min at 4°C in BioPS containing 50 μg/mL saponin. This was followed by 3 washes in MiR05, a respiration medium containing 0.5 mM EGTA, 3 mM MgCl2, 60 mM potassium-lactobionate, 20mM taurine, 10 mM KH2PO4, 20 mM 4-(2-hydroxyethyl)piperazine-1- ethanesulfonic acid (HEPES), 110 mM sucrose, and 1 g/L bovine serum albumin (BSA), essentially fatty acid–free (pH 7.1). Mitochondrial respiration was measured in triplicate (CV = 11%) (from 2 to 4 mg wet weight of muscle fibers) in MiR05 at 37°C by using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Oxygen concentration (in nanomoles per milliliter) and flux (in picomoles per second per milligram) were recorded with DatLab software (Oroboros). Reoxygenation by direct syringe injection of O_2 was necessary to maintain O_2 levels between 275 and 450 nmol/mL and to avoid a potential oxygen diffusion limitation.

3.4.4.2 Mitochondrial respiration protocol

A substrate–uncoupler–inhibitor titration (SUIT) protocol was used to determine the function of the mitochondrial electron transport system (ETS) and oxidative phosphorylation capacity. The SUIT sequence was as follows: octanoyl-carnitine (0.2 mM) and malate (2 mM) in the absence of adenylates were added for measurement of leak respiration (L) via electron transferring flavoprotein (ETF), (ETF)L, ADP (5 mM, saturating concentration) was added for measurement of oxidative phosphorylation capacity (P), (ETF)P. Pyruvate (5 mM) was then added for measurement through Complex 1 (CI), (ETF+CI)P, followed by addition of succinate (10 mM) for measurement of oxidative phosphorylation capacity (p) through complex 1 and 2 combined (ETF+CI+II)P. Cytochrome c (10 mM) was then added to test for outer mitochondrial membrane integrity (an oxygen flux increase of < 15% from (ETF+CI+II)p was considered acceptable). This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) titrations (0.75–1.5 mM), for the measurement of ETS capacity (E) through (ETF+CI+II)E. Rotenone (0.5 mM), an inhibitor of CI, was added to determine E through CII (ETF+CII)E, whereas addition of antimycin A (2.5 mM), an inhibitor of CIII, allowed measurement of and correction for residual oxygen consumption (ROX), which is indicative of non-mitochondrial oxygen consumption (**Figure 3.11**).

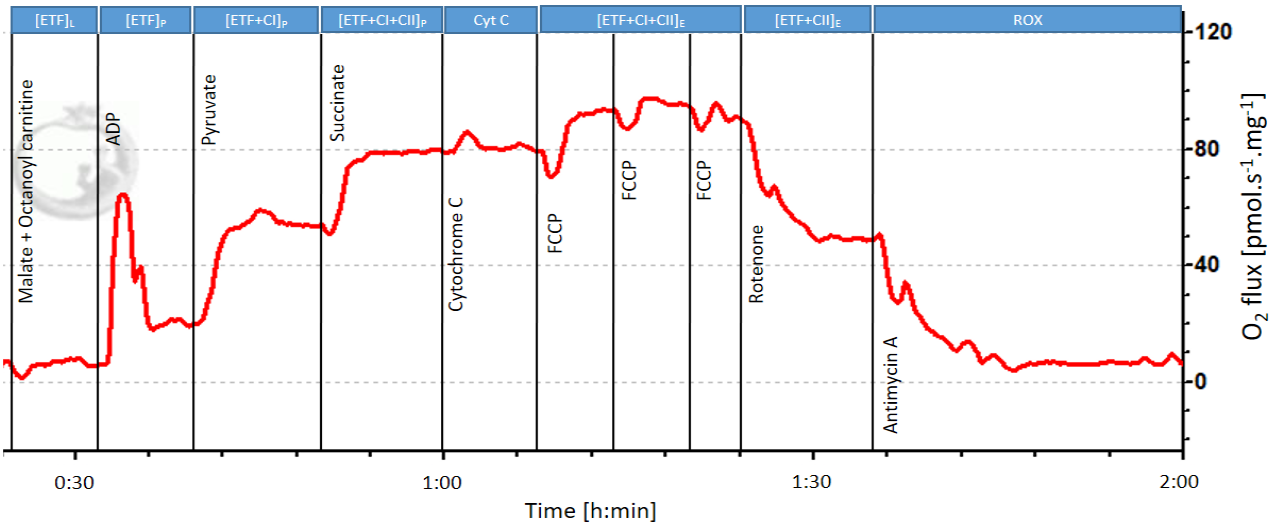

Figure 3.11 - Representative trace of high-resolution respirometry protocol with permeabilised fibres.
Human muscle biopsy taken from the vastus lateralis (2.39 mg wet weight; 37°C). Oxygen flux (pmol O2.s-1,mg-1 wet weight) during the substrate-uncoupler-inhibitor titration (SUIT) protocol. ETF – electron transferring flavoprotein, CI – complex 1, CII – complex 2, ROX – residual oxygen consumption after addition of electron transport system (ETS) inhibitors (rotenone and antimycin A), L – leak respiration, P – oxidative phosphorylation capacity at saturating ADP levels, E – uncoupled ETS.

3.4.5 Preparation of whole-muscle lysates for western blot and enzyme assays

Frozen muscle (10 to 20 mg) was homogenised in an ice-cold Auwerx lysis buffer (1:20 w/v) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, deionised water and a protease/phosphatase inhibitor cocktail [Cell Signaling Technology (CST), Danvers, MA, USA], adjusted to pH 7.4. Metal beads were added to all samples and run at 30 Hz for 2 minutes in a homogeniser (Tissue Lyser II, Qiagen), before samples were turned and homogenisation was repeated for a further 2 minutes at 30 Hz. Muscle homogenates were rotated at 4°C for 60 min and centrifuged at 15,000 g at 4°C for 20 min, and the supernatant was used for western blot analysis and enzyme activity assay. Protein concentration was determined in triplicate with a commercial colorimetric assay (Protein Assay kit-II; Bio-Rad, Gladesville, NSW, Australia), against bovine serum albumin standards (BSA, A9647; Sigma-Aldrich).

3.4.6 Western blotting

Muscle homogenate was diluted in 4X Laemmli buffer (0.25 M Tris, 4% SDS, 20% glycerol, 0.015% bromophenol blue and 10% 2-mercaptoethanol), and equal amounts of total protein (15 or 20 μg) were loaded in different wells on Criterion™ 4-20% TGX Stain-Free™ Precast Gels (Bio-Rad). Each participant's samples were loaded in adjacent lanes on the same gel. Each gel contained four to six internal standards of varying dilutions made from a mixed homogenate, taken from each sample in equal concentrations. These standards were used to form a calibration

curve, with density plotted against protein content. Protein abundance was then calculated from the measured band intensity for each sample on the gel, using the linear regression equation from the calibration curve (Murphy and Lamb 2013).

Gel electrophoresis was run for 20 minutes at 80 V and then for a further 60 to 90 minutes at 80 to 150 V. Transfer of proteins from the gel to 0.2 μm PVDF membrane at 25 V for 10 min was done via turbo transfer (Bio-Rad). Membranes were then blocked in 5% non-fat dry milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST) for 60 minutes. Membranes were then washed in TBST and incubated overnight at 4°C with the appropriate primary antibody (**Error! Reference source not found.**), prepared in TBST with 5% BSA and 0.02% sodium azide. Following four TBST washes, the membranes were then incubated at room temperature with the appropriate host species–specific secondary antibody for 60 min, before being exposed to a chemiluminescence solution. Images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Lab 5.0 software (Bio-Rad). Images are typically displayed with at least five bandwidths above and below the band of interest.

Table 3.5 –Western blot primary antibodies, and incubation conditions for all chapters.

Protein	Antibody	Dilution	Secondary Conditions
BMAL1	AB93806	1:5000 in 5%BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
Total OXPHOS	AB110413	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti mouse
PGC-1α	CST2178	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
DRP1	CST5341	1:1,000 in 5% BSA (in TBST)	1:5,000 in 5% SM (in TBST) – anti rabbit
MFN2	CST9482	1:1,000 in 5% BSA (in TBST)	1:5,000 in 5% SM (in TBST) – anti rabbit
p38 MAPK	CST9212	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-p38 MAPK Thr180/Tyr182	CST9211	1:1,000 in 5% BSA (in TBST	1:10,000 in 5% SM (in TBST) – anti rabbit
p53	CST2527	1:300 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST)– anti rabbit
LC3B	CST3868	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST)– anti rabbit
GLUT4	CST2213	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti mouse
AKT	CST9272	1:1,000 (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-AKT^{ser473}	CST9271	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
IRS1	CST2382	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
AS160	CST2447	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
mTOR	CST2983	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-mTOR$^{ser2448)}$	CST5586	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
Caspase-3	CST9662	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p70S6K (total)	CST9202	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
TSC2	CST4308	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-TSC2^{thr1462}	CST3617	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-4EBP1$^{thr37/46}$	CST2855	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
NFκB	CST8242	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit

BSA – bovine serum albumin, SM – skim milk

3.4.7 Citrate synthase activity assay

Citrate synthase (CS) activity was determined in triplicate on a 96-well microtiter plate by adding 7.5 µL of a 4 mg/mL muscle homogenate (freeze thawed in liquid nitrogen twice), 40 µL of 3mM acetyl CoA, 25 µL of 1mM 5,59-dithiobis(2-nitrobenzoic acid) (DTNB), 165 µL of 100 mM Tris

buffer (pH 8.3, kept at 30 °C). After addition of 15 µL of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate spectrophotometer (Bio-Rad) at 30°C, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity is reported as moles per hour per kilogram of protein.

3.4.8 Real-time quantitative polymerase chain reaction (RT-PCR)

3.4.8.1 RNA extraction

Frozen muscle samples (10 to 20 mg) were removed from -80 °C storage and placed on dry ice. 800 µL of TRIzol (Life Technologies, 15596-026) was added to each sample. One brand new stainless steel metal bead was then added to each tube and samples were then placed into the muscle homogeniser instrument (Tissue Lyser II, Qiagen) and run at 30 Hz for 2 min. Samples were then placed on ice for 5 min, before repeating homogenisation process for a further 2 min at 30 Hz. Muscle homogenate samples were then placed in -80 °C storage overnight. The following day, samples were spun at 15,800 RCF for 15 min, the supernatant was added to a new tube containing 250 µL of chloroform and left on ice for 5 min. Samples were centrifuged at 15,800 RCF for 15 min to achieve phase separation. The top phase was removed carefully (ensuring not to aspirate any of the interphase), added to a tube containing 400 µL of isopropanol (Sigma-Aldrich, 19516) and 10 µL of 5 M NaCl and left at room temperature for 10 min to precipitate. Samples were centrifuged at 15,800 RCF for 20 min at 4 °C. Isopropanol solution was removed and the RNA pellet was washed with 75% ethanol. Samples were centrifuged at 7,600 RCF for 8 min at 4 °C, ethanol was removed and the pellet was left to air dry for 5 min. 30 µL of DEPC water was added to each tube and all samples were stored at -80 °C. Prior to storage, separate aliquots were taken for RNA quantification and RNA integrity testing. Due to small muscle sample size, only samples from 7 participants in the Normal Sleep group were prepared for RT-PCR.

3.4.8.2 RNA quantification

RNA samples were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). 1 µL of each RNA sample was placed onto the spectrophotometer and readings for RNA concentration (ng/µL), the A260/280 ratio and A260/230 ratio was recorded (see **Appendix C**). No further dilution of RNA samples was used.

3.4.8.3 RNA integrity

Assessment of RNA integrity was performed using an automated microcapillary electrophoresis system (Experion, Bio-Rad Laboratories, Hercules, CA). This protocol was performed as per the manufacturer's instructions and generated an RNA quality Indicator score (RQI) from 1 to 10.

Any sample with an RQI greater than 7 was considered to be of good integrity and the RNA intact (Kuang, Yan et al. 2018).

3.4.8.4 cDNA synthesis

cDNA was synthesised using the iScript™ Reverse transcription supermix for RT-PCR kit (Bio-Rad Laboratories) (**Error! Reference source not found.**).

Table 3.6 - cDNA synthesis reaction components.

Component	Volume
iScript RT supermix	4 µL
RNA template	1 µg
Nuclease-free water	Up to 20 µL

The combined reaction components underwent priming at 5 min at 25°C, reverse transcription for 20 min at 46°C and reverse transcriptase inactivation for 1 min and 95°C in a thermal cycler. Five samples were selected at random and run with no reverse transcriptase (no RT control). DEPC water (180 µL) was added to all samples following cDNA synthesis and samples were stored at -20°C.

3.4.8.5 RT-PCR

Relative mRNA expression was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were designed using Primer-BLAST (Ye, Coulouris et al. 2012) to include all splice variants, and were purchased from Sigma-Aldrich (see Table 1 for primer details). All reactions were performed in duplicate on 384-well MicroAmp optical plates (4309849, Applied Biosystems) using an epMotion M5073 automated pipetting system (Eppendorf AG). Total reaction volume of 5 µL contained 2 µL of cDNA template, 2.5 µL of mastermix, and 0.3 µM or 0.9 µM primers. All assays ran for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

3.4.8.6 Primer design and data normalisation

The stability of six potential reference genes were determined using the web-based platform, RefFinder. This software compares four well-established methods for assessing the stability of reference genes and ranks them accordingly (Kuang, Yan et al. 2018). The three most stably expressed reference genes were TBP, B2M, and ACTB (**Table 3.7**) (Xie, Xiao et al. 2012). Expression of each target gene was normalised to the geometric mean of expression of the three reference genes (Vandesompele, De Preter et al. 2002), and using the $2^{-\Delta\Delta Ct}$ method (where Ct is the quantification cycle) (Schmittgen and Livak 2008). A list of housekeeping and target gene primer sequences are available in **Table 3.8**.

Table 3.7 - Evaluation of reference genes using RefFinder.

	Ranking					
Method	**1**	**2**	**3**	**4**	**5**	**6**
Delta CT	TBP	B2M	Cyclophilin	ACTB	18S	GAPDH
BestKeeper	ACTB	TBP	18S	GAPDH	B2M	Cyclophilin
NormFinder	TBP	B2M	ACTB	Cyclophilin	18S	GAPDH
Genorm	Cyclophilin	B2M	TBP	ACTB	18S	GAPDH
Recommended ranking	**TBP**	**B2M**	**ACTB**	**Cyclophilin**	**18S**	**GAPDH**

TBP – TATA-box binding protein, B2M – β-2-microglobulin, ACTB – actin β, 18S – 18S ribosomal 5 and GAPDH – glyceraldehyde-3-phosphate dehydrogenase

3.5 Statistical analysis

Statistical analyses were conducted using the statistical software package GraphPad Prism (V7.03). Prior to any statistical analysis, data was assessed for normal distribution using a Shapiro-Wilks test. Pre- to post-intervention changes in gene expression, protein content, mitochondrial respiratory function, glucose tolerance, citrate synthase activity, peripheral skin temperature and blood plasma hormone assessments were assessed for each group using a two-way repeated measures (RM) analysis of variance (ANOVA) (3 Groups x 2 Times). Significant effects of interaction (Group x Time), Time (Pre vs Post) and Group (NS vs SR vs SR+EX) are reported where effects are seen. Where there were significant effects, Bonferonni post-hoc testing was performed to locate the differences. All statistical analysis of gene expression and protein content data was run on raw values, and not fold-change data. Gene expression data in text is reported as percent fold-change from pre-intervention value ± % SD, 95% CI from fold-change data (as a percentage), with the *P* value from the raw data reported. Graphs represent fold-change from pre-intervention values, and individual responses are shown. A one-way RM ANOVA was used to assess differences between groups when it was not suitable to compare pre-intervention versus post-intervention values from within the same group. This was the case for myofibrillar/sarcoplasmic protein fractional synthetic rates, actigraphy and PSG sleep analysis data that was collected over the duration of the intervention period. Analysis of peripheral skin temperature amplitude and stability was performed by Prof. Karyn Esser and Assistant Prof. Elizabeth Schroeder, as previously described (Tranel, Schroder et al. 2015). All data in text, figures and tables are presented as mean ± standard deviation (SD), with P values ≤ 0.05 indicating statistical significance and confidence intervals are reported and set to 95% (unless otherwise stated).

Table 3.8 - RT-PCR primer sequences for all chapters.

Primer Name	Primer Sequence	Product Size (bp)	Efficiency (%)	Accession No.
Target Genes				
Bmal1	**F –** GCACGACGTTCTTTCTTCTGT **R –** GCAGAAGCTTTTTCGATCTGCTTTT	114	97.1	NM_001351820.1
Clock	**F** – CGTCTCAGACCCTTCCTCAAC **R** – GTAAATGCTGCCTGGGTGGA	71	92.5	NM_001267843.1
Cry1	**F –** ACTGCTATTGCCCTGTTGGT **R –** GACAGGCAAATAACGCCTGA	74	104.7	NM_004075.4
Per1	**F –** ATTCGGGTTACGAAGCTCCC **R –** GGCAGCCCTTTCATCCACAT	101	94.8	NM_002616
Per2	**F –** CATGTGCAGTGGAGCAGATTC **R –** GGGGTGGTAGCGGATTTCAT	109	93.8	NM_022817
Rev-erb α	**F –** ACAGATGTCAGCAATGTCGC **R –** CGACCAAACCGAACAGCATC	73	102.2	NM_005126
p53	**F –** GTTCCGAGAGCTGAATGAGG **R –** TTATGGCGGGAGGTAGACTG	123	101.8	NM_001126118.1
Tfam	**F –** CCGAGGTGGTTTTCATCTGT **R –** GCATCTGGGTTCTGAGCTTT	110	111.1	NM_003201.3
Drp1	**F –** CACCCGGAGACCTCTCATTC **R –** CCCCATTCTTCTGCTTCCAC	138	116.4	NM_001330380.1
Nrf2	**F –** AAGTGACAAGATGGGCTGCT **R –** TGGACCACTGTATGGGATCA	87	92.1	NM_001197297.1
Nrf1	**F –** CTACTCGTGTGGGACAGCAA **R –** AGCAGACTCCAGGTCTTCCA	143	102.5	NM_005011.5
Glut4	**F –** CTTCATCATTGGCATGGGTTT **R –** AGGACCGCAAATAGAAGGAAGA	75	85.4	NM_001042.2
Pgc-1α total	**F –** CAG CCT CTT TGC CCA GAT CTT **R –** TCACTGCACCACTTGAGTCCAC	101	103.6	NM_013261.3
Myostatin	**F –** GGAGAAGATGGGCTGAATCCG **R –** GCATCGTGATTCTGTTGAGTGC	111	98.9	NM_005259
β-Had	**F –** TGGACAAGTTTGCTGCTGAACAT **R –** TTTCATGACAGGCACTGGGT	137	80.6	NM_001184705.2
Pdk4	**F –** GCAGCTACTGGACTTTGGTT **R –** GCGAGTCTCACAGGCAATTC	84	99.7	NM_002612.4
Ampkα	**F –** CAGGGACTGCTACTCCACAGAGA **R –** CCTTGAGCCTCAGCATCTGAA	85	76.8	NM_001355028.1
Murf1	**F –** CCGTCGAGTGACCAAGGAGA **R –** CCAGGATGGCATACAACGTG	80	98.6	NM_032588
Mafbx	**F –** GCAGCTGAACAACATTCAGATCAC **R –** CAGCCTCTGCATGATGTTCAGT	97	99.5	NM_058229
Mfn1	**F –** CAGAAAGTGGTGTGGCACTTG **R –** TTTCACTGCTGACTGCGAGAT	104	112	NM_033540.2
Mfn2	**F –** CCCCCTTGTCTTTATGCTGATGTT **R –** TTTTGGGAGAGGTGTTGCTTATTTC	168	140	NM_014874.3
Foxo1	**F –** TGAGGGTTAGTGAGCAGGTTAC **R –** GGACTGCTTCTCTCAGTTCCT	73	108.1	NM_002015
Foxo3	**F –**TTGGTTTGAACGTGGGGAAC **R –**TGTGTCAGTTTGAGGGTCTGC	119	91.1	NM_001455.4
Mighty	**F –** CCAACTCCGGAGCAAATTTTTCA	106	94.7	NM_024595

	R – TCCGAAGCACAAGCTTCACT			
Lc3b	**F –** GAGAAGACCTTCAAGCAGCG **R –** TTATCACCGGGATTTTGGTTGGA	94	102.4	NM_022818.5
p62	**F –** AGAATCAGCTTCTGGTCCATCGG **R –** CTTTTCCCTCCGTGCTCCAC	129	103.2	NM_003900.5
Pink1	**F –** TGTGGAACATCTCGGCAGGT **R –** AGTGACTGCTCCATACTCCC	110	101.8	NM_032409.2
Park2	**F –** CACGACCCTCAACTTGGCTA **R –** GGTACCGGTTGTACTGCTCT	112	103.4	NM_013988.2
Housekeeping genes				
Gapdh	**F –** AATCCCATCACCATCTTCCA **R –** TGGACTCCACGACGTACTCA	82	106	NM_002046.7
B2M	**F –** TGCTGTCTCCATGTTTGATGTATCT **R –** TCTCTGCTCCCCACCTCTAAGT	86	98	NM_004048.2
ACTB	**F –** GAGCACAGAGCCTCGCCTTT **R –** TCATCATCCATGGTGAGCTGGC	70	107	NM_001101.3
TBP	**F –** CAGTGACCCAGCAGCATCACT **R –** AGGCCAAGCCCTGAGCGTAA	205	99	NM_003194.4
Cyclophilin	**F –** GTCAACCCCACCGTGTTCTTC **R –** TTTCTGCTGTCTTTGGGACCTTG	100	100	NM_021130.4
18S	**F –** CTTAGAGGGACAAGTGGCG **R –** GGACATCTAAGGGCATCACA	71	99	NR_003286.2

F – Forward primer, **R** – Reverse primer

Chapter 4. The Effect of Sleep Restriction, With or Without Exercise, on Measures of Peripheral Circadian Rhythms

The aim of this chapter was to investigate the effects of sleep restriction and exercise on circadian rhythms (as assessed via peripheral skin temperature and skeletal muscle clock gene expression and protein content). As demonstrated in the literature review, the consequences of circadian misalignment are now well established and far reaching. The data from this chapter will help to establish the effect of sleep loss on circadian rhythms and the potential of exercise to realign these effects. Data from this study has been presented at Exercise and Sport Science Australia (ESSA) Conference, 2018, the American College of Sports Medicine (ACSM) conference, 2018 and The Congress of the European College for Sports Sciences (ECSS), 2018.

4.1 Introduction

Circadian rhythms are the daily variations, which occur across approximately a 24-h period, to a range of physiological processes (such as metabolism, hormone secretion, body temperature, and the sleep/wake cycle). These circadian rhythms are generated at the molecular level in almost every tissue of the body via a transcriptional:translational feedback loop, comprised of core circadian clock genes (e.g., *Brain and muscle Arnt-like protein-1* (*Bmal1), Circadian locomotor output cycles kaput (Clock), Period 1 and 2 (Per1/2)* and *Cryptochrome 1 and 2* (*Cry1/2).* This feedback loop is regulated centrally via the suprachiasmatic nucleus (SCN) of the hypothalamus, which is entrained primarily via light input to the retina and the corresponding signals delivered to the SCN via the retinal hypothalamic tract (Dibner, Schibler et al. 2010). In turn, the SCN transmits this rhythmic information to peripheral tissues via both hormonal and neuronal pathways (Dibner, Schibler et al. 2010). However, circadian rhythms are also regulated independently within peripheral tissues (such as skeletal muscle) by their specific internal molecular clocks, which can be influenced by other external zeitgebers (time cues) such as meal timing and exercise (Zambon, McDearmon et al. 2003, Wolff and Esser 2012, Sato, Basse et al. 2019). Both the SCN and the peripheral clocks work in unison to synchronise the physiological processes at the whole-body level and allow the body to account for and adapt to variations in exogenous environmental stimuli. However, when the central or peripheral clocks fail to adapt to these changes in the environmental stimuli misalignment of normal circadian rhythms can occur. This misalignment occurs when the body's endogenous circadian rhythms are not aligned with exogenous zeitgebers, such as the sleep/wake cycle, feeding habits, and physical activity, causing a desynchrony between central and peripheral circadian processes (Leproult, Holmback et al. 2014).

Circadian rhythms can be assessed in a number of ways. The release of melatonin from the pineal gland is often assessed and is closely associated with sleep-onset (Cajochen, Krauchi et al. 2003). Further, increases in melatonin also correspond with a decrease in core body temperature and a subsequent increase in peripheral skin temperature; both of these parameters are good predictors for the onset of sleep and reliable models of circadian rhythm (Krauchi, Cajochen et al. 1999, Cajochen, Krauchi et al. 2003). Given the association between skin temperature and sleep onset, peripheral (wrist) skin temperature has been used and validated as a simple, non-invasive method for monitoring circadian rhythms over extended durations in human participants (Sarabia, Rol et al. 2008, Tranel, Schroder et al. 2015) and can offer insight into the effect of different interventions on circadian rhythms. Specifically, amplitude and stability, which are two descriptors of circadian rhythm that are often used to characterise biological processes, can be assessed from skin temperature (Stenvers, Jongejan et al. 2019). Circadian amplitude refers to the extent of daily oscillations and is defined as the difference between the mean value of the data and the maximum (or minimum) value, while the stability of the circadian rhythm refers to its phase homogeneity, during the intervention period. The greater the similarity between

oscillatory patterns each day, the higher the stability of the rhythm, which is indicative of greater alignment of circadian processes (Sarabia, Rol et al. 2008). Decreases in these two parameters have been associated with increased risk of a range of metabolic conditions (Corbalan-Tutau, Madrid et al. 2011, Tranel, Schroder et al. 2015, Harfmann, Schroder et al. 2017). Furthermore, at the molecular level, the expression of key skeletal muscle clock genes demonstrate a robust 24-h diurnal rhythm, with authors associating the disruption of these rhythms with the deterioration of metabolic health (van Moorsel, Hansen et al. 2016). Assessing changes in the expression of these genes at a given time-point may indicate the disruption of these diurnal rhythms. When assessed together, these measures can be used to determine changes to peripheral circadian rhythms in response to interventions, such as bouts of sleep restriction and potentially exercise.

The detrimental effect of sleep loss on a number of metabolic processes (e.g., insulin resistance and glucose tolerance) may occur, at least in part, via the misalignment of circadian rhythms (Cajochen, Jewett et al. 2003, Cedernaes, Osler et al. 2015, Cedernaes, Schonke et al. 2018). This is supported by the observation that one night of sleep deprivation (i.e., 40 h of sustained wakefulness) causes a phase-delay of melatonin release (Cajochen, Jewett et al. 2003). One night of sleep deprivation, such as that commonly experienced by 20% of the world's population who perform shift-work, can also alter the expression of core clock genes (i.e., *Bmal1* and *Cry1* gene expression; Cedernaes, Osler et al. (2015)) and the content of clock proteins (i.e., BMAL1; Cedernaes, Schonke et al. (2018)) in skeletal muscle. As more than 43% of genes that encode proteins display circadian rhythmicity somewhere in the body (often in an organ-specific manner; Zhang, Lahens et al. (2014)), and because a large number of genes are regulated via the transcription factor activity of the molecular clock proteins, alterations to their expression caused by sleep loss may have wide-ranging effects on aspects of metabolism (Liu, Li et al. 2007, Andrews, Zhang et al. 2010). The functional significance of disrupting the molecular clock has been shown in animal knockout models (i.e., homozygous knockout of *Clock* and *Bmal1* genes), which display reduced glucose tolerance, mitochondrial respiratory function, and contractile function (Andrews, Zhang et al. 2010, Pastore and Hood 2013). This study aimed to expand on the previous findings obtained from models of total sleep deprivation and investigate the effect of a sleep restriction protocol on the expression of skeletal muscle clock gene and protein expression.

The detrimental metabolic changes that occur in response to circadian misalignment may be ameliorated by performing exercise. Beyond its direct benefits, exercise is able to cause phase shifts of circadian rhythms in both humans and mice, as assessed via both melatonin release and clock gene expression (Van Reeth, Sturis et al. 1994, Wolff and Esser 2012, Youngstedt, Elliott et al. 2019). At the molecular level, it has been reported that resistance exercise may modulate and alter the expression of core circadian clock genes (Zambon, McDearmon et al. 2003). These findings suggest that exercise may have the potential to 'realign' circadian rhythms that have become misaligned due to interventions such

as sleep restriction. Exercise training has also been demonstrated to ameliorate deficits in exercise tolerance, mitochondrial content, and the content of mitochondrial signalling proteins observed in CLOCK knockout mice (Pastore and Hood 2013). However, whether endurance exercise is able to 'realign' or shift circadian rhythms that have been desynchronised by sleep loss has not been studied in humans. Accordingly, this study aimed to examine whether sleep restriction causes a shift in circadian rhythms, as assessed via gene and protein expression of the molecular clock, and the stability and amplitude measures of peripheral skin temperature. A second aim was to determine whether exercise is able to maintain or alter any observed changes in circadian rhythms. It was hypothesised that sleep restriction would cause the desynchronization of circadian rhythms and that exercise would have a stabilising influence on these measures. The extended application of such results may have further implications for therapeutic interventions, considering the connection between sleep loss-induced circadian misalignment and detrimental metabolic consequences, such as reductions in glucose tolerance.

4.2 Methodology and procedures

Full details of all experimental procedures can be found in **Chapter 3 General Methodology**. Details specific to the methodology and data presented in this chapter are outlined below.

4.2.1 Study overview

For an overview of the study protocol specified to this chapter, see **Figure 4.1**. Following the initial screening and assessment procedures, 24 healthy men underwent baseline fitness testing (see section 3.3.2) and a pre-study muscle biopsy to determine baseline mitochondrial respiration values (as outlined in Chapter 3). Participants were then allocated into one of three, matched experimental groups; Normal Sleep (NS), Sleep Restriction (SR) or Sleep Restriction and Exercise (SR+EX). Baseline participant characteristics for the three groups are shown in **Table 3.2**.

The experimental component of the study consisted of an eight-night stay within the sleep laboratory. The protocol for each of the three experimental groups consisted of an initial two nights of baseline sleep, followed by a five-night intervention period and then a final night of recovery sleep. During the baseline sleep period all participants spent 8 h TIB, between 23:00 h and 07:00 h. For the remaining 5 nights, the NS group spent 8 h TIB (between 23:00 h and 07:00 h), while both the SR and SR+EX groups spent 4 h TIB (between 03:00 and 07:00) each night. The SR and SR+EX group TIB (03:00 h – 07:00 h) was standardised to ensure all participants woke at the same time each morning and that the subsequent testing procedures were performed following the same time duration post waking. Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux to reduce the effect of lighting on circadian rhythms (Duffy and Wright 2005).

Two experimental testing sessions were conducted during the participant's stay. At 10:00 h on the morning of Day 3 (following 2 nights of baseline sleep), the pre-intervention resting skeletal muscle biopsy was collected as per section **3.3.3**, for the assessment of genes and proteins related to the regulation of the molecular circadian clock. A post-intervention muscle biopsy was then collected on the morning of Day 8 at 10:00 h (following the final night of the sleep intervention). Participants wore an iButton temperature recorder on their wrist for the duration of the study to assess the amplitude and stability of peripheral skin temperature rhythms (as per section **3.3.6**). For the duration of the study, each participant was provided with a standardised diet relative to body mass consisting of fixed proportions of carbohydrates (4.5 $g.kg^{-1}.d^{-1}$), protein (1.5 $g.kg^{-1}.d^{-1}$) and fat (1 $g.kg^{-1}.d^{-1}$). All meal times (there were six throughout the day) were kept constant throughout the study and participants were asked to eat all food provided. Meal timings were the same for all groups. An identical menu was provided on the days prior to both experimental sessions (an example of the daily diet is provided in **Appendix B**). Participants remained within the laboratory and were monitored throughout each study. To match

the activity count prior to the commencement of the study, participants were instructed to walk outside the facility at designated periods throughout the day, while being accompanied by a member of the research staff. Waking hours were spent watching television, reading, working on a computer, or talking to staff. Approval of the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee (HRE15-294).

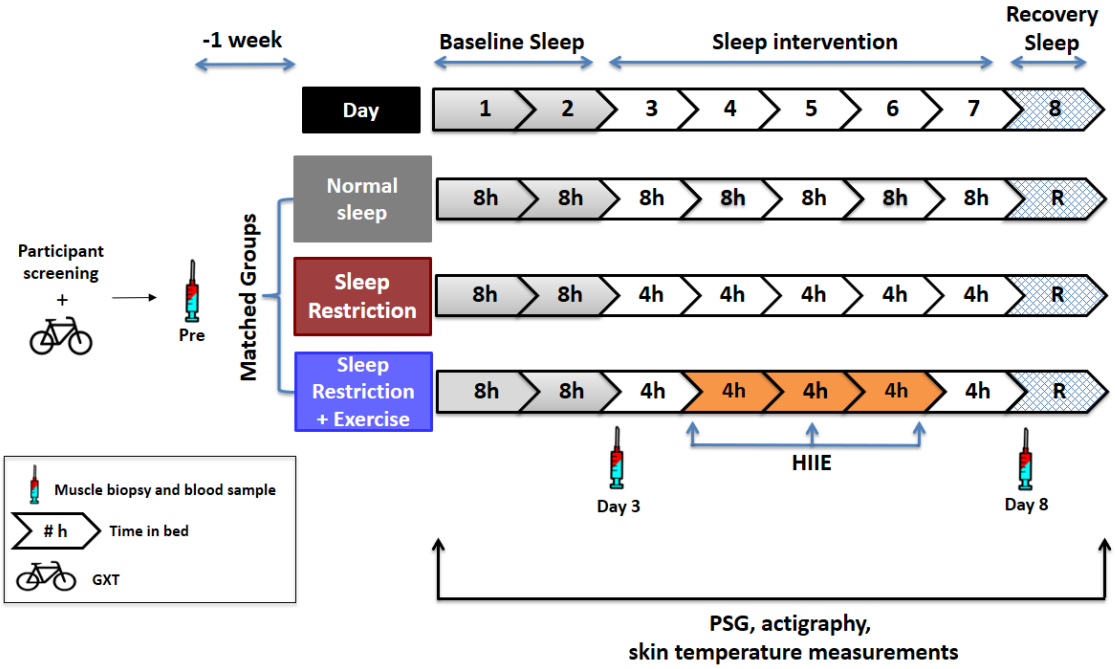

Figure 4.1 - Schematic representation of the study protocol in Chapter 4.
GXT – Graded exercise test, PSG – polysomnography sleep analysis, HIIE – high-intensity interval exercise, R – *ad libitum* recovery sleep, participant screening refers to medical questionnaires, exclusion criteria and, habitual sleep and physical activity monitoring.

4.2.2 High-intensity interval exercise (HIIE)

The high-intensity interval exercise sessions (consisting of 10 x 60-s intervals at 90% $\dot{W}_{peak}$, interspersed by 75-s of active recovery on a cycle ergometer) were conducted in the SR+EX group only, at 10:00 h on Day 4, 5 and 6 as reported in section **3.3.5.**

4.2.3 Sleep monitoring

Participants wore sleep actigraphy watches throughout the study to monitor parameters of sleep (including TST, WASO, SOL, and sleep efficiency). Polysomnography sleep monitoring data was also collected each night during the study. Analysis of the PSG data (n=4 in each group) from night 6 of the intervention is presented in **Table 4.4**. For further details regarding the sleep monitoring throughout the study see section **3.3.1.**

4.2.4 Wrist skin temperature measurements

The amplitude and stability of peripheral skin temperature was recorded every 10 minutes throughout the study using iButtons (as per section **3.3.6**).

4.2.5 Muscle sample analysis

Three muscle biopsies were collected throughout the study in accordance with section **3.3.3.** Muscle biopsies that were collected on Day 3 and Day 8 of the study protocol (i.e., pre- and post-intervention) were assessed according to the techniques described below.

4.2.5.1 Western blot analysis

Skeletal muscle protein content of the molecular clock protein BMAL1, was assessed as per the western blotting protocol outlined in section **3.4.6**. Details relating to the relevant antibody conditions are outlined in **Table 4.1**.

Table 4.1 – **Western blot primary antibodies, and incubation conditions for Chapter 4.**

Protein	Antibody	Dilution	Secondary Conditions
BMAL1	AB93806	1:5000 in 5%BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit

BSA – bovine serum albumin, SM – skim milk

4.2.5.2 Real-time quantitative polymerase chain reaction (RT-PCR)

The expression of genes relating to the regulation of the molecular clock and circadian rhythms were assessed as per the RT-PCR protocol outlined in section Real-time quantitative polymerase chain reaction (RT-PCR)**3.4.8.** Details relating to the specific primers used within this chapter are presented in **Table 4.2 - RT-PCR primer sequences for Chapter 4.Table 4.2.**

Table 4.2 - RT-PCR primer sequences for Chapter 4.

Primer Name	Primer Sequence	Product Size (bp)	Efficiency (%)	Accession No.
Target Genes				
Bmal1	**F –** GCACGACGTTCTTTCTTCTGT **R –** GCAGAAGCTTTTTCGATCTGCTTTT	114	97.1	NM_001351820.1
Clock	**F** – CGTCTCAGACCCTTCCTCAAC **R** – GTAAATGCTGCCTGGGTGGA	71	92.5	NM_001267843.1
Cry1	**F –** ACTGCTATTGCCCTGTTGGT **R –** GACAGGCAAATAACGCCTGA	74	104.7	NM_004075.4
Per1	**F –** ATTCGGGTTACGAAGCTCCC **R –** GGCAGCCCTTTCATCCACAT	101	94.8	NM_002616
Per2	**F –** CATGTGCAGTGGAGCAGATTC **R –** GGGGTGGTAGCGGATTTCAT	109	93.8	NM_022817
Rev-erb α	**F –** ACAGATGTCAGCAATGTCGC **R –** CGACCAAACCGAACAGCATC	73	102.2	NM_005126

F – Forward primer, **R** – Reverse primer

4.2.6 Statistical analysis

Statistical analyses were conducted using the statistical software package GraphPad Prism (V7.03). Pre- to post-intervention changes in gene expression, protein content, and peripheral skin temperature were assessed for each group using a two-way repeated measures (RM) analysis of variance (ANOVA) (3 Groups x 2 Times). Significant effects of interaction (Group x Time), Time (Pre vs Post) and Group (NS vs SR vs SR+EX) are reported where effects are seen. Where there were significant effects, Bonferonni post-hoc testing was performed to locate the differences. All statistical analysis of gene expression and protein content data was run on raw values, and not fold-change data. Gene expression data in text is reported as percent fold-change from pre-intervention value ± % SD, 95% CI from fold-change data (as a percentage), with the *P* value from the raw data reported. Graphs represent fold-change from pre-intervention values, and individual responses are shown. A one-way RM ANOVA was used to assess differences between groups when it was not suitable to compare pre-intervention versus post-intervention values from within the same group. This was the case for actigraphy and PSG sleep analysis data that was collected over the duration of the intervention period. Analysis of peripheral skin temperature amplitude and stability was performed by Prof. Karyn Esser and Assistant Prof. Elizabeth Schroeder, as previously described (Tranel, Schroder et al. 2015). All data in text, figures and tables are presented as mean ± standard deviation (SD), with P values ≤ 0.05 indicating statistical significance and confidence intervals are reported and set to 95% (unless otherwise stated).

4.3 Results

4.3.1 Actigraphy Sleep Analysis

There were no significant differences for baseline values of total sleep duration (min), sleep onset latency (SOL), wake after sleep onset (WASO), or sleep efficiency between any of the groups (P>0.05) (**Table 4.3**).

The NS group showed no significant change in sleep parameters between the baseline and intervention period (P>0.05). The SR group had a reduction in total sleep duration (mean difference ± SD min, 95% CI min, P value) (-224 ± 20 min, CI [-241, -203 min], P<0.001), SOL (-18 ± 18 min, CI [-30, -6 min], P<0.001) and WASO (-16 ± 9 min, CI [-25, -7 min], P<0.001) in the intervention period, compared to baseline values. The SR+EX group had a similar reduction in sleep duration for the intervention period, compared to baseline values (-223 ± 9 min, CI [-242, -204 min], P<0.001), WASO (-16 ± 13 min, CI [-25, -7 min], P<0.001) and SOL (-11 ± 8 min, CI [-23, 1 min], P=0.06).

Total sleep duration during the intervention period was significantly reduced in the SR group compared to the NS group (-219 ± 8 min, CI [-236, -202 min], P<0.001), and the SR+EX group compared to the NS group (-214 ± 8 min, CI [-231.5, -197 min], P<0.001). Further, SOL (-14 ± 4 min, CI [-23, -7 min], P<0.001) and WASO (-26 ± 4 min, CI [-37, -16 min], P<0.001) were significantly reduced during the sleep intervention in the SR group, and the SR+EX group (SOL -16 ± 4 min, CI [-25, -8 min], P=0.001; WASO -18 ± 5.0 min, CI [-30, -8 min], P=0.001), compared to the NS group. There were no significant differences between the SR and SR+EX groups during the intervention period for total sleep duration (-5 ± 3 min, CI [-23 to 12 min], P=0.718), SOL (1.0 ± 1 min, CI [-6 to 10 min], P=0.851) or WASO (-7.0 ± 4 min, CI [-18 to 3 min], P=0.228).

Table 4.3 - Actigraphy sleep analysis for both the baseline (average of two nights) and the intervention period (average of 5 nights) for each group.

	NS		SR		SR+EX	
	Baseline	**Intervention**	**Baseline**	**Intervention**	**Baseline**	**Intervention**
Sleep Duration (min)	448.0 ± 25	449 ± 22	452 ± 17	230 ± 5*#	459 ± 9	235 ± 5*#
SOL (min)	13 ± 8	17 ± 9	21 ± 8	3 ± 2*#	13 ± 8	1 ± 1 #
WASO (min)	29 ± 12	34 ± 7	25 ± 12	9 ± 3*#	32 ± 14	10 ± 5 *#
Sleep efficiency %	87 ± 6	86 ± 6	88 ± 3	92. ± 3	89 ± 4	93 ± 3

Values are mean ± SD, Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction + Exercise (SR+EX), Sleep Onset Latency (SOL), Wake After Sleep Onset (WASO), * P<0.05 compared to within group baseline, # P<0.05 compared to control (NS) during the intervention period.

4.3.2 Polysomnography sleep analysis from night 6

Polysomnography sleep analysis was conducted on night 6 of the sleep intervention and used to assess the sleep architecture (sleep stages) of the participants sleep (n=4, in each group), as an objective indicator of sleep quality (**Table 4.4**). Total sleep time (TST) was significantly reduced (mean difference ± SD min, 95% CI min, *P* value) in the SR (-207 ± 10 min, CI [-231, -184 min], *P*<0.001) and SR+EX (-204 ± 10 min, CI [-229, -181 min], *P*<0.001) groups, compared to the NS group. There was no significant difference for TST between SR and SR+EX (2 ± 2 min, CI [-26, 21 min], *P*>0.999). Total nREM sleep duration (N1, N2 and N3 stages combined) (mean difference nREM ± SD min, 95% CI min, *P* value) was significantly lower in the SR (-169 ± 14 min, CI [-205, -134 min], *P*<0.001) and SR+EX (-160 ± 12 min, CI [-195, -124 min], *P*<0.001) groups compared to the NS group, which resulted from reductions in the time spent in N1 (SR: -18 ± 5 min, CI [-31, -6 min], *P*=0.005 and SR+EX: -22 ± 5 min, CI [-34, -10 min], *P*=0.002) and N2 stages of sleep (SR: -154 ± 23 min, CI [-211, -96 min], *P*<0.001 and SR+EX: -137 ± 17 min, CI [-194, -80 min], *P*<0.001). Time spent in REM sleep was significantly reduced in the SR (-38 ± 12 min, CI [-71, -5 min], *P*=0.024) and SR+EX (-45 ± 9 min, CI [-78, -12 min], *P*=0.009) groups, compared to the NS group. Despite differences in TST between NS and both the SR and SR+EX groups, there was no significant difference in the absolute amount of the N3 stage of nREM sleep between any of the groups. However, when measured relatively (i.e., percentage TST spent in N3) (mean difference TST in N3 ± SD %, 95% CI, *P* value), both the SR (16 ± 4%, CI [4, 27 %], *P*=0.012) and SR+EX (14 ± 3%, CI [2, 26%], *P*=0.025) groups had a higher percentage of TST spent in N3, compared to the NS group. There was no significant difference for the percentage of TST in N3 between the SR and SR+EX groups (2 ± 5 %, CI [-10, 14 %] *P*>0.999).

4.3.3 Actigraphy and PSG comparison

A comparison of TST values as assessed by PSG and wrist watch actigraphs were compared for night 6 of the sleep study protocol (n=4, in each group). There was no significant difference in values for TST between PSG and actigraphy for any of the groups (mean difference ± SD min, 95% CI min, *P* value), NS – 27 ± 47 min, CI [-14, 69 min], *P*=0.270, SR – 3 ± 7 min, CI [-39, 45 min], *P*>0.999 and SR+EX – 7 ± 10 min, CI [-35, 49 min], *P*>0.999 (**Figure 4.2**).

Table 4.4 - Polysomnography sleep analysis from night 6 of the study.

	NS (n=4)	SR (n=4)	SR+EX (n=4)
TST (min)	442 ± 19	235 ± 4 #	237 ± 1 #
REM (min)	103 ± 14	65 ± 20 #	58 ± 12 #
REM (% of TST)	23 ± 3	28 ± 8	25 ± 5
nREM (min)	339 ± 20	170 ± 18 #	179 ± 12 #
nREM (% of TST)	77 ± 3	72 ± 8	75 ± 5
N1 (min)	27 ± 10	9 ± 2 #	5 ± 2 #
N1 (% of TST)	6 ± 2	4 ± 1	2 ± 1 #
N2 (min)	240 ± 32	86 ± 34 #	103 ± 9 #
N2 (% of TST)	54 ± 7	37 ± 15	43 ± 4
N3 (min)	72 ± 17	75 ± 18	71 ± 14
N3 (% of TST)	16 ± 3	32 ± 7 #	30 ± 6 #
Wake (min)	18 ± 15	4 ± 4	2 ± 1
Wake (% of TST)	4 ± 4	2 ± 2	1 ± 1
WASO (min)	18 ± 15	4 ± 4	2 ± 1
Sleep efficiency (%)	93 ± 5	98 ± 2	99 ± 1
Sleep latency (min)	13 ± 15	1 ± 1	1 ± 1

Values are mean ± SD NS – Normal Sleep group, SR – Sleep Restriction, SR+EX – Sleep Restriction + Exercise, TST – total sleep time, REM – Rapid eye-movement, nREM – non-rapid eye-movement, N1 – nREM stage 1, N2 – nREM stage 2, N3 – nREM stage 3, WASO – Wake after sleep onset. # denotes significantly different from the NS group (P<0.05).

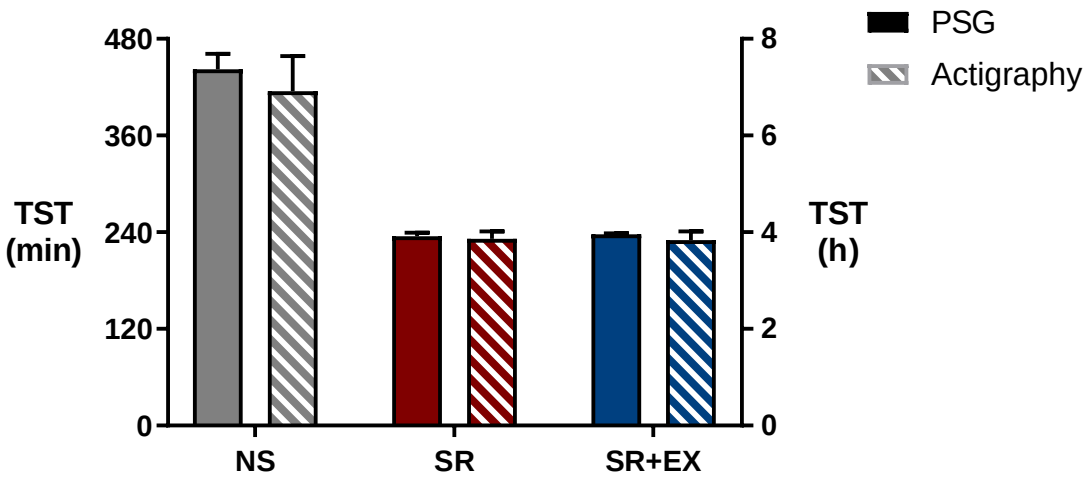

Figure 4.2 - Comparison of Day 6 sleep analysis between polysomnography and sleep watch actigraphy total sleep time (TST) analysis.
NS – Normal sleep, SR – Sleep restriction, SR+EX – Sleep restriction + exercise.

4.3.4 Wrist skin temperature analysis

There was a significant interaction (*P*= 0.042) and time (*P*<0.001*)* effect for wrist skin temperature amplitude, which showed (mean amplitude change ± SD °C, 95% CI °C, *P* value) a significant decrease in both the SR (0.83 ± 0.72°C, CI [0.20, 1.45 °C], *P*=0.007) and SR+EX groups (0.87± 0.70°C, CI [0.20, 1.54°C], *P*=0.018), but not the NS group (0.04 ± 0.67°C, CI [-0.59, 0.66°C], *P*>0.999) between baseline values and the sleep intervention period (i.e. Day 3 to Day 8) (**Figure 4.3**). There was no interaction (*P*=0.374) or time effect (*P*=0.437)) for wrist skin temperature stability between baseline values and the duration of the intervention.

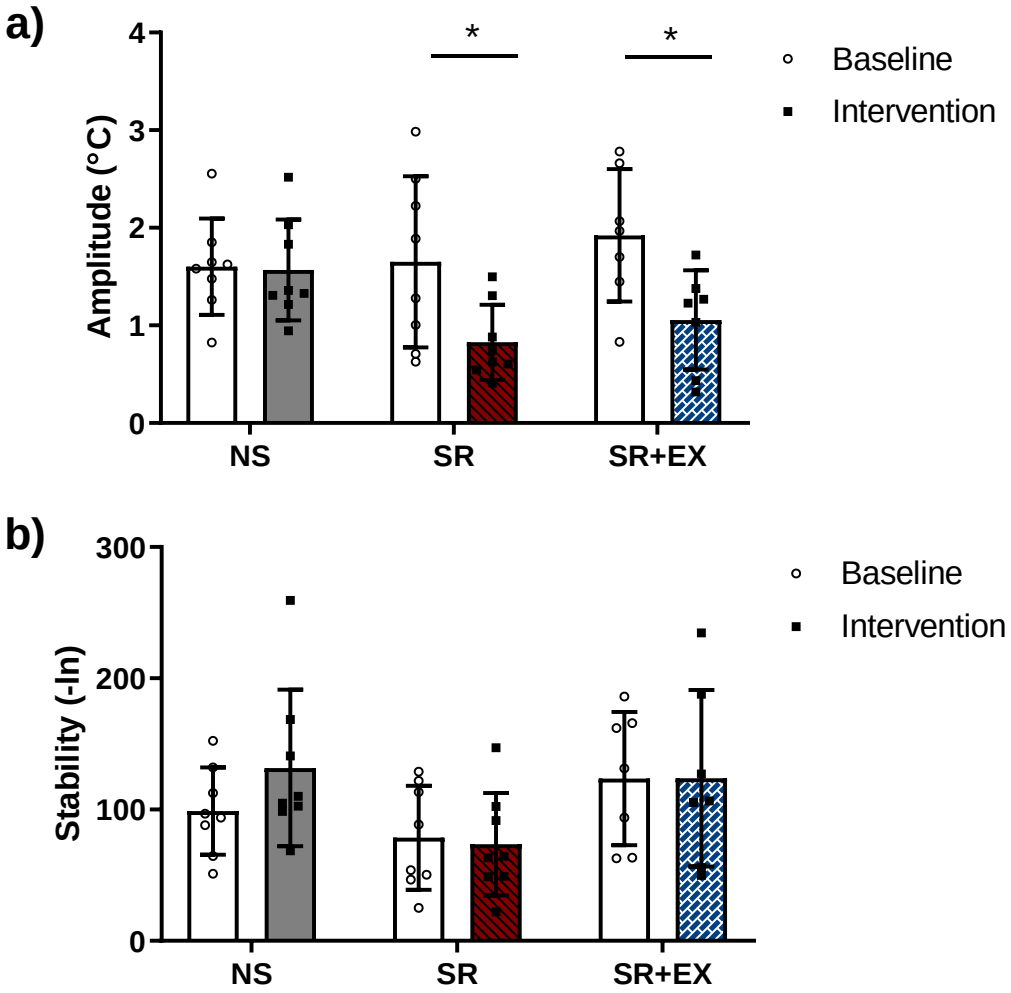

Figure 4.3 - Amplitude and stability of skin temperature measurements at baseline and throughout the sleep intervention.
a) Amplitude and b) stability. Baseline measurements are Day 1, 2 and 3 (until 23:00 h) and the intervention period is Day 3 (from 23:00 h) to Day 8 (08:00 h). NS – Normal Sleep (n=8), SR – Sleep Restriction (n=8), and SR+EX – Sleep Restriction and Exercise (n=7). * Denotes significant difference between baseline and intervention values for each group (*P*<0.05).

4.3.5 mRNA expression of key circadian clock genes

There were no significant interaction effects for *Bmal1* (*P*=0.154), *Clock* (*P*=0.775), *Per1* (*P*=0.527), *Per2* (*P*=0.719), *Cry1* (*P*=0.466) or *Rev-Erb α* (*P*=0.583) mRNA expression in skeletal muscle samples from pre- to post-intervention. However, there was a significant effect of time for *Bmal1* (*P*=0.041), with post-hoc analysis indicating a significant reduction in mRNA expression from pre to post intervention in the SR group (-29 ± 33%, CI [-6, 64%] *P*=0.031). This change was not seen in either the NS group (2 ± 22%, CI [-40, 35%], *P*>0.999) or the SR+EX group (7 ± 51%, CI [-29, 43%], *P*>0.999) (**Figure 4.4**).

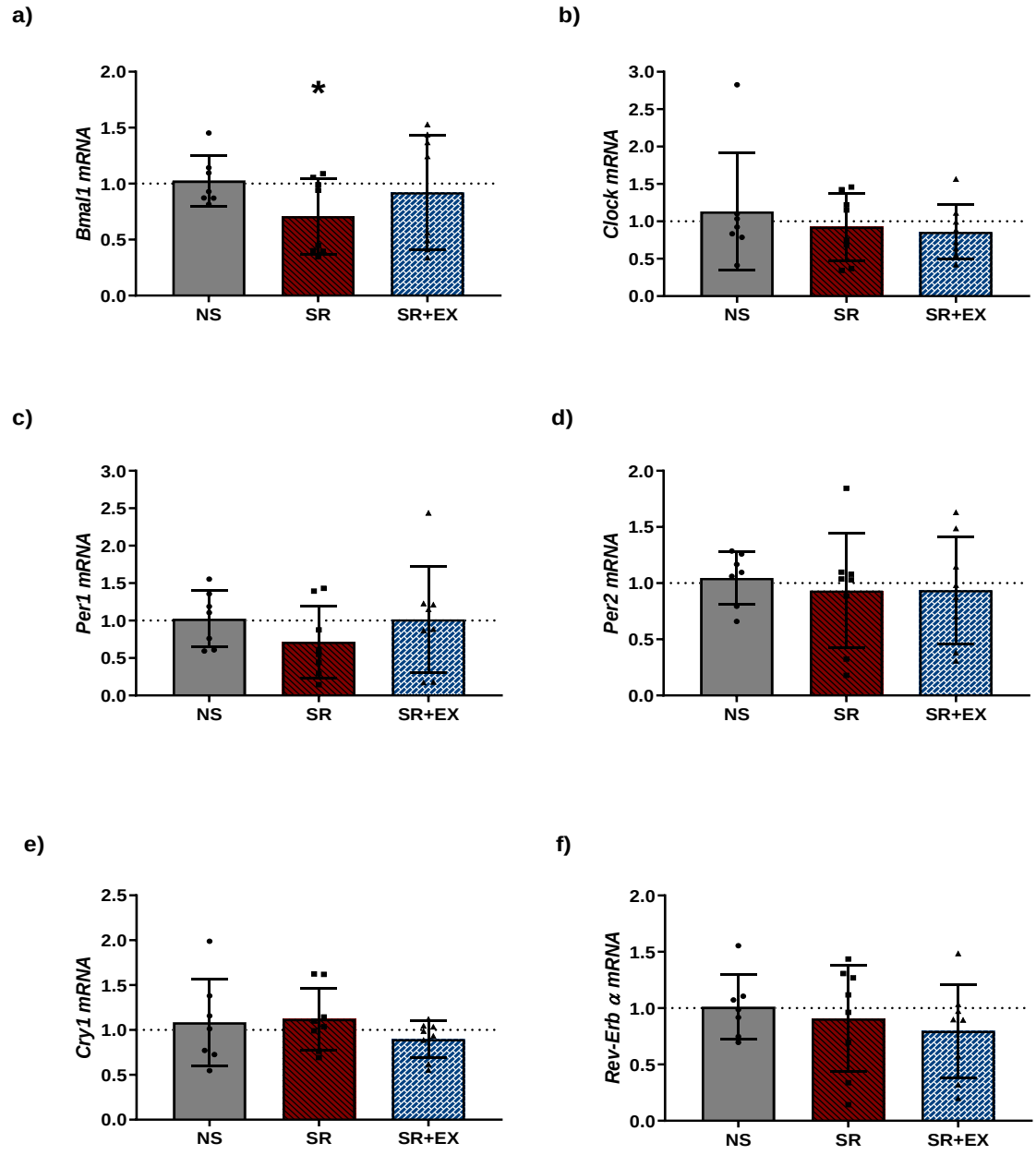

Figure 4.4 - Skeletal muscle mRNA expression of key clock genes.
Data is normalised to pre-intervention values (i.e., fold change from pre-intervention). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). * Denotes significant difference for each group from pre- to post-intervention (p<0.05). n=7 in the NS group, n=8 in the SR and SR+EX groups.

4.3.6 BMAL1 Protein Content

There was no significant interaction or time effect (P=0.778 and P=0.079, respectively) for BMAL1 protein content from pre- to post-intervention (**Figure 4.5**).

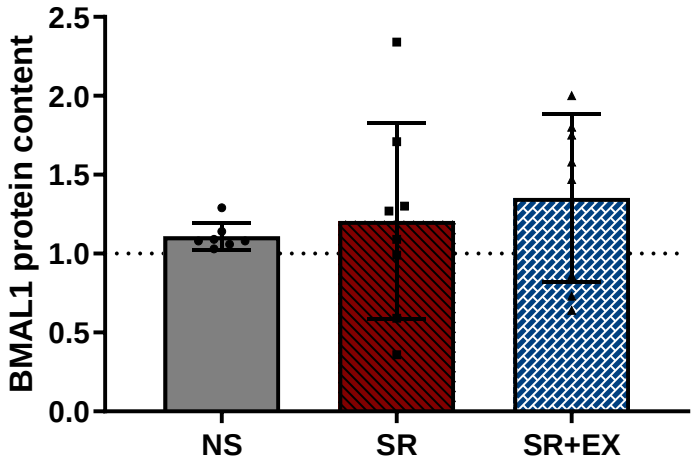

Figure 4.5 - Skeletal muscle Brain and muscle ARNT like protein-1 (BMAL1) protein content.
Values are normalised to with-in group pre-intervention values (i.e., fold change from pre intervention). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). n=8 in all groups.

4.4 Discussion

4.4.1 Main Findings

The aim of this chapter was to investigate the effect of a sleep restriction protocol (5 nights, 4 h TIB each night) on markers of peripheral circadian rhythms (i.e., skin temperature, and skeletal muscle clock gene expression and protein content) and the influence of HIIE on these markers. Sleep duration (i.e., TST) was significantly reduced in both the SR and SR+EX groups during the intervention period, compared to the NS group. This was comprised of a reduction in N1, N2, and REM, but not N3, stages of sleep. The assessment of peripheral skin temperature showed a reduction in amplitude in both the SR and SR+EX groups from pre- to post-intervention. There was a significant decrease in *Bmal1* mRNA gene expression from pre- to post-intervention in the SR group only, but this was not reflected by changes in BMAL1 protein content. These findings highlight a detrimental effect of sleep restriction on indices of peripheral circadian rhythms, and indicate that HIIE was able to mitigate these effects at a molecular level.

4.4.2 Sleep quantity and quality

It has been suggested that changes to a person's regular sleep/wake cycle can influence circadian rhythms (Moller-Levet, Archer et al. 2013). The sleep intervention in this study reduced TIB from 8 h to 4 h, by delaying scheduled sleep onset from 23:00 h to 03:00 h in the SR and SR+EX groups. Total sleep time (TST) was significantly reduced in both the SR and SR+EX groups, as assessed via both wrist watch actigraphy (assessed for the duration of the study) and PSG (assessed only on night 6). As previously reported following similar sleep restriction protocols, the reduction in TST was typified by a decrease in the time spent in the REM, N1 and N2 sleep stages, while the duration of time spent in N3 (slow wave sleep) was conserved (Spiegel, Leproult et al. 1999, Buxton, Pavlova et al. 2010, Rao, Neylan et al. 2015). This is consistent with previous research that indicates that the proportion of time spent in the N3 sleep stage is increased in the hours immediately following sleep onset, however, as sleep duration increases REM becomes more prominent and time spent in the N3 sleep stage is reduced (Rao, Neylan et al. 2015). Slow wave sleep is thought to be under tight homeostatic regulation and is considered the 'restorative' stage of sleep, with previous studies indicating a role for N3 sleep in metabolic function and glucose tolerance (Tasali, Leproult et al. 2008). In summary, these results confirm the effectiveness of the intervention protocol for reducing sleep duration in the SR and SR+EX groups.

Previous research has associated exercise with improvements in aspects of sleep quality and duration, particularly in clinical cases (e.g., insomnia and sleep apnea) (Kubitz, Landers et al. 1996, King, Oman et al. 1997, Kredlow, Capozzoli et al. 2015, Dolezal, Neufeld et al. 2017), although much of this has relied upon subjective questionnaires and self-reported assessments. In the present study, there was no

difference in sleep quality between the SR and SR+EX groups based on time spent in the different sleep stages, WASO, SOL, or sleep efficiency. The PSG data showed no differences in the amount of time spent in N3 (slow-wave sleep) between the SR and SR+EX groups on night six of the sleep study (the night immediately following the final session of HIIE). These results suggest there was no improvement in sleep quality as a result of performing exercise, which may be a reflection of the fixed TIB. The effect of exercise on sleep quality when sleep duration is not restricted, as it was here, requires further investigation.

4.4.3 Sleep, exercise, and peripheral skin temperature circadian rhythm

The effect of the sleep restriction on circadian rhythms was assessed by measuring peripheral skin temperature. Comparison of data collected during the intervention period (i.e., Day 3 (from 23:00 h) to Day 8) with data collected during the baseline period (i.e., on Days 1, 2 and 3 (Day 3 prior to 23:00 h)) indicate that skin temperature amplitude was significantly reduced in both the SR and SR+EX groups, while temperature stability was unchanged by the intervention. Amplitude is a parameter commonly used to assess the circadian nature of biological processes (e.g., temperature, gene expression, hormone release). Although changes in skin temperature following sleep restriction have not previously been reported, Moller-Levet, Archer et al. (2013) have reported a reduced amplitude of core clock gene expression in white blood cells following seven nights of sleep restriction (6 h TIB each night). While exercise has previously been shown to cause a phase shift of melatonin profiles, no change in amplitude of the melatonin profile was observed (Van Reeth, Sturis et al. 1994), which is consistent with the finding in this study that exercise did not restore the decrease in skin temperature amplitude induced by sleep restriction. These findings suggest that a period of sleep restriction reduces the amplitude of circadian rhythms and these reductions are not influenced by exercise.

4.4.4 Sleep and clock genes

Given the changes in amplitude, and the results from Moller-Levet et al. (2013) and Cedernaes et al. (2015, 2018), the potential effect of sleep restriction on the expression of skeletal muscle clock genes and proteins was also investigated. Analysis of core circadian clock genes indicated a significant reduction in *Bmal1* mRNA from pre- to post-intervention in the SR group, but not in the SR+EX group. There were no changes in the other measured core clock genes (i.e., *Clock, Cry1, Per1, Per2* and *Rev-erb* α), and no change in BMAL1 protein content despite the change in mRNA. Previously, Cedernaes et al. (2015) reported a decrease in *Bmal1* and *Cry1* mRNA expression following a night of simulated shift work (i.e., 24 h of sleep deprivation) (Cedernaes, Osler et al. 2015) and later reported an increase in BMAL1 protein content following a similar intervention (Cedernaes, Schonke et al. 2018). These findings support the reductions in *Bmal1* mRNA expression reported in this study (a 29% decrease in this study and an 18% decrease in Cedernaes et al. 2015). However, the difference for the change in

BMAL1 protein content between the studies, while potentially a consequence of the duration/severity of each intervention, requires further consideration.

Due to the limited sampling time-points and the diurnal nature of *Bmal1* gene expression (van Moorsel, Hansen et al. 2016), it cannot be discerned whether there is simply a reduction in *Bmal1* gene expression overall, or, a reduction in *Bmal1* gene expression at the time of sampling (which would be indicative of a shift in the diurnal rhythm of *Bmal1 gene* expression). Examples of this shift in diurnal clock gene expression have been demonstrated previously, with delayed meal timing causing a delay in the acrophase (i.e., peak) of *Per2* expression in human white adipose tissue (Wehrens, Christou et al. 2017), and afternoon glucocorticoid administration causing a (~10 h) phase delay of the diurnal pattern of *Bmal1* mRNA in white blood cells from humans (Cuesta, Cermakian et al. 2015). Together, this suggests that the oscillating pattern of peripheral clock gene expression can be shifted by different zeitgebers and that a shift in expression, rather than a decrease, of *Bmal1* mRNA expression may have occurred in the current study. Additional research with multiple sample collection throughout a 24-h period is needed to elucidate this hypothesis. Indeed, the variation in response of *Bmal1* mRNA expression among participants to the intervention may suggest that the response of each individual may be influenced by their existing circadian rhythm. Nonetheless, the present study indicates that sleep restriction (5 nights of 4 h TIB) reduces the expression of *Bmal1* mRNA expression, compared to pre-intervention.

The alterations to *Bmal1* gene expression described in this study may be indicative of disruption of the internal skeletal muscle circadian clock in the SR group. While it is possible to compensate for the loss of other components of the molecular clock (Schiaffino, Blaauw et al. 2016, van Moorsel, Hansen et al. 2016), BMAL1 is essential for the maintenance of behavioural and molecular circadian rhythms (Bunger, Wilsbacher et al. 2000). Loss of function of the BMAL1 protein (via whole-body homozygous mice models - *Bmal1*$^{-/-}$) also causes a reduction in lifespan, and has deleterious effects on muscle function via reductions in muscle fibre cross-sectional area (CSA), mitochondrial content, and mitochondrial respiratory function (Schiaffino, Blaauw et al. 2016). Furthermore, both whole-body and skeletal-muscle-specific *Bmal1*$^{-/-}$ ablation causes reductions in insulin sensitivity (Andrews, Zhang et al. 2010, Dyar, Ciciliot et al. 2014, Schiaffino, Blaauw et al. 2016). However, the influence of reduced *Bmal1* mRNA expression, without a change in BMAL1 protein content, as is the case in this study, is less clear. Furthermore, whether prolonged sleep restriction alters BMAL1 protein content, as previously described with sleep deprivation (Cedernaes, Schonke et al. 2018), is unknown. Despite this, the present findings suggest that sleep restriction can cause a disruption to the molecular circadian clock, which over extended durations has been associated with negative metabolic consequences (such as insulin resistance).

4.4.5 Sleep, exercise and clock genes

Light exposure and food intake both have a well-characterised ability to regulate circadian rhythms; however, exercise and physical activity are also regarded as potent zeitgebers (i.e., circadian time cues) (Wolff and Esser 2012). In the current study, and in contrast to the SR group, the SR+EX group demonstrated no change in *Bmal1* mRNA expression from pre- to post-intervention. Resistance exercise has been shown to cause a phase-shift of the expression of clock genes (including *Bmal1*), which in turn shifted the pattern of expression of other diurnally expressed genes (Zambon, McDearmon et al. 2003). Previous studies have also demonstrated that exercise is able to cause both phase-advances and delays, with differing metabolic effects, depending on the time of day the exercise is performed (Van Reeth, Sturis et al. 1994, Wolff and Esser 2012, Savikj, Gabriel et al. 2018, Sato, Basse et al. 2019, Youngstedt, Elliott et al. 2019). This may suggest that the exercise protocol in this study was able to realign, or mitigate the disruption of circadian rhythms induced by sleep restriction, in a similar fashion to phase-shifts of clock genes reported previously (Cuesta, Cermakian et al. 2015, Wehrens, Christou et al. 2017). The beneficial effect of exercise in mice with a dysfunctional CLOCK protein (*Clock*$^{-/-}$) has also been reported, with the reductions in mitochondrial content and exercise tolerance observed in the KO animals, ameliorated with exercise (Pastore and Hood 2013). Future studies that clarify the effect of exercise performed at different times of the day, as well as changes in the underlying regulatory mechanisms (i.e., clock gene expression), will help with the implementation and optimisation of exercise strategies, to realign changes in circadian rhythms induced by inadequate sleep.

4.4.6 Limitations

Melatonin and/or cortisol concentrations (in saliva, plasma or urine) are often considered the gold-standard for the measurement of circadian rhythm (Cajochen, Krauchi et al. 2003). However, these methods can be invasive, costly, and disruptive (with frequent sampling needed and therefore not suitable while participants are asleep). Therefore, the use of iButtons to measure skin temperature, non-invasively, with high frequency, and over the duration of the study was considered appropriate in this instance. Importantly, it is a commonly used and validated method, which is inversely related to core body temperature (Corbalan-Tutau, Madrid et al. 2011). Previous studies have demonstrated the diurnal variation of clock gene expression in skeletal muscle via multiple sampling time points over a 24-h period (van Moorsel, Hansen et al. 2016). While this was not feasible in this study, it would help to elucidate if the changes observed in *Bmal1* gene expression were a result of a phase-shift *or* a reduction in its expression. Furthermore, the effect of exercise to shift circadian rhythms may depend on the time of day that the exercise occurs (Youngstedt, Elliott et al. 2019). In this study the chronotype of each participant was assessed using the MEQ (see **Table 3.4** for results). Of the 24 participants, only one participant was classified as being of a ‘definite morning’ chronotype, while the rest were classified as either intermediate, or moderate morning/evening chronotypes. Without prior information regarding the

circadian rhythms of each participant (i.e., melatonin release throughout the day), it is difficult to predict the effect that exercise will have in relation to causing either a phase advance or delay (which can be achieved by generating a phase-response curve). The different circadian rhythms of each participant may contribute to the individual responses observed to the intervention, and potentially the between group differences observed. In the future, a better understanding of the effect of exercise on circadian rhythms, and an in-depth profile of an individual participant's chronotype and circadian rhythms will be important for assessing responses to sleep interventions.

Polysomnography (PSG) sleep monitoring is considered the 'gold-standard' for assessing sleep quality and quantity. Ideally, this study would include a summary of the PSG data that was collected from each night of the study; however, due to the cost, expertise, and time-commitment required to do this, only data from night 6 is included. By assessing the night 6 PSG data, the difference in TST between the groups shown via wrist-watch actigraphy can be confirmed. Further, assessment of night 6 PSG data also provides insight into the potential effect of exercise on sleep quality, as this was the night immediately following the final session of HIIE in the SR+EX group. Considering that no differences in sleep quality were observed on this night, and that wrist watch actigraphy has been validated as a reliable and accurate measure of sleep (Krystal and Edinger 2008), the analysis of the remaining PSG data was deemed unnecessary.

4.4.7 Conclusions

The misalignment of circadian rhythms is linked to significant adverse metabolic factors, such as insulin resistance and the development of other risk factors for T2DM and metabolic syndrome (Harfmann, Schroder et al. 2017, Bescos, Boden et al. 2018). The results of this study suggest that sleep restriction can cause the misalignment of circadian rhythms (as assessed via skin temperature and skeletal muscle clock gene expression), which may provide some mechanistic insight as to how sleep loss causes detrimental metabolic effects such as reduced glucose tolerance and increased risk for developing T2DM. Further research investigating the effect of sleep loss on the diurnal expression of clock genes, and the subsequent metabolic consequences is warranted. Furthermore, the effect of exercise at different times throughout the day, and its influence on circadian rhythms, deserves further attention, so as to better understand how to optimise the therapeutic potential of exercise in a variety of conditions that are underpinned by circadian misalignment.

Chapter 5. The Effect of Sleep Restriction, With or Without Exercise, on Glucose Tolerance and Mitochondrial Function

The results of Chapter 4 described the effect of sleep restriction on peripheral circadian rhythms and the possibly stabilising influence of exercise. Following on from this, the aim of this chapter was to investigate the mechanisms underlying the previously reported sleep-loss-induced reductions in glucose tolerance, with a particular emphasis on examining changes to the mitochondria. Furthermore, based on the hypothesised mechanisms for these reductions in glucose tolerance, this chapter will examine the efficacy of high-intensity interval exercise to mitigate these detrimental effects. Aspects of this study have been presented orally at the Exercise and Sport Science Australia (ESSA) Conference, 2018, the American College of Sports Medicine (ACSM) conference, 2018 and The Congress of the European College for Sports Sciences (ECSS) 2018. These results will also be presented by Professor David Bishop in an invited session at the ACSM World Congress on The Basic Science of Exercise, Circadian Rhythms and Sleep in the USA, 2019.

5.1 Introduction

The detrimental effects of sleep loss on health have now been comprehensively established with both epidemiological and clinical studies acknowledging sleep loss as a risk factor for the development of a number of chronic diseases, including type 2 diabetes mellitus (T2DM) (Anothaisintawee, Reutrakul et al. 2015). In fact, sleep loss was identified as a comparable risk factor for the development of T2DM to traditionally associated risk factors, such as physical inactivity (Anothaisintawee, Reutrakul et al. 2015). The link between sleep loss and T2DM has been attributed to the disruption of regular glucose metabolism, and, consequently, increases in blood glucose concentration and insulin resistance (Spiegel, Leproult et al. 1999).

The effect of sleep loss on glucose metabolism is increasingly well-documented within laboratory settings. Many studies have shown that periods of sleep restriction (reduced time in bed, TIB), typically with a sleep opportunity of 4 to 5 h per night, cause significant reductions in a range of indices related to the ability to metabolise glucose. Among the first studies to exhibit this finding was Spiegel et al. (1999), who reported that 4 h TIB each night for 6 nights significantly reduced insulin sensitivity (Spiegel, Leproult et al. 1999). Similar findings have now been reported in a number of subsequent studies (Buxton, Pavlova et al. 2010, Nedeltcheva, Kilkus et al. 2010, Reynolds, Dorrian et al. 2012, Rao, Neylan et al. 2015). It has since been shown that just one night of restricted sleep (4 h TIB) or one night of sleep deprivation (i.e., no sleep) can reduce insulin sensitivity, demonstrating the severity of sleep loss on glucose metabolism (Donga, van Dijk et al. 2010, Cedernaes, Lampola et al. 2015, Cedernaes, Osler et al. 2015, van den Berg, Mook-Kanamori et al. 2016, Cedernaes, Schonke et al. 2018). Furthermore, the effects of sleep restriction on glucose metabolism are not necessarily reversed by a period of recovery sleep (Eckel, Depner et al. 2015, Depner, Melanson et al. 2019). Despite these findings, there is limited data explaining the physiological and molecular mechanisms that underlie these changes. Gaining a better understanding of these mechanisms may help to tailor specific interventions aimed at counteracting the detrimental effects of sleep loss.

The mechanisms underlying the impairment of glucose tolerance following sleep restriction are likely to be multifactorial. Previous studies have demonstrated that sleep loss reduces the activation (phosphorylation) of Protein kinase B (Akt) (in *ex vivo* cultured adipose tissue samples), a crucial step in the stimulation of the insulin-signalling pathway (Broussard, Ehrmann et al. 2012, Sweeney, Jeromson et al. 2017). Other studies have linked alterations to regular hormone and cytokine secretion (i.e., TNF-α and interleukin-6), as well as circadian misalignment (Spiegel 1999, Mullington 2009, Cedernaes 2015), to the reduction of glucose tolerance. While not having been investigated in the context of sleep loss previously, the development of insulin resistance has been associated with a reduction in mitochondrial content or respiratory function. Kelley et al. (2002) previously demonstrated a positive correlation between increased mitochondrial area and improved insulin sensitivity, while a

reduction in citrate synthase activity and mitochondrial respiratory function has also been reported in T2DM patients compared to obese non-diabetics, suggesting a link between mitochondrial changes and the development of insulin resistance and T2DM (Kelley, He et al. 2002, Mogensen, Sahlin et al. 2007). Therefore, sleep-loss-induced reductions in glucose tolerance may, in part, be a consequence of changes in mitochondrial content, function, or the processes that regulate these properties (including mitochondrial dynamics and mitochondrial protein synthesis). One previous study examined the effect of 120 h of total sleep deprivation on mitochondrial enzymes in human skeletal muscle and reported a 24% reduction in citrate synthase activity (Vondra et al. 1981). Additionally, 72 h of sleep deprivation in rats significantly decreased electron transfer from mitochondrial complex 1 to complex 3 in the hypothalamus (Andreazza et al. 2010). However, how these results are applied in the context of the sleep loss commonly experienced by humans remains to be determined. Further investigation of these mechanisms is warranted to help elucidate potential beneficial therapeutic interventions.

Regular endurance exercise is an effective strategy to improve glucose tolerance and to subsequently prevent and treat T2DM. There are improvements in insulin sensitivity and glucose tolerance in the 24 to 48 h following exercise, which result from increased activation of the insulin-independent signalling pathway (contraction pathway) (Henriksen 2002). This subsequently causes increased translocation of glucose transporter type 4 (GLUT4) to the cell membrane and increased glucose uptake into the muscle (Richter and Hargreaves 2013). Exercise training also improves insulin sensitivity and glucose tolerance via the upregulation of the insulin-stimulated signalling pathway in skeletal muscle, via increased content of signalling proteins such as insulin receptor substrate-1 (IRS1), Akt substrate of 160 kDa (AS160), and GLUT4 (Frosig and Richter 2009, Richter and Hargreaves 2013). Endurance exercise is a potent stimulus for the induction of mitochondrial biogenesis (Bishop, Botella et al. 2019), with increases in sarcoplasmic and mitochondrial protein synthesis, and both mitochondrial content and mitochondrial respiratory function (Holloszy 1967, Holloszy and Booth 1976, Di Donato, West et al. 2014, Bell, Seguin et al. 2015, Granata, Oliveira et al. 2016, MacInnis, Zacharewicz et al. 2017). Traditional moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT) are both well documented to improve markers of mitochondrial content and function (MacInnis, Zacharewicz et al. 2017), whilst also improving aspects of glycaemic control (Little, Gillen et al. 2011). Consequently, endurance exercise may be able to mitigate the detrimental effects of sleep loss on glucose metabolism by providing the stimulus for increases in mitochondrial content and function (Saner, Bishop et al. 2018). To date, however, this hypothesis has not been investigated.

Accordingly, the aim of this study was to expand on the previous literature that has demonstrated the effect of sleep loss on glucose tolerance, but with the intention of examining for the first time the underlying cellular mechanisms and potential changes in the mitochondria that may contribute to these changes. These findings may support the use of exercise to counteract these detrimental effects,

potentially with less of a time commitment than obtaining extra sleep. The results of this study may be of significance for the development of future health recommendations, by improving our understanding of how and why exercise may mitigate the effects of sleep loss on glucose tolerance, particularly in populations who commonly experience sleep loss (such as shift-workers). It was hypothesised that sleep restriction would reduce mitochondrial respiratory function, concomitantly with a reduction in glucose tolerance, but that three sessions of high-intensity interval exercise (HIIE) would ameliorate the detrimental reductions in mitochondrial function and glucose tolerance that are induced with sleep-restriction protocols.

5.2 Methodology and procedures

Full details of all experimental procedures can be found in **Chapter 3 General Methodology**. Details specific to the methodology and data presented in this chapter are outlined below.

5.2.1 Study overview

For an overview of the study protocol specified to this chapter, see **Figure 5.1**. Following the initial screening and assessment procedures, 24 healthy men underwent baseline fitness testing (see section 3.3.2) and a pre-study muscle biopsy to determine baseline mitochondrial respiration values and background skeletal muscle D_2O enrichment (as outlined in Chapter 3). Participants were then allocated into one of three, matched experimental groups; Normal Sleep (NS), Sleep Restriction (SR) or Sleep Restriction and Exercise (SR+EX). Baseline participant characteristics for the three groups are shown in **Table 3.2**.

The experimental component of the study consisted of an eight-night stay within the sleep laboratory. The protocol for each of the three experimental groups consisted of an initial two nights of baseline sleep, followed by a five-night intervention period and then a final night of recovery sleep. During the baseline sleep period all participants spent 8 h TIB, between 23:00 h and 07:00 h. For the remaining 5 nights, the NS group spent 8 h TIB (between 23:00 h and 07:00 h), while both the SR and SR+EX groups spent 4 h TIB (between 03:00 and 07:00) each night. The SR and SR+EX group TIB (03:00 h – 07:00 h) was standardised to ensure all participants woke at the same time each morning and that the subsequent testing procedures were performed following the same time duration post waking. Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux to reduce the effect of lighting on circadian rhythms (Duffy and Wright 2005).

Upon arrival all participants consumed 150 mL of D_2O for the assessment of SarcPS throughout the study. Further, two experimental testing sessions were conducted during the participant's stay. At 08:00 h on the morning of Day 3 (following 2 nights of baseline sleep), the pre-intervention OGTT was performed to assess glucose tolerance (**3.3.8**). Following this, a skeletal muscle biopsy was obtained as per section **3.3.3**, for the assessment of mitochondrial respiratory function, mitochondrial content, sarcoplasmic protein synthesis (SarcPS), and genes and proteins related to the regulation of mitochondrial function and glucose metabolism. This experimental testing session was repeated on the morning of Day 8 (following the final night of the sleep intervention). For the duration of the study, each participant was provided with a standardised diet relative to body mass consisting of fixed proportions of carbohydrates (4.5 $g.kg^{-1}.d^{-1}$), protein (1.5 $g.kg^{-1}.d^{-1}$) and fat (1 $g.kg^{-1}.d^{-1}$). All meal times (there were six throughout the day) were kept constant throughout the study and participants were asked to eat all food provided. Meal timings were kept the same for all groups. An identical menu was

provided on the days prior to both experimental sessions (an example of the daily diet is provided in **Appendix B**). Participants remained within the laboratory and were monitored throughout each study. To match the activity count prior to the commencement of the study, participants were instructed to walk outside the facility at designated periods throughout the day, while being accompanied by a member of the research staff. Waking hours were spent watching television, reading, working on a computer, or talking to staff. Approval of the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee (HRE15-294).

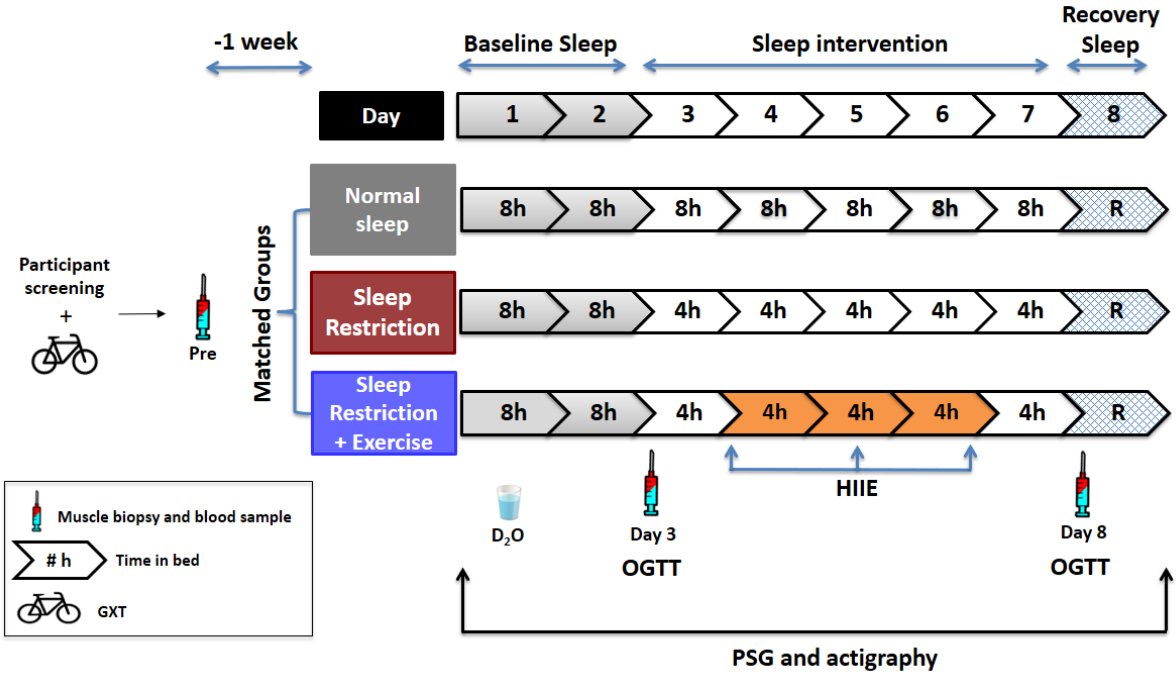

Figure 5.1 - Schematic representation of the study protocol for Chapter 5.
GXT – Graded exercise test, D2O – deuterium oxide ingestion, OGTT – oral glucose tolerance test, PSG – polysomnography sleep analysis, HIIE – high-intensity interval exercise, R – *ad libitum* recovery sleep, participant screening refers to medical questionnaires, exclusion criteria and, habitual sleep and physical activity monitoring.

5.2.2 High-intensity interval exercise (HIIE)

The high-intensity interval exercise sessions (consisting of 10 x 60-s intervals at 90% $\dot{W}_{peak}$, interspersed by 75-s of active recovery on a cycle ergometer) were conducted in the SR+EX group only, at 10:00 h on Day 4, 5 and 6 as reported in section **3.3.5.**

5.2.3 Oral glucose tolerance testing (OGTT)

To assess changes in glucose tolerance from pre- to post-intervention an OGTT was conducted at 08:00 h on Day 3 and Day 8 of the experimental protocol. Following ingestion of 75 g of glucose, venous blood samples were collected at the 0, 10, 20, 30, 60, 90, and 120 minute time-points, in accordance with section **3.3.8**.

5.2.4 Insulin and IL-6 ELISA assays

Plasma samples were collected prior to, and throughout each OGTT at the designated time-points. The concentration of plasma IL-6 was assessed for the samples collected prior to the OGTT and plasma insulin concentrations were assessed for all samples collected during the OGTT. The ELISA assays were performed as per the manufacturer's instructions and further details can be found in sections **3.4.1** and **3.4.2**.

5.2.5 Muscle sample analysis

Three muscle biopsies were collected throughout the study in accordance with section **3.3.3.** Muscle biopsies that were collected on Day 3 and Day 8 of the study protocol (i.e., pre- and post-intervention) were assessed according to the techniques described below.

5.2.5.1 Mitochondrial respiratory function analysis

Mitochondrial respiratory function was assessed in permeabilised skeletal muscle fibres using the high-resolution Oxygraph-2K (Oroboros, Innsbruck, Austria). Details regarding the preparation of muscle fibres, the SUIT protocol used, and the analysis of these results are available in section **3.4.4.**

5.2.5.2 Citrate synthase activity assay

Citrate synthase (CS) activity was assessed via enzymatic assay and used as a marker of mitochondrial content. For details regarding the assay preparation and analysis see section **3.4.7**.

5.2.5.3 Sarcoplasmic protein synthesis (SarcPS)

Deuterium oxide (D_2O) was ingested on Day 1 of the study protocol to allow for the assessment of SarcPS during the intervention period. Details regarding D_2O ingestion, assessment of ^2H body water enrichment (via saliva sample collection), preparation of sarcoplasmic fractions from the muscle biopsies, analysis, and calculations of the fractional synthetic rate are available in section **3.4.3**. The citrate synthase activity of whole-muscle, sarcoplasmic and myofibrillar fractions was also assessed and can be viewed in section **3.4.3.8**.

5.2.5.4 Western blot analysis

Skeletal muscle protein content for proteins involved in the regulation of mitochondrial content, function, dynamics, mitophagy, and glucose metabolism were assessed as per the western blotting protocol outlined in section **3.4.6**. Details relating to the relevant antibody conditions are outlined in **Table 5.1**.

Table 5.1 – Western blot primary antibodies and incubation conditions for Chapter 5.

Protein	Antibody	Dilution	Secondary Conditions
Total OXPHOS	AB110413	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti mouse
PGC-1α	CST2178	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
DRP1	CST5341	1:1,000 in 5% BSA (in TBST)	1:5,000 in 5% SM (in TBST) – anti rabbit
MFN2	CST9482	1:1,000 in 5% BSA (in TBST)	1:5,000 in 5% SM (in TBST) – anti rabbit
p38 MAPK	CST9212	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-p38 $MAPK^{Thr180/Tyr182}$	CST9211	1:1,000 in 5% BSA (in TBST	1:10,000 in 5% SM (in TBST) – anti rabbit
p53	CST2527	1:300 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST)– anti rabbit
LC3B	CST3868	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST)– anti rabbit
GLUT4	CST2213	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti mouse
AKT	CST9272	1:1,000 (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-AKT^{ser473}	CST9271	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
IRS1	CST2382	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
AS160	CST2447	1:1,000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit

BSA – bovine serum albumin, SM – skim milk

5.2.5.5 Real-time quantitative polymerase chain reaction (RT-PCR)

The expression of genes relating to the regulation of the mitochondrial biogenesis, mitochondrial dynamics, mitophagy, and glucose metabolism were assessed as per the RT-PCR protocol outlined in section Real-time quantitative polymerase chain reaction (RT-PCR)**3.4.8.** Details relating to the specific primers used within this chapter are presented in **Table 4.2 - RT-PCR primer sequences for Chapter 4.Table 5.2.**

5.2.6 Statistical analysis

Statistical analyses were conducted using the statistical software package GraphPad Prism (V7.03). Pre- to post-intervention changes in gene expression, protein content, mitochondrial respiratory function, glucose tolerance, citrate synthase activity, and blood plasma hormone assessments were assessed for each group using a two-way repeated measures (RM) analysis of variance (ANOVA) (3 Groups x 2 Times). Significant effects of interaction (Group x Time), Time (Pre vs Post) and Group (NS vs SR vs SR+EX) are reported where effects are seen. Where there were significant effects, Bonferonni post-hoc testing was performed to locate the differences. All statistical analysis of gene expression and protein content data was run on raw values, and not fold-change data. Gene expression data in the text is reported as percent mean change from pre-intervention value ± SD %, 95% CI from fold-change data (as a decimal), with the *P* value from the raw data reported. Graphs represent fold-change from pre-intervention values, and individual responses are shown. A one-way RM ANOVA was used to assess differences between groups when it was not suitable to compare pre-intervention versus post-intervention values from within the same group. This was the case for sarcoplasmic protein fractional synthetic rate data that was collected over the duration of the intervention period. All data in text, figures and tables are presented as mean ± standard deviation (SD), with P values ≤ 0.05 indicating statistical significance and confidence levels are reported and set to 95% (unless otherwise stated).

Table 5.2 - RT-PCR primer sequences for Chapter 5.

Primer Name	Primer Sequence	Product Size (bp)	Efficiency (%)	Accession No.
Target Genes				
p53	**F –** GTTCCGAGAGCTGAATGAGG **R –** TTATGGCGGGAGGTAGACTG	123	101.8	NM_001126118.1
Tfam	**F –** CCGAGGTGGTTTTCATCTGT **R –** GCATCTGGGTTCTGAGCTTT	110	111.1	NM_003201.3
Drp1	**F –** CACCCGGAGACCTCTCATTC **R –** CCCCATTCTTCTGCTTCCAC	138	116.4	NM_001330380.1
Nrf2	**F –** AAGTGACAAGATGGGCTGCT **R –** TGGACCACTGTATGGGATCA	87	92.1	NM_001197297.1
Nrf1	**F –** CTACTCGTGTGGGACAGCAA **R –** AGCAGACTCCAGGTCTTCCA	143	102.5	NM_005011.5
Glut4	**F –** CTTCATCATTGGCATGGGTTT **R –** AGGACCGCAAATAGAAGGAAGA	75	85.4	NM_001042.2
Pgc-1α total	**F –** CAG CCT CTT TGC CCA GAT CTT **R –** TCACTGCACCACTTGAGTCCAC	101	103.6	NM_013261.3
β-Had	**F –** TGGACAAGTTTGCTGCTGAACAT **R –** TTTCATGACAGGCACTGGGT	137	80.6	NM_001184705.2
Pdk4	**F –** GCAGCTACTGGACTTTGGTT **R –** GCGAGTCTCACAGGCAATTC	84	99.7	NM_002612.4
Ampkα	**F –** CAGGGACTGCTACTCCACAGAGA **R –** CCTTGAGCCTCAGCATCTGAA	85	76.8	NM_001355028.1
Mfn1	**F –** CAGAAAGTGGTGTGGCACTTG **R –** TTTCACTGCTGACTGCGAGAT	104	112	NM_033540.2
Mfn2	**F –** CCCCCTTGTCTTTATGCTGATGTT **R –** TTTTGGGAGAGGTGTTGCTTATTTC	168	140	NM_014874.3
Lc3b	**F –** GAGAAGACCTTCAAGCAGCG **R –** TTATCACCGGGATTTTGGTTGGA	94	102.4	NM_022818.5
p62	**F –** AGAATCAGCTTCTGGTCCATCGG **R –** CTTTTCCCTCCGTGCTCCAC	129	103.2	NM_003900.5
Pink1	**F –** TGTGGAACATCTCGGCAGGT **R –** AGTGACTGCTCCATACTCCC	110	101.8	NM_032409.2
Park2	**F –** CACGACCCTCAACTTGGCTA **R –** GGTACCGGTTGTACTGCTCT	112	103.4	NM_013988.2

F – Forward primer, **R** – Reverse primer

5.3 Results

5.3.1 Plasma glucose during OGTT

An overview of plasma glucose responses to the OGTT is displayed in **Figure 5.2** and **Table 5.3**. There was a significant interaction effect (*P*=0.002) for total glucose AUC, with an increase in the SR group from pre- to post-intervention (mean change ± SD, 95% CI, *P* value) (149 ± 115 A.U., CI [54, 243 A.U.], *P*=0.002), but not the NS group (-59 ± 122 A.U., CI [-36, 154 A.U.], *P*=0.356), or the SR+EX group (67 ± 57, CI [-162 to 28 A.U.], *P*=0.239). There was no significant difference between groups for pre-intervention glucose AUC values (*P*=0.771) (**Figure 5.3**).

There was a significant interaction effect (*P*=0.010) for plasma glucose concentration (mmol.L^{-1}) at the 30-minute time-point of the OGTT, indicating an increase in the SR group from pre- to post-intervention (mean increase ± SD mmol.L^{-1}, 95% CI, *P* value) (1.56 ± 1.80 mmol.L^{-1}, CI [0.29, 2.84 mmol.L^{-1}], *P*=0.013), but not the NS group (0.61 ± 1.30 mmol.L^{-1}, CI [-0.66 to 1.89 mmol.L^{-1}], *P*=0.674) or the SR+EX group (0.34 ± 0.69 mmol.L^{-1}, CI [-0.94 to 1.62 mmol.L^{-1}], *P*>0.999).

A significant interaction effect was also seen at the 60- and 90-minute time-points of the OGTT for plasma glucose concentration (*P*=0.010 and 0.008, respectively). There was a significant increase in both the SR (2.54 ± 1.94 mmol.L^{-1}, CI [1.15, 3.94 mmol.L^{-1}], *P*<0.001) and SR+EX (1.51 ± 2.90 mmol.L^{-1}, CI [0.11, 2.91 mmol.L^{-1}] *P*=0.031) groups, but no change in the NS group (-0.01 ± 0.34 mmol.L^{-1}, CI [-1.39 to 1.40 mmol.L^{-1}], *P*>0.999) at the 60-minute time-point. The SR+EX group had an increased plasma glucose concentration at the 90-minute time-point (1.18 ± 0.65 mmol.L^{-1}, CI [0.13, 2.23 mmol.L^{-1}], *P*=0.024), whereas there was no change in the SR (0.77 ± 1.07 mmol.L^{-1}, CI [-0.27, 1.83 mmol.L^{-1}], *P*=0.203) or NS (0.72 ± 1.53 mmol.L^{-1}, CI [-1.77, 0.34 mmol.L^{-1}], *P*=0.273) groups from pre- to post-intervention.

Table 5.3 - Analysis of plasma glucose (mmol.L^{-1}) measurements during the oral glucose tolerance test (OGTT), pre- and post-intervention

Time (min)	NS		SR		SR+EX	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*
0	5.1 ± 0.4	5.0 ± 0.7	5.2 ± 0.3	5.0 ± 0.2	5.2 ± 0.2	5.0 ± 0.2
10	5.9 ± 0.8	6.0 ± 1.2	5.8 ± 0.9	5.7 ± 0.8	5.6 ± 0.3	5.3 ± 0.4
20	6.9 ± 1.2	7.0 ± 1.6	6.6 ± 0.9	7.2 ± 1.3	6.8 ± 0.6	6.3 ± 0.7
30	7.3 ± 1.9	6.7 ± 1.8	6.9 ± 1.1	8.5 ± 1.3*	7.3 ± 1.0	7.0 ± 0.9
60	5.6 ± 2.1	4.9 ± 1.4	5.9 ± 1.4	8.4 ± 1.3*	5.1 ± 1.2	6.6 ± 1.5*
90	4.8 ± 1.8	4.1 ± 1.1	5.1 ± 1.2	5.9 ± 1.1	4.1 ± 0.9	5.2 ± 0.9*
120	4.3 ± 1.4	4.0 ± 1.2	4.2 ± 0.9	5.1 ± 1.5	4.6 ± 0.6	4.7 ± 0.9
Mean glucose	5.7 ± 1.2	5.4 ± 1.1	5.7 ±0.2	6.5 ± 0.4*	5.5 ± 0.3	5.7 ± 0.4
Total AUC	676.5 ± 188.6	617.3 ± 136.0	678.4 ±32.4	827.2 ± 55.5*	638.4 ± 49.8	705.4 ± 49.8

Values are mean ± SD mmol.L^{-1}. Area under the curve (AUC), Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction + Exercise (SR+EX). *Denotes significant effect from pre-intervention (*P*<0.05). n=8 per group.

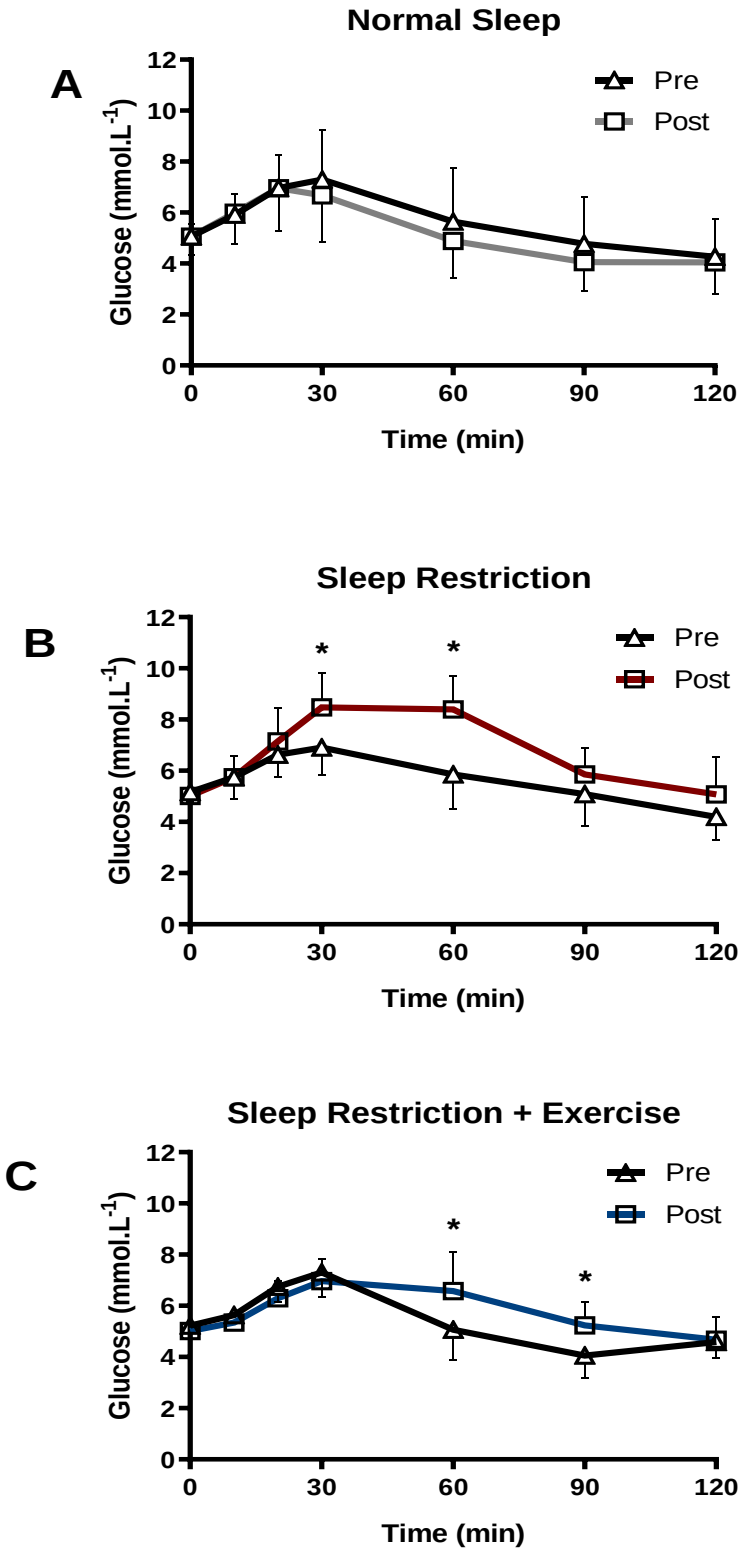

Figure 5.2 - Plasma glucose measurements during the pre- and post-intervention oral glucose tolerance test (OGTT).
Plasma glucose concentration throughout the 120-minute OGTT in the (A) Normal Sleep (NS), (B) Sleep Restriction (SR), (C) Sleep Restriction + Exercise (SR+EX) groups. Values are mean ± SD, * Denotes significant within-group differences from pre- to post-intervention ($p<0.05$). n=8 per group.

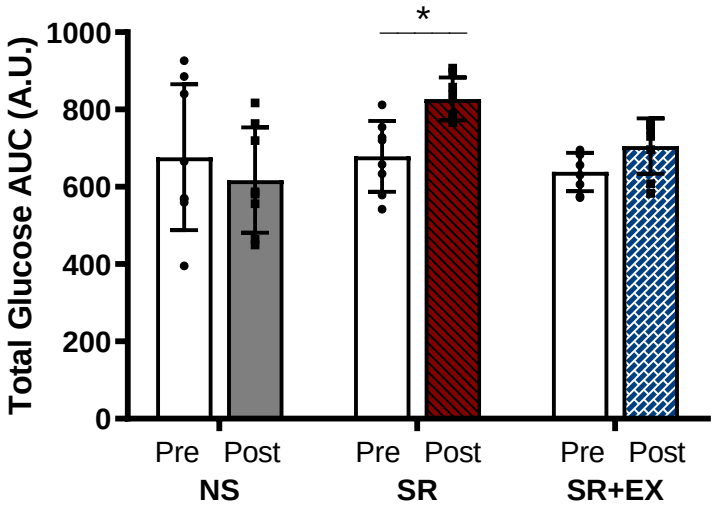

Figure 5.3 - Total glucose area under the curve (AUC) during the pre- and post-intervention oral glucose tolerance test (OGTT).
Values are mean ± SD, individual data points are shown, * Denotes significant within-group differences from pre- to post-intervention ($p<0.05$). n=8 per group.

5.3.2 Plasma insulin concentration during the OGTT

An overview of plasma insulin responses to the OGTT is displayed in **Figure 5.4**. There was no interaction effect (*P*=0.085) for the change in insulin AUC for any group pre- to post-intervention (NS: -581 ± 1797 A.U., CI [-2037, 874 A.U.], *P*=0.933; SR: 1275 ± 1787 A.U., CI [-180.4, 2731 A.U.], *P*=0.100 and SR+EX: 518 ± 1043 A.U., CI [-937, 1975 A.U.], *P*>0.999). Although not statistically significant, there was a 29% increase in insulin AUC in the SR group following the intervention. There was no significant difference between the pre-intervention insulin AUC values between any of the groups (*P*=0.137) (**Figure 5.5**). However, there was a significant interaction effect (*P*=0.025) for plasma insulin levels, such that the 60-minute time-point of the OGTT increased from pre- to post-intervention, in the SR group (mean change ± SD µIU/mL, 95% CI, *P* value) (25.63 ± 25.46 µIU/mL, CI [8.20, 43.05 µIU/mL], *P*=0.001), but not the NS (-8.84 ± 40.80 µIU/mL, CI [-31.19, 13.66 µIU/mL], *P*>0.999) or SR+EX group (9.94 ± 8.33 µIU/mL, CI [8.121, 28.01 µIU/mL], *P*=0.901).

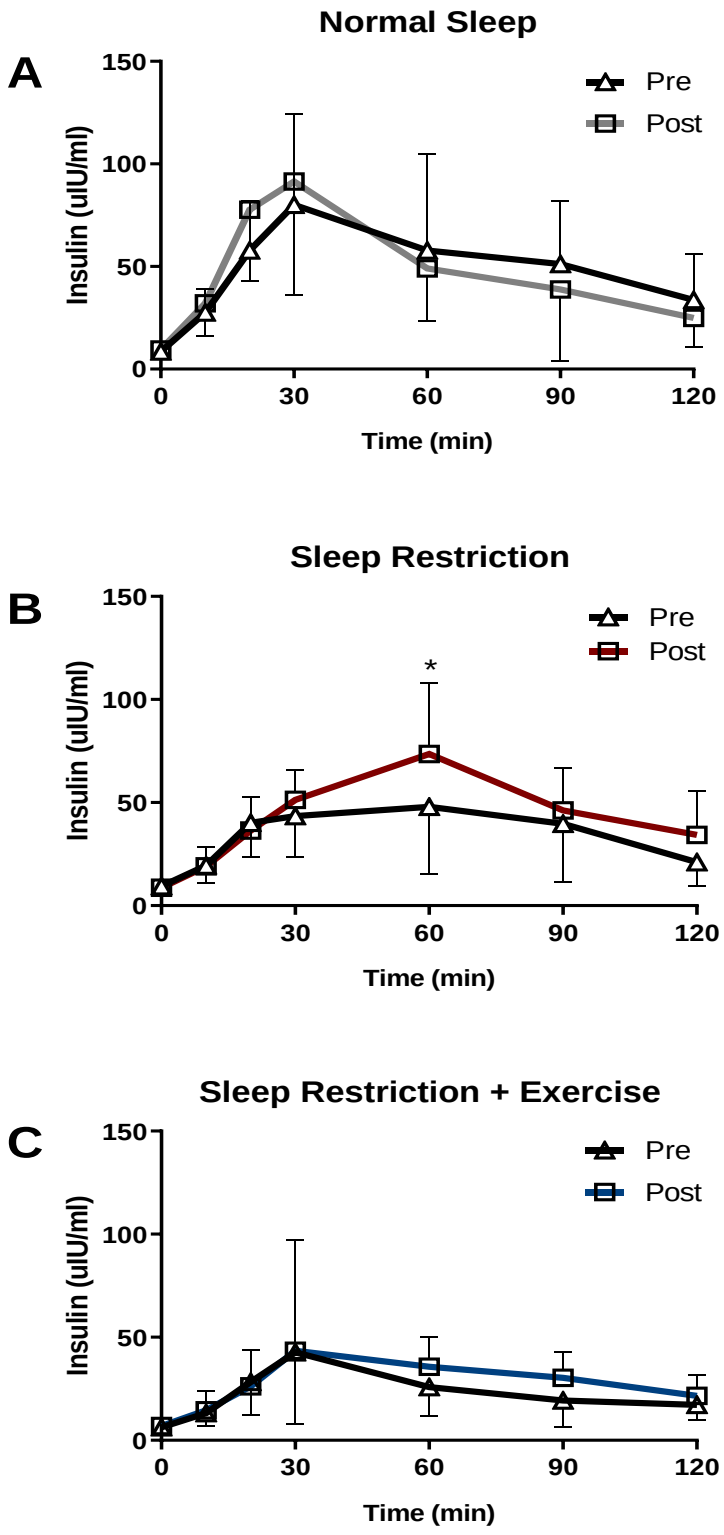

Figure 5.4 - Plasma insulin levels during the pre- and post-intervention OGTT.
Plasma insulin concentration throughout the 120-minute OGTT for the (A) Normal Sleep, (B) Restricted Sleep, (C) Restricted Sleep + Exercise groups. Values are mean ± SD. * Denotes significant within-group differences from pre- to post-intervention ($P<0.05$). n=8 per group.

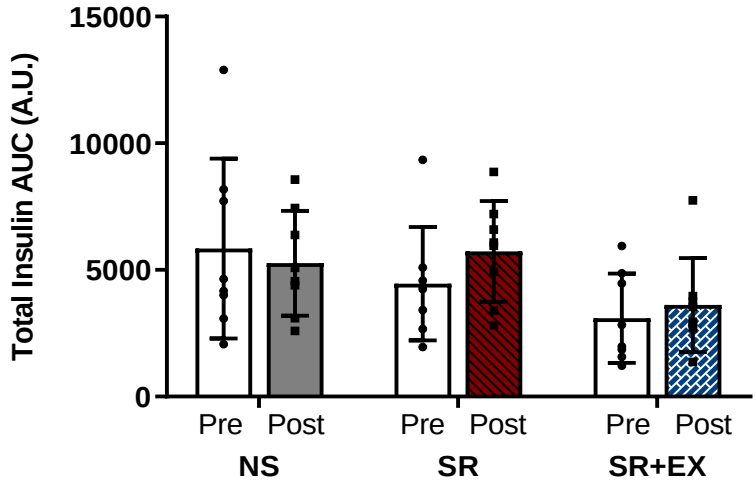

Figure 5.5 - Total plasma insulin area under the curve (AUC) during the oral glucose tolerance test (OGTT). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction + Exercise (SR+EX). Values are mean ± SD. n=8 per group.

5.3.3 Mitochondrial respiratory function

There was a significant interaction effect (P=0.032) for mitochondrial respiration $(ETF+CI+CII)_P$, which revealed a reduction from pre- to post-intervention (mean change ± SD pmol $O_2.s^{-1}.mg^{-1}$, 95% CI, P value) in the SR group (-15.9 ± 12.4 pmol $O_2.s^{-1}.mg^{-1}$, CI [-25.6, -6.1 pmol $O_2.s^{-1}.mg^{-1}$], P=0.001) (**Figure 5.6**). This was not evident in the NS (8.1 ± 6.9 pmol $O_2.s^{-1}.mg^{-1}$, CI [-1.6 to 17.9 pmol $O_2.s^{-1}.mg^{-1}$], P=0.122) or SR+EX group (0.6 ± 11.8 pmol $O_2.s^{-1}.mg^{-1}$, CI [-9.1, 10.4 pmol $O_2.s^{-1}.mg^{-1}$], P=0.997). Further, while there was no significant interaction effect (P=0.067), there was a significant effect of time (P=0.003) for $(ETC+CI)_P$ mitochondrial respiration, with post-hoc analysis indicating a significant reduction in the SR group (-8.6 ± 8.4 pmol $O_2.s^{-1}.mg^{-1}$, CI [-14.8, -2.4 pmol $O_2.s^{-1}.mg^{-1}$], P=0.005), which was not seen in either the NS (5.1 ± 5.1 pmol $O_2.s^{-1}.mg^{-1}$, CI [-1.1, 11.3 pmol $O_2.s^{-1}.mg^{-1}$], P=0.125) or the SR+EX (0.3 ± 6.3 pmol $O_2.s^{-1}.mg^{-1}$, CI [-5.9, 6.5 pmol $O_2.s^{-1}.mg^{-1}$], P>0.999) groups. There was a significant decrease in $(ETS)_E$ over time (P=0.021); however, there was no specific difference detected from pre- to post-intervention in any of the groups (NS; P=0.662, SR; P=0.083, SR+EX; P=0.746).

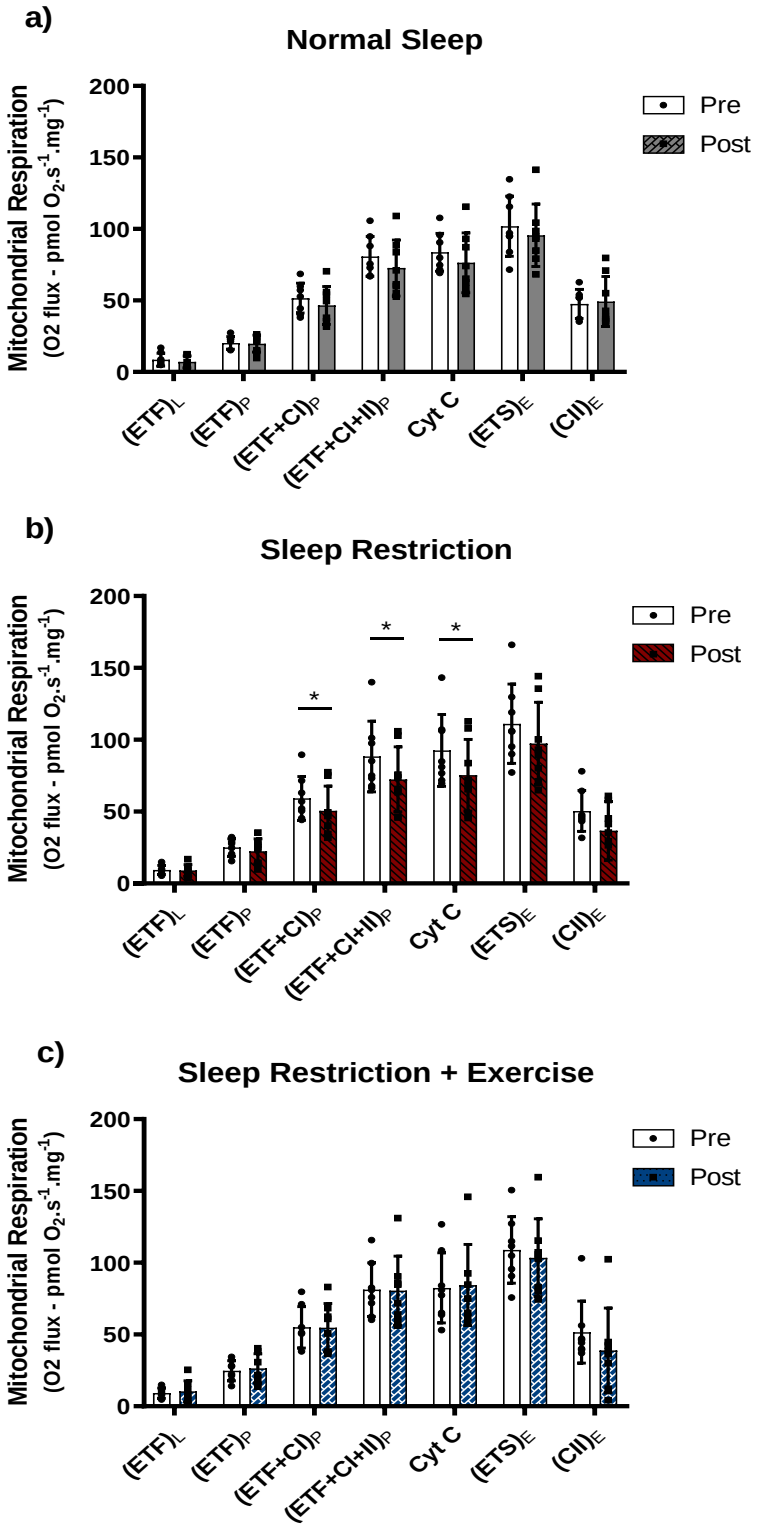

Figure 5.6 - Mitochondrial respiratory function from pre-intervention compared to post-intervention.
(a) Normal Sleep, (b) Sleep Restriction and (c) Sleep Restriction + Exercise. $(ETF)_L$ – leak respiration through ETF; $(ETF)_P$ – maximal coupled mitochondrial respiration through ETF; $(ETF+CI)_P$ – maximal coupled mitochondrial respiration through ETF and CI; $(ETF+CI+CII)_P$- maximal coupled mitochondrial respiration through ETF,CI and CII; Cyt C test – outer mitochondrial membrane integrity test; $(ETS)_E$ – maximal uncoupled mitochondrial respiration through ETF, CI and CII, $(CII)_E$ – maximal uncoupled mitochondrial respiration through CII. Pre – pre-intervention, Post – post-intervention. * Denotes significant difference within group from pre- to post-intervention ($p<0.05$). n=8 per group.

5.3.4 Markers of mitochondrial content

There was no change in CS activity from pre- to post-intervention (interaction effect, *P*=0.972) for any of the groups (NS 0.10 ± 0.45 mol/h/kg protein, CI [-0.24, 0.44 mol/h/kg protein], *P*>0.999); (SR 0.13 ± 0.29 mol/h/kg protein, CI [-0.21, 0.47 mol/h/kg protein], *P*=0.992) and (SR+EX 0.08, ± 0.35 mol/h/kg protein, CI [-0.26, 0.43 mol/h/kg protein], *P*>0.999) (**Figure 5.7**). As a further validation of changes in mitochondrial content, protein content for subunits of mitochondrial complexes was assessed. No significant interaction effects were observed in protein content for complex 1 (*P*= 0.116), Complex 2 (*P*=0.649), Complex 3 (*P*=0.621), Complex 4 (*P*=0.718) or Complex 5 (*P*=0.158) (**Figure 5.8**).

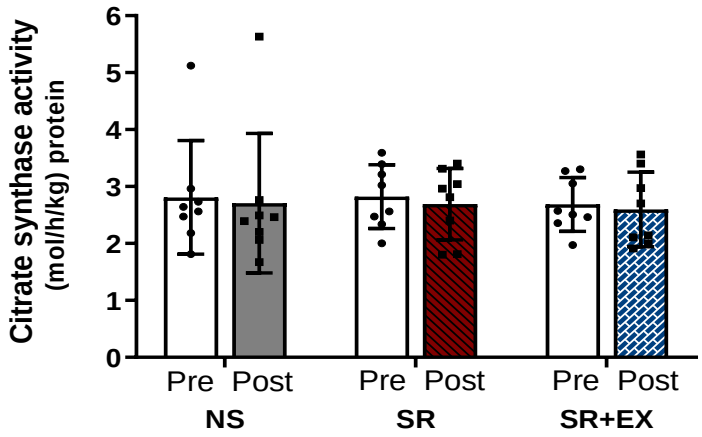

Figure 5.7 - Citrate synthase activity pre-intervention and post-intervention.
Normal Sleep (NS), Sleep Restriction (SR), and Sleep Restriction and Exercise (SR+EX).

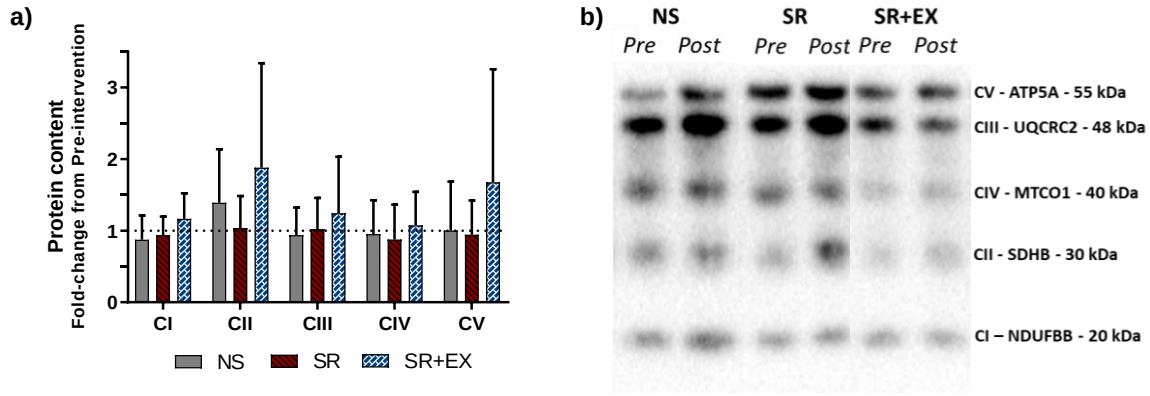

Figure 5.8 - Fold-change of protein content for subunits of mitochondrial complexes (I – V) from pre- and post-intervention.
a) protein content of mitochondrial complex subunits; Mitochondrial complex 1 (CI) - NDUFB8, Complex 2 (CII) - SDHB, Complex 3 (CIII) - Core protein 2 (UQCCRC2), Complex 4 (CIV) – MTCO, Complex 5 (CV) – ATP5A, Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). b) Representative blot of mitochondrial complex subunits. n=8 per group.

5.3.5 Sarcoplasmic protein synthesis

An overview of the muscle sarcoplasmic protein fractional synthetic rate (FSR) is shown in **Figure 5.9**. Compared to the NS group, sarcoplasmic FSR was significantly reduced in the SR group (between groups difference FSR %/day ± SD, 95% CI, *P* value, -0.62 ± 0.11%, CI [-0.90, -0.33], *P*<0.001). Further, sarcoplasmic FSR was significantly reduced in the SR group compared to the SR+EX group (-0.66 ± 0.12%, CI [-0.95, -0.37], *P*<0.001). There was no difference in sarcoplasmic FSR between the NS and SR+EX group (0.04 ± 0.10%, CI [-0.33, 0.24], *P*>0.999).

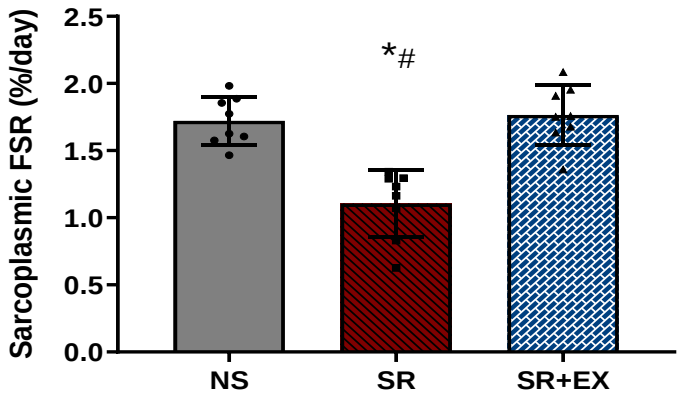

Figure 5.9 - Fractional synthetic rate (FSR) of sarcoplasmic protein synthesis during the sleep intervention. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX) groups (n=8, each group). * Denotes significantly different from NS, # denotes significantly different from SR+EX (both *P*<0.05). n=8 per group.

5.3.6 Mitochondrial-related gene expression

There was no significant interaction effect for *Pgc1α* (*P*=0.552), *p53* (*P*=0.523), *Tfam* (*P*=0.816), *NrfF2* (*P*=0.777), *Drp1* (*P*=0.937), *Mfn2* (*P*=0.562), *Mfn1* (*P*=0.414), *Lc3b* (*P*=0.486), *p62/SQSTM1* (*P*=0.088), *Pink1* (*P*=0.895), or *Park2* (*P*=0.444) mRNA from pre- to post-intervention (**Figure 5.10**). There was, however, a significant effect of time for *Mfn2* (*P*=0.002), with post-hoc analysis revealing a significant reduction of *Mfn2* mRNA gene expression from pre- to post intervention (mean change ± SD %, 95 % CI, *P* value) in the SR group (-35 ± 28 %, CI [-7, 79 %], *P*=0.044), but not the NS (18 ± 28 %, CI [-27, 64 %], *P*=0.125) or SR+EX (2 ± 68 %, CI [-40, 46 %], *P*=0.737) groups. There was a decrease in *Tfam* mRNA expression with time (*P*=0.035), but there were no specific group differences from pre- to post-intervention.

5.3.7 Mitochondrial-related protein content

There was no significant interaction effect for PGC-1α (*P*=0.257), p53 (*P*=0.294), DRP1 (*P*=0.642), MFN2 (*P*=0.768), LC3BI (*P*=0.096), LC3BII (*P*=0.205), LC3II:I ratio (*P*=0.884), or total p38MAPK (*P*=0.229) protein content from pre- to post-intervention. However, there was a significant interaction effect for p-p38 MAPK$^{Thr180/Tyr182}$ (*P*=0.027), such that there was a significant increase in p-p38 MAPK $^{Thr180/Tyr182}$ (mean difference ± SD A.U., 95% CI, *P* value) in the SR+EX group (0.51 ± 0.64 A.U., CI [0.05, 0.97 A.U.], *P*=0.027), but not in the NS (-0.08 ± 0.43 A.U., CI [-0.54, 0.38 A.U.], *P*>0.999) or SR (-0.16 ± 0.37 A.U., CI [-0.62, 0.29], *P*>0.999) groups. Further, there was a significant interaction effect for the ratio between p-p38 MAPK $^{Thr180/Tyr182}$ and total p38 MAPK (*P*=0.030) where there was a significant increase in the SR+EX group (0.70 ± 0.94 A.U., CI [0.07, 1.33 A.U.], *P*=0.027), but no change in either the NS (0.00 ± 0.46 A.U., CI [-0.63, 0.63 A.U.], *P*>0.999) or SR (-0.26 ± 0.56 A.U., CI [-0.89, 0.37 A.U.], *P*=0.888) groups (**Figure 5.11**).

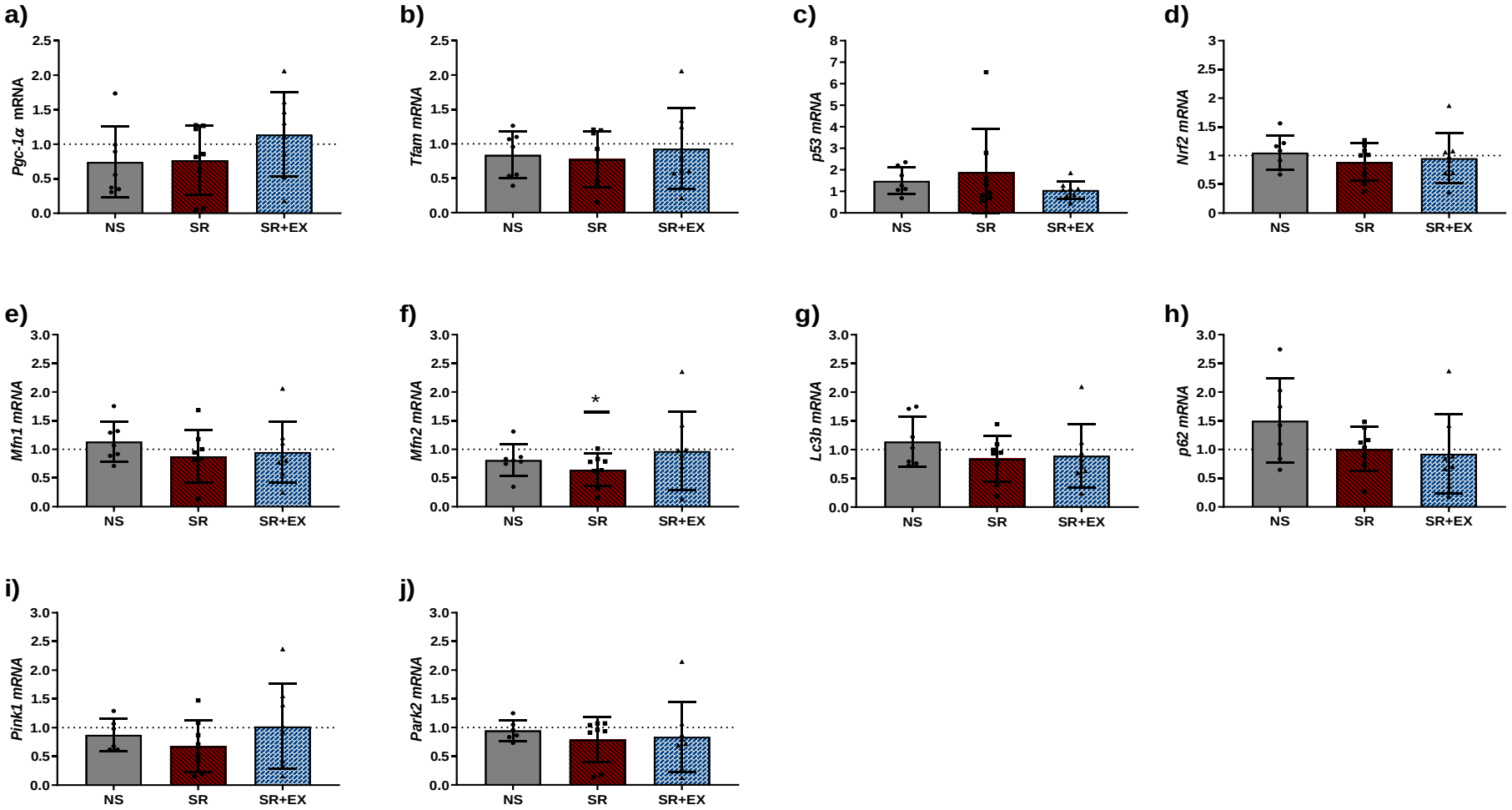

Figure 5.10 - Skeletal muscle mRNA expression of genes related to mitochondrial biogenesis, mitochondrial dynamics and mitophagy.
Normalised to pre-intervention values (i.e. fold change from pre-intervention). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). * Denotes significant change from pre-intervention (P<0.05). n= 7 in NS group, n= 8 in SR and SR+EX groups.

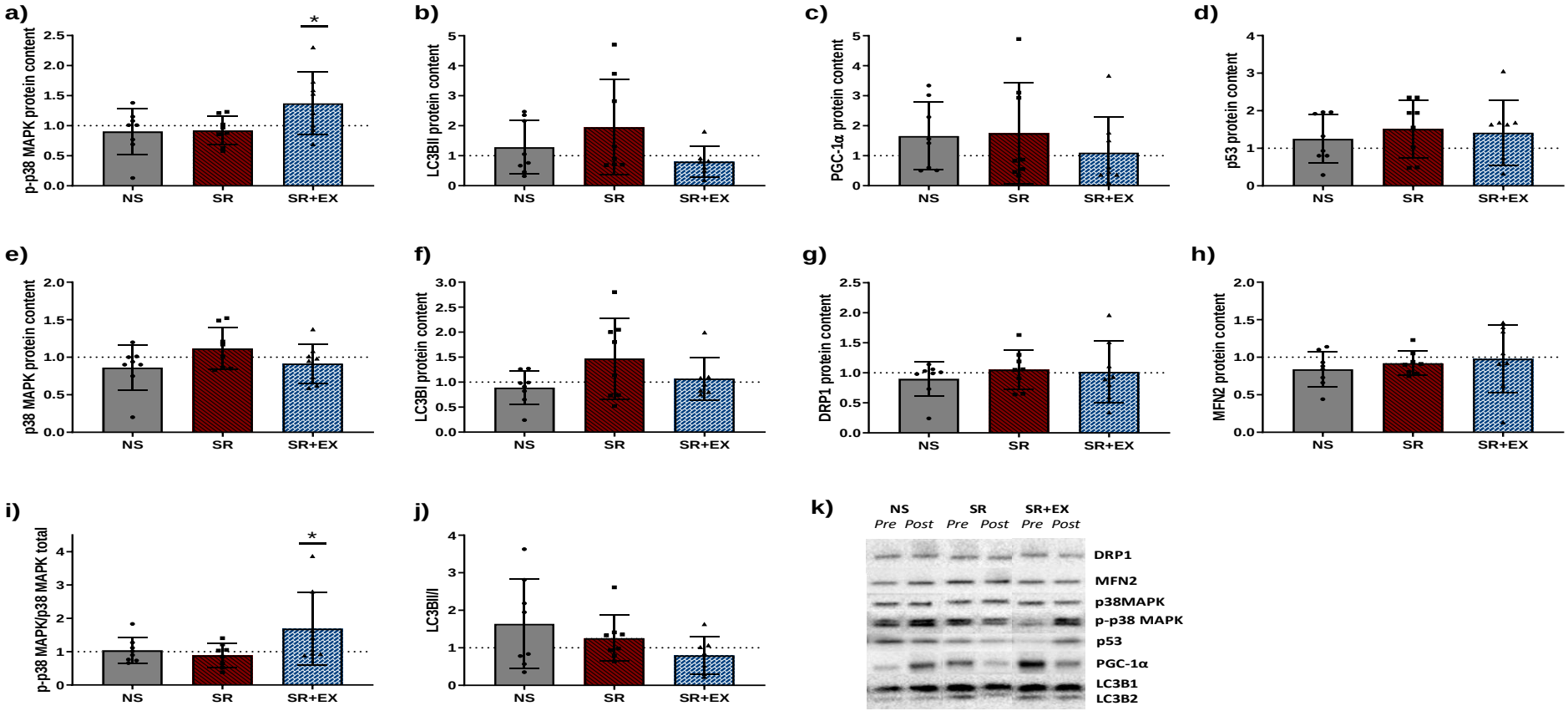

Figure 5.11 - Skeletal muscle protein content of proteins involved in mitochondrial biogenesis, dynamics, autophagy and signalling.
Values are normalised to Pre-intervention values (i.e. fold change from pre-intervention). a) p-p38MAPK Thr180/Tyr182, b) LC3BII, c) PGC-1α, d) p53, e) p38MAPK (Total), f) LC3BI, g) DRP1 (Total), h) MFN2, i) p-p38MAPK Thr180/Tyr182/p38MAPK (Total) ratio j) LC3BII/LC3BI ratio and k) representative western blot images. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). * Denotes significant change from pre-intervention ($P<0.05$). n=8 per group.

5.3.8 Glucose metabolism related mRNA expression in skeletal muscle

For *Glut4* mRNA expression there was no significant interaction effect (*P*=0.192), but there was a significant time effect (*P*=0.022), that revealed a reduction in *Glut4* mRNA expression from Pre- to Post-intervention (mean change ± SD %, 95% CI, *P* value) in the SR group (-38 ± 33%, CI [-13, 88%], *P*=0.020), but not in the NS (-14 ± 18%, CI [-39, 68%], *P*=0.840) or SR+EX groups (16 ± 83%, CI [-67, 33%], *P*=0.924), (**Figure 5.12**). There was no significant interaction (*P*=0.952) or time (*P*=0.060) effect for *Ampk1α* mRNA from pre- to post-intervention. Likewise, there was no significant interaction effect for *Pdk4* (*P*=0.862) or *β-Had* (*P*=0.872) mRNA. However, there was a significant effect of time *for β-Had* mRNA (*P*=0.011), with post-hoc testing showing no change within groups from pre- to post-intervention.

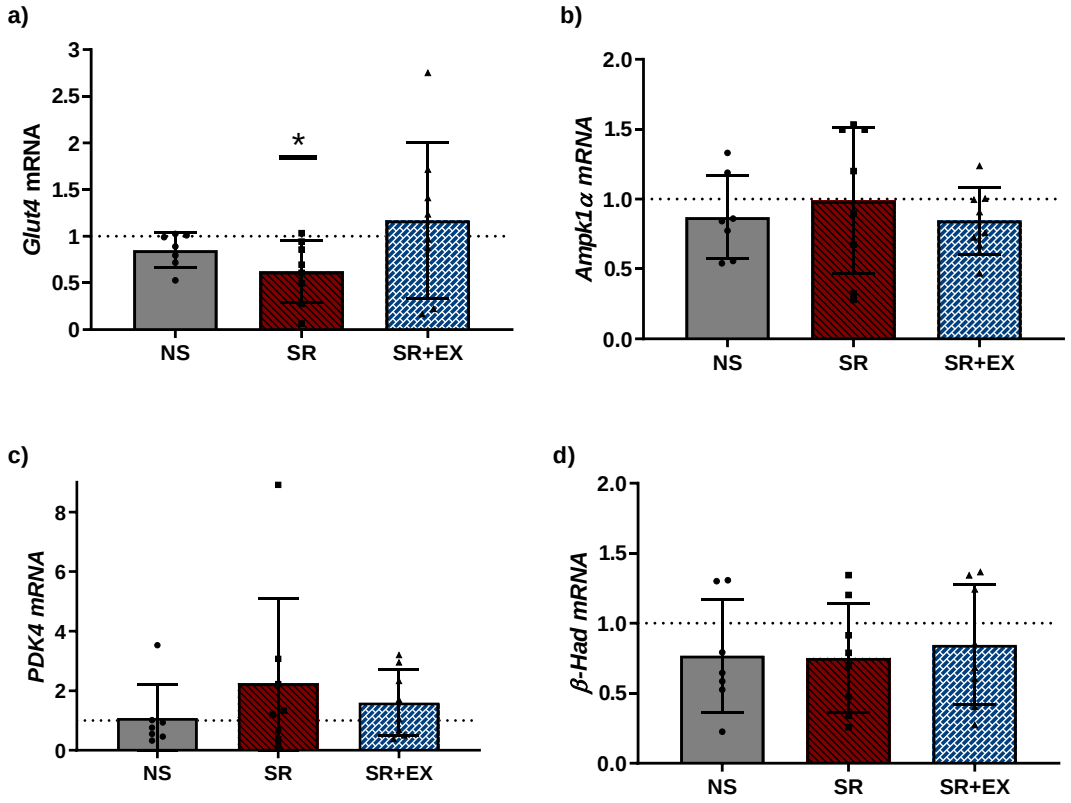

Figure 5.12 - Skeletal muscle mRNA expression of glucose uptake related genes.
Normalised to pre-intervention values (i.e. fold change from pre-intervention) for a) *GLUT4* mRNA expression, b) *Ampk1α* mRNA expression, c) *Pdk4* mRNA expression, and d) *ß-Had* mRNA expression. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). * Denotes significant within-group difference from pre- to post-intervention (p<0.05). n=7 in NS group and n=8 in SR and SR+EX groups.

5.3.9 Insulin signalling pathway proteins

There was no significant interaction effect for GLUT4 protein content (*P*=0.4658), total AKT (*P*=0.313), p-AKT ^{ser473} (*P*=0.058), p-AKT/AKT (Total) (*P*=0.148), total IRS-1 protein content (*P*=0.744), AS160 protein content (*P*=0.056), p-AS160^{thr642} (*P*=0.100) and p-AS160^{thr642}/AS160 (Total) (*P*=0.715) from pre- to post intervention (**Figure 5.13**).

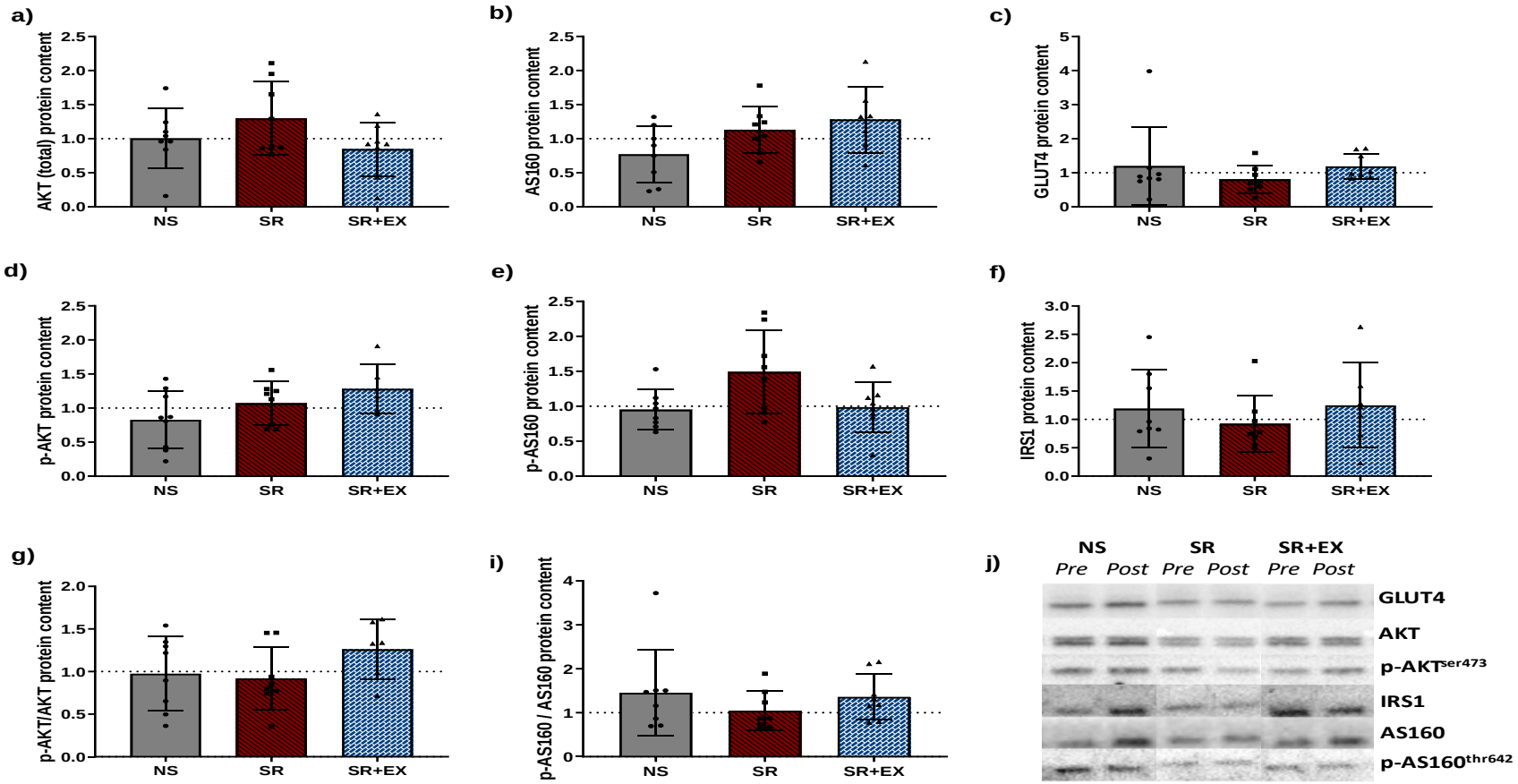

Figure 5.13 - Skeletal muscle protein content of proteins involved in skeletal muscle glucose uptake.
a) AKT (total) b) AS160 (Total), c) GLUT4, d) p-AKT^{ser473}, e) p-AS160^{thr642}, f) IRS1 (Total), g) p-AKT ser473/AKT (Total) ratio, i) p-AS160^{thr642}/AS160 (Total) and j) representative western blots. Values normalised to with-in group pre-intervention values (i.e. fold change from pre-intervention). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). Individual changes from pre-intervention are shown and bars represent mean values ± SD. n= 8 per group.

5.3.10 Plasma interleukin-6 concentrations

There was no significant change from pre- to post-intervention for plasma interleukin-6 concentrations in any of the groups (interaction effect, *P*= 0.306), (mean change pg/mL ± SD, 95% CI, P value), NS (0.08 ± 0.76 pg/mL, CI [-0.38, 0.49 pg/mL], *P*>0.999), SR (-0.28 ± 0.37 pg/mL, CI [-0.65, 0.16 pg/mL], *P*=0.375) and SR+EX (-0.23 ± 0.23 pg/mL, CI [-0.60, 0.21 pg/mL], *P*=0.631) (**Figure 5.14 - Plasma interleukin-6 concentrations**).

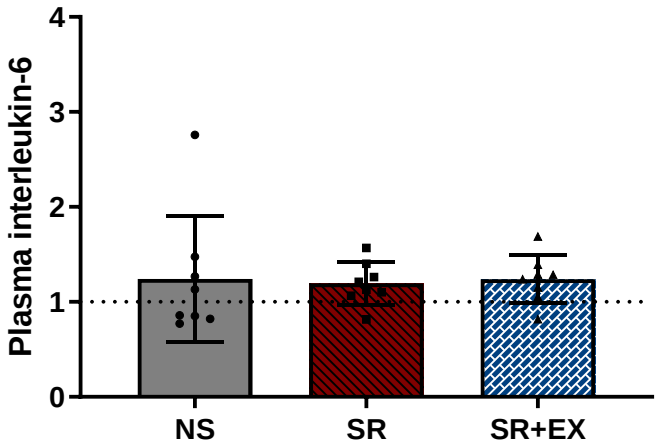

Figure 5.14 - Plasma interleukin-6 concentrations
Presented as fold-changes from pre- to post-intervention. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX). n=8 per group.

5.4 Discussion

5.4.1 Main findings

The aim of this chapter was to further our understanding of the mechanisms that lead to sleep-loss-induced reductions in glucose tolerance, and to explore the potential of exercise as an intervention to mitigate these effects. The sleep restriction protocol significantly reduced glucose tolerance, skeletal muscle mitochondrial respiratory function, and sarcoplasmic protein synthesis (SarcPS) – an indicator of mitochondrial protein synthesis. However, performing three sessions of high-intensity interval exercise (HIIE) during the sleep restriction intervention partially mitigated the reductions that were observed in the SR group. Further, there were differences between the SR and SR+EX groups in the expression and content of important glucose and mitochondrial-related regulatory markers, which may help to elucidate the mechanisms underpinning the physiological changes observed in the context of the sleep restriction protocol. While adding further support to the negative physiological impact of sleep loss on skeletal muscle health, these results show for the first time that sleep restriction reduces mitochondrial respiratory function. Importantly, by performing HIIE during the sleep restriction intervention the reductions on glucose tolerance and mitochondrial function can be mitigated.

5.4.2 Sleep restriction and glucose tolerance

The sleep restriction protocol used in this study (consisting of 4 h TIB, each night, for 5 consecutive nights), resulted in a significant decrease in glucose tolerance, as assessed via total area under the curve (AUC) from an OGTT. This outcome was reflected by significantly increased blood glucose levels at the 30- and 60-minute time points of the OGTT. While differing methodological approaches have led to slight variations of results, previous research using similar sleep restriction protocols has comprehensively demonstrated similar detrimental effects on glucose metabolism (Spiegel, Leproult et al. 1999, Buxton, Pavlova et al. 2010). Both Rao et al. (2015) and Reynolds et al. (2012) used a similar sleep restriction protocol, (i.e., 5 nights, with a 4 h sleep opportunity each night) and reported a 25% decrease in whole-body insulin sensitivity using a hyperinsulinaemic-euglycaemic clamp (HEC), and reduced glucose tolerance using continuous glucose monitoring (Reynolds, Dorrian et al. 2012, Rao, Neylan et al. 2015). Although Rao et al. (2015) also performed an OGTT at the end of both the regular sleep and sleep restriction protocol, they did not detect a change in glucose AUC (they did report changes in insulin AUC). This may be explained by differences in experimental conditions (i.e., diet or other circadian influences such as the sleep/wake schedules) or the day-to-day variability previously reported with the OGTT (CV 13.4%) (Gordon, Fraser et al. 2011). While the OGTT is a validated, commonly used technique, which is well correlated with the gold standard HEC, it provides only a snapshot of blood glucose and insulin at certain time-points following a glucose challenge and

cannot account for the contribution of hepatic glucose production; this means it provides only limited mechanistic information and sensitivity to changes in glucose metabolism (DeFronzo, Tobin et al. 1979, Matsuda and DeFronzo 1999, Mari, Pacini et al. 2001, Muniyappa, Lee et al. 2008). Nonetheless, the changes in glucose AUC observed (i.e., a 22% increase with sleep restriction) in this study are in line with previous research and solidify the evidence supporting the detrimental effect of even short periods of sleep restriction on glucose tolerance. Given that even acute post-prandial hyperglycaemia, such as has been demonstrated here, is considered a risk factor for microvascular complications, these findings could be considered clinically meaningful (Danne, Nimri et al. 2017).

The insulin response during an OGTT can also reflect changes in glucose metabolism. In the current study, plasma insulin concentrations were significantly elevated at the 60-minute time-point for the SR group only. However, whilst the average insulin total AUC from pre- to post-intervention increased by 29% (likely a meaningful physiological change), this did not reach statistical significance. Increases in plasma insulin concentrations have previously been reported (Spiegel, Leproult et al. 1999, Rao, Neylan et al. 2015, Sweeney, Jeromson et al. 2017) with Rao et al. (2015), for example, reporting a significant 20% increase following 5 nights of 4 h TIB. Increases in insulin concentrations to a standard glucose load are typically indicative of an increase in insulin resistance (generally defined as a decreased responsiveness to the metabolic actions of insulin) (Muniyappa, Lee et al. 2008). Similar to the glucose changes, discrepancies between the results from this study and previous studies may be explained by experimental protocol variations, such as diet, timing of sampling, and with individual variability in response to the glucose challenge. Despite no significant change in AUC, the elevated insulin concentrations at the 60-minute time point in the SR group, combined with the glucose results, suggest a detrimental effect of sleep restriction on glucose metabolism.

5.4.3 Sleep restriction, glucose tolerance and exercise

A novel aspect of this study was to examine the effect of three sessions of HIIE during the sleep restriction protocol. In contrast to the SR group, and despite a small increase in glucose at the 60- and 90-minute time points of the OGTT, there was no change in glucose AUC post-intervention for the SR+EX group. Likewise, there was no significant increase in plasma insulin for the SR+EX group in terms of AUC or individual OGTT time points. While the role of exercise in mitigating the detrimental effects of sleep loss has not been extensively examined in this context, there is considerable evidence that demonstrates the beneficial effect of exercise on glucose metabolism (Richter and Hargreaves 2013). In the context of sleep loss, a recent study demonstrated a protective effect of HIIE on glucose metabolism when exercise is performed in the weeks prior to a night of sleep deprivation (de Souza, Dattilo et al. 2017). Furthermore, Van

Helder et al. (1993) also suggested that physical activity (in the form of a 6 h hike), performed during a 60-h period of sleep deprivation, was able to improve the insulin response compared to those not performing the physical activity (VanHelder, Symons et al. 1993). However, the results of both of these studies may be influenced by the acute effects of exercise on glucose tolerance (which are known to persist for up to 48 h post-exercise), with testing being performed in the hours immediately post exercise (Richter and Hargreaves 2013). Therefore, this study provides novel data to indicate that performing HIIE during a period of sleep restriction can mitigate the negative effects on glucose tolerance. The potential mechanisms underlying these findings, however, have not previously been investigated.

5.4.4 Sleep loss and mitochondria

For the first time, we have observed a reduction in skeletal muscle mitochondrial respiratory function in response to sleep restriction. A reduction in $(ETF+CI)_P$ and $(ETF+CI+CII)_P$ mitochondrial respiration was observed in the SR group, but not the NS or SR+EX groups. It has previously been reported that following 72 h of total sleep deprivation, the activity of complexes I to III within the mitochondrial electron transport system (measured via spectrophotometric methods) was reduced in the hypothalamus of rats (Andreazza, Andersen et al. 2010). Additionally, a study investigating a single night of 4 h TIB in humans reported a decrease in insulin sensitivity with a concomitant increase in plasma acylcarnitines, suggested to be indicative of both reduced fatty acid oxidation and mitochondrial function (van den Berg, Mook-Kanamori et al. 2016). In contrast to the SR group, the SR+EX group maintained mitochondrial respiratory function following the intervention. This can be explained by previous findings demonstrating the potency of HIIE for improving mitochondrial function (Granata, Oliveira et al. 2016, MacInnis, Zacharewicz et al. 2017). The changes in mitochondrial function provide a potential mechanism for the reported decrease in glucose metabolism observed in the SR group. Indeed, reductions in mitochondrial respiratory function have previously been associated with the development of insulin resistance (via a range of mechanisms) (Kelley, He et al. 2002, Mogensen, Sahlin et al. 2007, Fazakerley, Minard et al. 2018), although this has been disputed by others (Pospisilik, Knauf et al. 2007, Holloszy 2013). While these results do not allow us to establish a causal link between decreases in mitochondrial function and glucose tolerance, it is interesting to note that sleep restriction was associated with a decrease in both mitochondrial respiratory function and glucose tolerance, and both of these changes were mitigated by exercise.

5.4.5 Mechanisms for decreases in mitochondrial respiratory function

5.4.5.1 Sarcoplasmic protein synthesis

Mitochondria are dynamic organelles that are constantly regulated in response to changing cellular conditions. The synthesis of new mitochondrial proteins has been suggested as the best measure of mitochondrial biogenesis, with the understanding that changes to the mitochondrial reticulum also involve processes of mitochondrial remodelling and protein breakdown (Miller and Hamilton 2012, Bishop, Botella et al. 2019). In the current study there was a reduced SarcPS, which can be considered a proxy for mitochondrial protein synthesis (MitoPS) (as suggested by the high citrate synthase activity reported in the sarcoplasmic fraction – see **Figure 3.10**), in the SR group compared to both the NS and SR+EX groups. These findings could potentially contribute to the observed changes in mitochondrial respiration via the decreased production of new mitochondrial proteins and/or the reduced replacement of damaged mitochondrial proteins, with a subsequent impact on oxidative phosphorylation capacity (Miller and Hamilton 2012). However, while changes in MitoPS and mitochondrial respiratory function have been shown to occur concomitantly, a direct link is yet to be established between the two (Robinson, Dasari et al. 2017, Groennebaek, Jespersen et al. 2018, Bishop, Botella et al. 2019)

While SarcPS has not previously been reported in the context of sleep loss, HIIE has consistently been shown to increase both sarcoplasmic and mitochondrial protein synthesis and this likely accounts for the higher SarcPS in the SR+EX group compared to the SR group (Scalzo, Peltonen et al. 2014, Bell, Seguin et al. 2015). Indeed, previous reports indicate that SarcPS remains elevated for 48 h following endurance exercise (Miller, Olesen et al. 2005), which is a similar time-frame to when the muscle samples were collected following the final HIIE session in this study. Although, combining exercise with sleep restriction maintained comparable levels of SarcPS with the NS group, it is unclear whether sleep restriction (4 h TIB) actually attenuated the protein synthetic response induced by the exercise. That said, an additional group that combined normal sleep (8 h TIB) with the exercise intervention (i.e., NS+EX) would help to elucidate this. Indeed, it has been shown that a period of step reduction induces a state of 'anabolic resistance', whereby the increase in myofibrillar protein synthesis in response to an infusion of amino acids is reduced compared to a period of normal activity (Breen, Stokes et al. 2013). Whether sleep restriction has a similar effect to this, but on SarcPS, is unknown. However, to determine if the changes in SarcPS may have influenced the changes in mitochondrial respiration, the processes involved in the remodelling of mitochondrial morphology (i.e., mitochondrial fission and fusion), or mitochondrial content must also be considered.

5.4.5.2 Markers of mitochondrial content

Considering the changes observed for mitochondrial respiration and SarcPS, the effect of sleep restriction on markers of mitochondrial content was also assessed. In the present study there were no changes to the protein content of mitochondrial complex subunits or CS activity - both of which are markers of mitochondrial content (Larsen, Nielsen et al. 2012). While reduced CS activity has previously been reported, this was in response to an extreme sleep deprivation intervention (i.e., 120 h of continuous wakefulness) (Vondra, Brodan et al. 1981), making a direct comparison of these findings difficult. Despite no change in the SR+EX group, exercise is well known to increase mitochondrial content (Holloszy 1967, Hoppeler, Howald et al. 1985). It's plausible that the time frame of the intervention and the exercise training volume (30 minutes of exercise, across 5 days and in 3 HIIE sessions) of our study was insufficient to observe changes in CS activity. Others have reported increases in CS activity following 3 sessions of HIIE, although the total volume of exercise was considerably higher (10 x 4 min intervals at 90% $\dot{V}O_{2peak}$, interspersed by 2 min of rest) (Perry, Lally et al. 2010). Together, these results suggest that the changes in mitochondrial respiratory function and reduced SarcPS occurred independently of detectable changes in mitochondrial content. This dissociation between changes in mitochondrial content and respiratory function has previously been reported and it has been suggested that these properties may be differentially regulated (Rowe, Patten et al. 2013, Granata, Oliveira et al. 2016, Granata, Jamnick et al. 2018, Groennebaek, Jespersen et al. 2018, Meinild Lundby, Jacobs et al. 2018). Increases in MitoPS and mitochondrial respiratory function, without increases in CS activity have previously been reported (although this was following a resistance training intervention) (Groennebaek, Jespersen et al. 2018). One explanation for the observations in this study may involve the processes regulating mitochondrial dynamics/remodelling (i.e., fission and fusion), which can alter the efficiency and oxidative capacity of the mitochondria, without necessarily altering mitochondrial content (Drake, Wilson et al. 2016, Nielsen, Gejl et al. 2017). Future studies would benefit from the addition of transmission electron microscopy (TEM) (considered the 'gold-standard' for the assessment of mitochondrial content) to confirm these findings and to assess potential alterations to mitochondrial morphology that may be indicative of remodelling processes and functional measurements.

5.4.5.3 Molecular mechanisms regulating mitochondrial content and function

Some of the potential molecular mechanisms that might contribute to the changes in mitochondrial function and sarcoplasmic protein synthesis with sleep restriction were also investigated. This included genes and proteins known to regulate mitochondrial dynamics and biogenesis. In the current study there was a reduction in Mitofusin-2 (*Mfn2)* mRNA expression in the SR group, suggesting that sleep restriction may have implications for mitochondrial fusion, although no change in MFN2 protein content was observed, potentially due to the short duration

of the study. Considering the previously reported positive correlation between increased skeletal muscle *Mfn2* mRNA expression and insulin sensitivity, and that MFN2 null mice display reduced mitochondrial respiratory function (without changes in mitochondrial content), concomitant with reduced insulin sensitivity, (Bach, Naon et al. 2005, Sebastian, Hernandez-Alvarez et al. 2012), the reduced *Mfn2* gene expression in this study may have contributed to the reduced mitochondrial respiratory function and glucose tolerance observed in the SR group.

Exercise may also influence mitochondrial dynamics and mitophagy, with previous studies demonstrating changes to the genes and proteins involved in the regulation of the mitochondrial reticulum (via fission, fusion and mitophagy) following both a single session of exercise and exercise training (Perry, Lally et al. 2010, Fealy, Mulya et al. 2014, Konopka, Suer et al. 2014, MacInnis, Zacharewicz et al. 2017, Trewin, Berry et al. 2018). In the current study *Mfn2* gene expression was maintained in the SR+EX group from pre- to post-intervention. As *Mfn2* has previously been shown to be elevated 24 h post an endurance exercise session (Cartoni, Leger et al. 2005) and muscle samples were collected 48-h post the final HIIE session in our study, this may explain why *Mfn2* mRNA levels were maintained in the SR+EX group.

Exercise is also known to upregulate pathways responsible for the induction of mitochondrial biogenesis. The SR+EX group had an increase in p-p38 MAPK$^{Thr180/Tyr182}$ post-intervention. This is novel data as the signalling mechanisms regulating different aspects of mitochondrial content and function have not been measured in the context of sleep restriction protocols. The increases in p-p38 MAPK$^{Thr180/Tyr182}$ in the SR+EX group is in accordance to the responses often seen following exercise. Although this increase is often seen acutely in the hours immediately following exercise (Bartlett, Hwa Joo et al. 2012), there have also been reports of increased phosphorylation 24 h post-exercise (Little, Safdar et al. 2011). p38 MAPK activity is associated with the activation of further downstream targets (i.e., PGC-1α and p53) of signalling pathways associated with mitochondrial biogenesis (Akimoto, Pohnert et al. 2005), although this was not seen in this study. This result suggests the upregulation of molecular signalling pathways involved in mitochondrial biogenesis by HIIE, which may have contributed to the maintenance of mitochondrial respiratory function and higher SarcPS in the SR+EX group.

5.4.6 Molecular mechanisms involved in glucose metabolism

Together with, or independent of, changes in mitochondrial respiratory function, sleep-restriction may have influenced a range of genes and proteins known to be involved in glucose metabolism and skeletal muscle glucose uptake. Glucose transporter 4 (GLUT4) is the key glucose transporter isoform, responsible for the facilitated diffusion of glucose into skeletal muscle (Birnbaum 1989).

In this study, a significant decrease in *Glut4* mRNA expression was observed in the SR group, while no change was observed in the SR+EX group. In contrast, Cedernaes et al. (2018) reported no change in *Glut4* mRNA expression or GLUT4 protein content following one night of sleep deprivation (Cedernaes, Schonke et al. 2018). The differences between studies may be explained by the differing sleep loss interventions. As an increase in *Glut4* mRNA expression is commonly observed in the hours immediately following exercise (although levels usually return to baseline within 24 h of exercise), this may explain why a similar decrease to the SR group was not observed in the SR+EX group (Ren, Semenkovich et al. 1994, Kraniou, Cameron-Smith et al. 2004, Kraniou, Cameron-Smith et al. 2006). In some cases, reductions in GLUT4 expression (mRNA and protein) have been associated with reductions in glucose tolerance; however, this effect is not consistent and appears to be tissue-type and method specific (i.e., whole-body or tissue specific knockout) (Fam, Rose et al. 2012). Further, *Glut4* mRNA and protein content is maintained in the skeletal muscle of type 2 diabetics, with the reduction in glucose tolerance strongly associated with GLUT4 translocation to the plasma membrane, as opposed to changes in overall protein content (Shepherd and Kahn 1999, Richter and Hargreaves 2013). The phosphorylation of Akt substrate of 160 kDa (AS160 or Tbc1D4) is responsible for activating GLUT4 translocation and we observed no change in p-AS160^{thr642} in this study, suggesting GLUT4 translocation was not influenced (Bogan 2012). Aside from the changes to *Glut4* expression, there were no changes to an array of insulin signalling or substrate metabolism genes and proteins. There is a gap within the literature for sleep-restriction-induced deficits of the insulin signalling pathway, with the only other studies to investigate this showing a decrease in Akt activity *in vitro* (Broussard, Ehrmann et al. 2012, Sweeney, Jeromson et al. 2017) (although no changes in total or p-AKT were shown in the current study). The results of the current study, while not conclusively linking *Glut4* mRNA expression to changes in glucose tolerance, may be an early indication of disruption to the insulin/glucose uptake signalling pathways, which contribute to the detrimental effect of sleep restriction on glucose metabolism. Further studies are needed to investigate these pathways in this context.

5.4.7 Sleep loss, inflammation and insulin resistance:

The development of insulin resistance and T2DM has previously been associated with higher plasma levels of the pro-inflammatory marker interleukin-6 (IL-6) (Kern, Ranganathan et al. 2001), whilst at lower levels IL-6 may improve glucose metabolism in skeletal muscle (Kim, Bachmann et al. 2009). There was no change in plasma IL-6 concentrations from pre- to post-intervention in any of the groups in the present study. The IL-6 response to previous sleep restriction protocols has produced varying results (Mullington, Simpson et al. 2010). Similar protocols to the current study (5 nights, 4 h TIB) have been associated with increases in IL-6 (van Leeuwen, Lehto et al. 2009), while other findings are in line with these results and demonstrate

no change (Faraut, Boudjeltia et al. 2012). These differing results may be explained by a number of factors, including timing of sample collection, inter-individual differences, and the variations in sleep-restriction protocols. Previous studies have reported a strong correlation (r = 0.71) between plasma IL-6 and insulin sensitivity (Kern, Ranganathan et al. 2001). Further, research has shown that IL-6 exposure in various cell models can inhibit the insulin signalling pathway (Senn, Klover et al. 2002). However, in contrast to this, some research has also shown that infusion of IL-6 may even increase glucose disposal rates during exercise (Febbraio, Hiscock et al. 2004). It has been shown that skeletal muscles can produce and release IL-6 into the blood during exercise, which could indicate a beneficial effect of IL-6 on glucose tolerance (Febbraio and Pedersen 2002). No change in IL-6 was observed in the SR+EX group, which may be a reflection of the 48 h gap between the final exercise session and sample collection. Increased blood sampling throughout the protocol (i.e., multiple times each day), rather than just pre- and post-intervention may help to elucidate any role of IL-6 in the changes to glucose tolerance observed and help to track any potential changes in plasma concentration that may occur in during the protocol.

5.4.8 Limitations

While our sample size is common to similarly invasive and logistically challenging studies (Donga, van Dijk et al. 2010, Reynolds, Dorrian et al. 2012, Rao, Neylan et al. 2015), it may not have provided sufficient power to detect significance for some changes. Nonetheless, the study was adequately powered to detect changes in glucose tolerance and mitochondrial respiratory function following 5 nights of 4 h TIB. Another potential limitation of the study was the use of an OGTT, rather than a hyperinsulinaemic-euglycaemic clamp, to assess glucose metabolism. However, the OGTT is a validated, commonly used, cost-effective, and simple method for assessing glucose tolerance (and is regularly used in clinical populations) (DeFronzo, Tobin et al. 1979, Matsuda and DeFronzo 1999). While the hyperinsulinaemic-euglycaemic clamp provides information regarding the contributions of both the peripheral tissues (i.e., skeletal muscle) and the liver to whole-body insulin sensitivity, it also has some drawbacks (Muniyappa, Lee et al. 2008). During a clamp, the hyperinsulinaemic conditions created may not reflect the physiological conditions associated with the ingestion of a regular mixed meal, and hence the value of the findings may be somewhat limited in application (Muniyappa, Lee et al. 2008). Nevertheless, its use in this study, or that of continuous glucose monitoring, may have allowed for a deeper investigation of glycaemic control, and the potential role of the insulin signalling pathway in sleep-restriction-induced decreases in glucose tolerance. It should also be noted that the ingestion of glucose (from the OGTT) prior to the collection of muscle biopsies may influence the acute muscle signalling response (particularly the insulin signalling pathway). However, as no change

in insulin signalling markers were observed (i.e., p-AKT^{ser473} and p-AS160^{thr642}) in any groups this is unlikely.

Another limitation of this study was the analysis of SarcPS as an indicative measure for mitochondrial protein synthesis (MitoPS). While both this study and others (Bell, Seguin et al. 2015) have used the sarcoplasmic fraction as a surrogate for MitoPS, and sarcoplasmic fractions are thought to be comprised heavily of mitochondria, it is acknowledged that this fraction contains a number of other cellular proteins and is not a 'pure' mitochondrial fraction (Haun, Vann et al. 2019). However, as demonstrated by the CS activity of each fraction, the sarcoplasmic samples were significantly enriched in citrate synthase, compared to both the whole-muscle and myofibrillar fractions. As we have not directly measured mitochondrial FSR, we can only speculate to the similarities between MitoPS and SarcPS. To assess MitoPS, a large amount of tissue is required (approximately 80 to 100 mg) and thus was not feasible in this study.

5.4.9 Conclusion

In summary, this study has provided the first direct evidence of a concomitant decrease in mitochondrial respiratory function and glucose tolerance following a sleep restriction protocol (5 nights of 4 h TIB). This coincided with a decrease in sarcoplasmic protein synthesis (indicative of decreased mitochondrial protein synthesis), but no changes in a range of markers associated with mitochondrial content. Furthermore, alterations in mRNA expression of select genes involved in mitochondrial dynamics and glucose uptake were observed. Collectively, these results highlight some potential mechanisms by which sleep restriction may lead to reductions in glucose tolerance, although studies of longer duration may help to elucidate these mechanisms further and allow detection of changes in regulatory proteins to be observed. Additionally, methods that allow further mechanistic insight into the effect of sleep loss on the insulin signalling pathway (such as the HEC (with pre- and post-muscle biopsies) should be considered. Of note, HIIE, mitigated the detrimental changes, potentially via the upregulation of signalling pathways involved in mitochondrial biogenesis. These data provide a basis for the development of evidence-based health guidelines and recommendations for those experiencing inadequate sleep by highlighting the molecular mechanisms that can be targeted via interventions such as exercise.

Chapter 6. The Effect of Sleep Restriction, With or Without Exercise, on Myofibrillar Protein Synthesis

The results of Chapter 5 demonstrated the detrimental effect of sleep restriction on glucose tolerance, SarcPS – an estimate of MitoPS, and mitochondrial respiratory function in human skeletal muscle. As hypothesised, performing exercise was able to counteract some of these negative effects. The aim of the results presented in this chapter was to expand the investigation into the detrimental effects of sleep restriction by examining the mechanisms underlying previously reported sleep-loss-induced reductions in muscle mass, by measuring changes in myofibrillar protein synthesis and some of the molecular signalling processes by which it is regulated. Furthermore, the efficacy of using high-intensity interval exercise to counteract the effects of sleep restriction was examined.

6.1 Introduction

Sleep contributes to many physiological functions, with roles in glucose regulation, physical recovery, hormone regulation, learning, memory consolidation, and cognitive performance (Spiegel, Leproult et al. 1999, Van Dongen, Maislin et al. 2003). In recent times, sleep loss has also been causatively implicated in the maintenance of muscle mass (Nedeltcheva, Kilkus et al. 2010, Dattilo, Antunes et al. 2012). Large human cohort studies indicate an increased likelihood of both lower skeletal muscle mass and sarcopenia in those reporting insufficient or poor quality sleep (Chien, Wang et al. 2015, Buchmann, Spira et al. 2016, Hu, Jiang et al. 2017). Furthermore, during an energy deficit a disproportionately greater loss of muscle mass has been reported in humans following sleep restriction (5.5 h TIB, for 14 days), compared to normal sleep (Nedeltcheva, Kilkus et al. 2010), and muscle atrophy has been observed in animal models of sleep deprivation (Dattilo, Antunes et al. 2012, Monico-Neto, Antunes et al. 2015, Monico-Neto, Giampa et al. 2015, de Sa Souza, Antunes et al. 2016). Given the important metabolic and structural roles of skeletal muscle, and the increasing prevalence of sleep loss in modern society (Ford, Cunningham et al. 2015), there are clear health implications to mitigating reductions in muscle mass as a result of inadequate sleep. Thus, the aim of this study was to elucidate the potential mechanisms by which sleep loss may induce reductions in muscle mass. Furthermore, potential therapeutic interventions, such as exercise, which help to mitigate these detrimental effects, may have benefits for the considerable proportions of modern society reported to experience inadequate sleep (Ford, Cunningham et al. 2015, Sleep Health Foundation 2017).

There are a number of potential mechanisms by which sleep loss may lead to muscle atrophy; however, these have not been comprehensively studied in humans. Changes in muscle mass are determined by the balance between the rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Rennie 1985, Gibson, Halliday et al. 1987), which may be influenced by sleep loss. In the post-absorptive (fasted) state, MPB exceeds MPS, resulting in a net loss of protein, while, in comparison, in the fed state MPS increases above MPB, resulting in a net gain of protein (Rennie, Edwards et al. 1982). Indeed, previous research showed a reduced protein turnover (either reduced protein synthesis or an increase in protein degradation, or both), following 72 h of sleep deprivation in humans, as assessed by an increased urea excretion (Kant, Genser et al. 1984). Additionally, Cedernaes et al. (2018) also report increases in urea metabolites in both muscle and plasma following a night of simulated shift work. Despite these previous findings being from interventions of extended periods of wakefulness, they may also be relevant in instances of sleep loss commonly experienced by the wider population. Although no study has investigated the effects of sleep loss on either MPS or MPB, research with other interventions known to negatively affect muscle mass (e.g. muscle disuse/immobilisation and step reduction) typically infer a reduction in MPS, rather than an increase in MPB (Symons, Sheffield-Moore et al. 2009, Breen, Stokes et al. 2013, Rudrappa, Wilkinson et al. 2016). Measurements of MPS can be specified to sub-fractions of muscle, and these previous studies have highlighted a reduction in

myofibrillar protein synthesis (MyoPS - fractions enriched in contractile proteins, such as actin and myosin) in particular (Breen, Stokes et al. 2013). Accordingly, a reduction in MPS, in both the fed and fasted state, may also explain sleep-loss-induced changes to muscle mass; however, further research is required to test this hypothesis.

Reductions in MPS may be caused by a downregulation of the molecular pathways governing protein synthesis. At the molecular level, protein synthesis is regulated by the mammalian Target of Rapamycin (mTOR), which integrates nutritional and mechanical stimuli to generate an appropriate molecular response. Activation of the mTOR complex 1 (mTORC1) subsequently leads to the concurrent phosphorylation and activation of 70-kDa ribosomal S6 protein kinase (p70S6K) and inhibition of 4E-binding protein 1 (4EBP1), which in turn leads to an increase in protein synthesis and increases in muscle mass and strength (Bodine, Stitt et al. 2001). In contrast, myostatin acts as a negative regulator of protein synthesis, with its inhibition resulting in increases in muscle mass (Amthor, Macharia et al. 2007). How these pathways are influenced by sleep restriction and its potential effect on MPS has not been investigated.

While modification of protein synthesis is thought to be the driving force undermining changes in muscle mass, changes to protein degradation pathways, such as the ubiquitin proteasome system (UPS) and the autophagy pathway, can also be assessed to compliment MPS data. Molecular markers of these pathways are often measured as a proxy for MPB, due to the significant technical challenges associated with measuring MPB *in vivo* (Tipton, Hamilton et al. 2018). Activation of the UPS pathway is governed by the activity of AKT, and subsequent regulation of the transcription factors forkhead box O 1/3 (FOXO1 and FOXO3), which control the expression of ligases responsible for regulating protein degradation (i.e., muscle-specific E3 ubiquitin ligases, muscle RING-Finger-1 (MuRF1) and muscle atrophy F-box (MAFbx1)) (Sandri 2008). Animal models have shown an increased level of ubiquitinated proteins and markers of protein degradation (e.g., p-FOXO3) and autophagy pathways (e.g., LC3 and p62/SQSTM1) following 96 h of sleep deprivation in rats, with concomitant evidence of muscle atrophy (as assessed via muscle fibre cross-sectional area (CSA) (Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016). While yet to be investigated in humans, the molecular signalling pathways regulating MPS and MPB may contribute to sleep loss-induced changes in muscle mass.

Although resistance exercise is perhaps the most well-documented exercise known to stimulate protein synthesis, endurance exercise has also been shown to increase the activity of the protein synthesis pathway, with a subsequent increase in MyoPS (Di Donato, West et al. 2014, Bell, Seguin et al. 2015). Indeed, Bell et al. (2015) demonstrated that HIIE, consisting of 10 x 1-minute intervals at 90% $\dot{V}O_{2max}$ on a cycle ergometer, increased MyoPS (Bell, Seguin et al. 2015). While this increase was to a lesser

extent than resistance exercise (and aerobic exercise at 55-60% $\dot{V}O_{2peak}$ did not increase MyoPS), it nevertheless demonstrates the potential of HIIE for promoting MPS. Furthermore, following 30 minutes of cycling (at 60% W_{max}), Di Donato et al. (2014) reported an increase in p-mTOR^{ser2448} content, with a corresponding increase in MyoPS, suggesting the protein synthesis signalling pathways were involved (Di Donato, West et al. 2014). Therefore, HIIE may be able to counteract the detrimental effects of sleep loss on muscle mass by increasing the activity of the protein synthesis pathway and MyoPS, although this has not previously been investigated in humans.

The aim of this study was to investigate the effect of five nights of sleep restriction, with or without three sessions of HIIE, on MyoPS and the molecular signalling pathways that regulate it. HIIE was chosen because of the increases in MyoPS previously reported (Miller, Olesen et al. 2005, Di Donato, West et al. 2014, Bell, Seguin et al. 2015) and its other proven clinical benefits (such as improvements in insulin sensitivity and oxidative capacity) (Little, Gillen et al. 2011). Secondary to this, the molecular mechanisms that underpin potential changes in the protein synthesis and degradation pathways were investigated. These findings may help to direct future health recommendations on the topic by elucidating the mechanisms underpinning sleep loss-induced changes in muscle mass and designing targeted interventions to counteract these mechanisms.

6.2 Study methodology

Full details of all experimental procedures can be found in **Chapter 3 General Methodology**. Details specific to the methodology and data presented in this chapter are outlined briefly below.

6.2.1 Study overview

For an overview of the study protocol specified to this chapter, see **Figure 6.1**. Following the initial screening and assessment procedures, 24 healthy men underwent baseline fitness testing (see section 3.3.2) and a pre-study muscle biopsy to determine baseline mitochondrial respiration values and background skeletal muscle D_2O enrichment (as outlined in Chapter 3). Participants were then allocated into one of three, matched experimental groups; Normal Sleep (NS), Sleep Restriction (SR) or Sleep Restriction and Exercise (SR+EX). Baseline participant characteristics for the three groups are shown in **Table 3.2**.

The experimental component of the study consisted of an eight-night stay within the sleep laboratory. The protocol for each of the three experimental groups consisted of an initial two nights of baseline sleep, followed by a five-night intervention period and then a final night of recovery sleep. During the baseline sleep period all participants spent 8 h TIB, between 23:00 h and 07:00 h. For the remaining 5 nights, the NS group spent 8 h TIB (between 23:00 h and 07:00 h), while both the SR and SR+EX groups spent 4 h TIB (between 03:00 and 07:00) each night. The SR and SR+EX group TIB (03:00 h – 07:00 h) was standardised to ensure all participants woke at the same time each morning and that the subsequent testing procedures were performed following the same time duration post waking. Between 23:00 h and 03:00 h, lighting was dimmed to below 15 lux to reduce the effect of lighting on circadian rhythms (Duffy and Wright 2005).

Upon arrival all participants consumed 150 mL of D_2O for the assessment of MyoPS throughout the study. Further, two experimental testing sessions were conducted during the participant's stay. At 10:00 h on the morning of Day 3 (following 2 nights of baseline sleep), the pre-intervention resting skeletal muscle biopsy was collected as per section **3.3.3**, for the assessment of genes and proteins related to protein synthesis and degradation signalling pathways. A post-intervention muscle biopsy was then collected on the morning of Day 8 at 10:00 h (following the final night of the sleep intervention). For the duration of the study, each participant was provided with a standardised diet relative to body mass consisting of fixed proportions of carbohydrates (4.5 $g.kg^{-1}.d^{-1}$), protein (1.5 $g.kg^{-1}.d^{-1}$) and fat (1 $g.kg^{-1}.d^{-1}$). All meal times (there were six throughout the day) were kept constant throughout the study and participants were asked to eat all food provided. Meal timings were kept the same for all groups. An identical menu was provided on the days prior to both experimental sessions (an example of the daily diet is provided in **Appendix B**). Participants remained within the laboratory and were monitored throughout each study. To match the activity count prior to the commencement of the study, participants

were instructed to walk outside the facility at designated periods throughout the day, while being accompanied by a member of the research staff. Waking hours were spent watching television, reading, working on a computer, or talking to staff. Approval of the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee (HRE15-294).

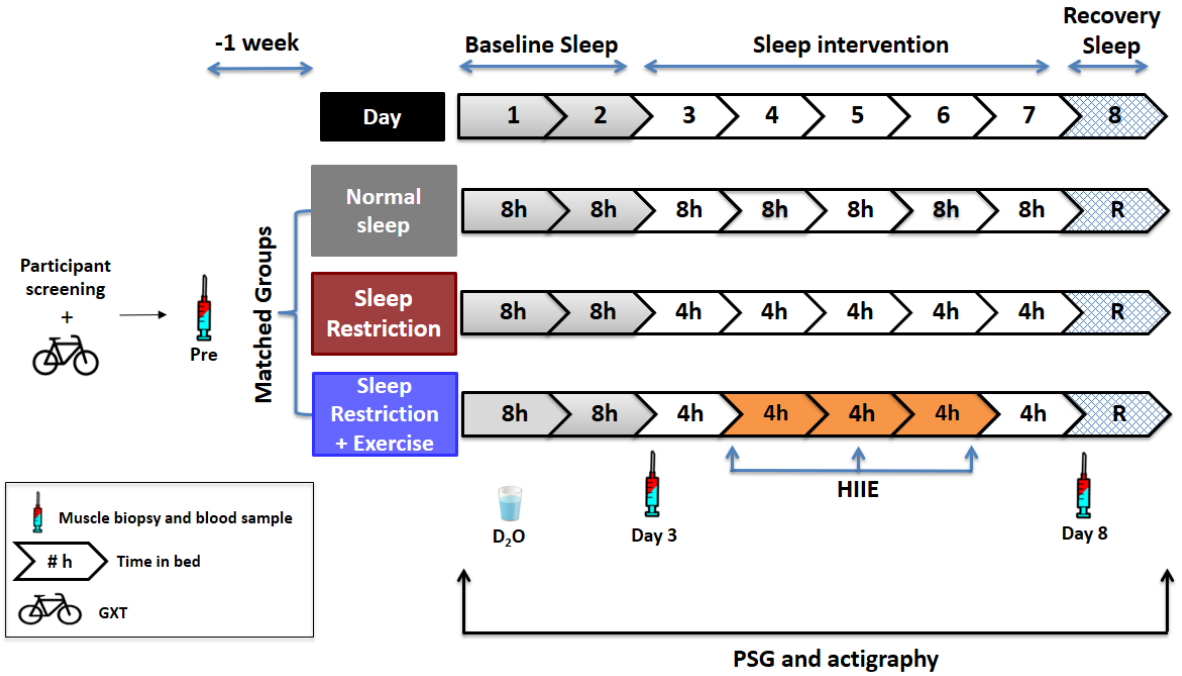

Figure 6.1- Schematic representation of the study protocol for Chapter 6.
GXT – Graded exercise test, D2O – deuterium oxide ingestion, PSG – polysomnography sleep analysis, HIIE – high-intensity interval exercise, R – *ad libitum* recovery sleep, participant screening refers to medical questionnaires, exclusion criteria and, habitual sleep and physical activity monitoring.

6.2.2 High-intensity interval exercise (HIIE)

The high-intensity interval exercise sessions (consisting of 10 x 60-s intervals at 90% $\dot{W}_{peak}$, interspersed by 75-s of active recovery on a cycle ergometer) were conducted in the SR+EX group only, at 10:00 h on Day 4, 5 and 6 as reported in section **3.3.5.**

6.2.3 Muscle sample analysis

Three muscle biopsies were collected throughout the study in accordance with section **3.3.3.** Muscle biopsies that were collected on Day 3 and Day 8 of the study protocol (i.e., pre- and post-intervention) were assessed according to the techniques described below.

6.2.3.1 Myofibrillar protein synthesis (MyoPS)

Deuterium oxide (D_2O) was ingested on Day 1 of the study protocol to allow for the assessment of MyoPS during the intervention period. Details regarding D_2O ingestion, assessment of body water

enrichment (via saliva sample collection), preparation of myofibrillar fractions from the muscle biopsies, analysis, and calculations of the fractional synthetic rate are available in section **3.4.3**.

6.2.3.2 Western blotting

Skeletal muscle protein content was assessed via western blot (see section **3.4.6**) for markers of protein synthesis signalling pathways and markers of protein degradation pathways. Details relating to the relevant antibody conditions are outlined in **Table 6.1**.

Table 6.1 – Western blot primary antibodies, and incubation conditions for Chapter 6.

Protein	Antibody	Dilution	Secondary Conditions
mTOR	CST2983	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-mTOR^{ser2448}	CST5586	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
Caspase-3	CST9662	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p70S6K (total)	CST9202	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
TSC2	CST4308	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-TSC2^{thr1462}	CST3617	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
p-4EBP1$^{thr37/46}$	CST2855	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit
NFκB	CST8242	1:1000 in 5% BSA (in TBST)	1:10,000 in 5% SM (in TBST) – anti rabbit

BSA – bovine serum albumin, SM – skim milk

6.2.3.3 Real-time quantitative polymerase chain reaction (RT-PCR)

The expression of genes relating to the regulation of protein synthesis and protein degradation were assessed as per the RT-PCR protocol outlined in section Real-time quantitative polymerase chain reaction (RT-PCR)**3.4.8.** Details relating to the specific primers used within this chapter are presented in **Table 4.2 - RT-PCR primer sequences for Chapter 4.Table 6.2.**

Table 6.2 - RT-PCR primer sequences for Chapter 6.

Primer Name	Primer Sequence	Product Size (bp)	Efficiency (%)	Accession No.
Target Genes				
Myostatin	**F –** GGAGAAGATGGGCTGAATCCG **R –** GCATCGTGATTCTGTTGAGTGC	111	98.9	NM_005259
Murf1	**F –** CCGTCGAGTGACCAAGGAGA **R –** CCAGGATGGCATACAACGTG	80	98.6	NM_032588
Mafbx	**F –** GCAGCTGAACAACATTCAGATCAC **R –** CAGCCTCTGCATGATGTTCAGT	97	99.5	NM_058229
Foxo1	**F –** TGAGGGTTAGTGAGCAGGTTAC **R –** GGACTGCTTCTCTCAGTTCCT	73	108.1	NM_002015
Foxo3	**F –**TTGGTTTGAACGTGGGGAAC **R –**TGTGTCAGTTTGAGGGTCTGC	119	91.1	NM_001455.4
Mighty	**F –** CCAACTCCGGAGCAAATTTTTCA **R –** TCCGAAGCACAAGCTTCACT	106	94.7	NM_024595

F - Forward primer, **R** - Reverse primer

6.2.4 Statistical analysis

Statistical analyses were conducted using the statistical software package GraphPad Prism (V7.03). Pre- to post-intervention changes in gene expression and protein content were assessed for each group using a two-way repeated measures (RM) analysis of variance (ANOVA) (3 Groups x 2 Times). Significant effects of interaction (Group x Time), Time (Pre vs Post) and Group (NS vs SR vs SR+EX) are reported where effects are seen. Where there were significant effects, Bonferonni post-hoc testing was performed to locate the differences. All statistical analysis of gene expression and protein content data was run on raw values, and not fold-change data. Gene expression data in text is reported as percent fold-change from pre-intervention value ± % SD, 95% CI from fold-change data (as a percentage), with the *P* value from the raw data reported. Graphs represent fold-change from pre-intervention values, and individual responses are shown. A one-way RM ANOVA was used to assess differences between groups when it was not suitable to compare pre-intervention versus post-intervention values from within the same group. This was the case for myofibrillar protein fractional synthetic rates, data that was collected over the duration of the intervention period. All data in text, figures and tables are presented as mean ± standard deviation (SD), with P values ≤ 0.05 indicating statistical significance and confidence levels are reported and set to 95% (unless otherwise stated).

6.3 Results

6.3.1 Myofibrillar protein synthesis

Compared to the NS group, the fractional synthetic rate (FSR) of myofibrillar proteins was significantly reduced in the SR group (difference between groups FSR %/day ± SD, 95% CI, *P* value); 0.29 ± 0.08 FSR %/day, CI [0.09, 0.50 FSR %/day], *P*=0.004) (**Figure 6.2**). Further, the myofibrillar FSR was significantly reduced in the SR group compared to the SR+EX group (0.37 ± 0.09 FSR %/day, CI [-0.58, -0.17 FSR %/day], *P*<0.001). There was no difference in myofibrillar FSR between the NS and SR+EX group (0.08 ± 0.06 FSR %/day, CI [-0.29, 0.12 FSR%/day], *P*=0.947).

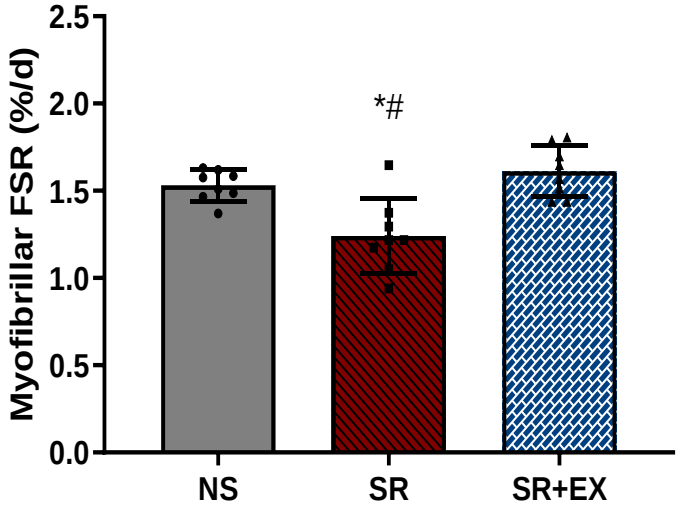

Figure 6.2- Fractional synthetic rate (FSR) of myofibrillar proteins throughout the sleep intervention. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction + Exercise (SR+EX) groups. *Denotes statistical significance from the NS group, # Denotes statistical significance from the SR+EX group, one-way ANOVA (P<0.05).

6.3.2 Protein synthesis related mRNA gene expression targets

There was no significant interaction effect for mRNA gene expression of *Foxo1* (*P*=0.691), *Foxo3* (*P*=0.744), *Myostatin* (*P*=0.195), *Mafbx* (*P*=0.236) or *Mighty* (*P*=0.814) compared to pre-intervention values. There was a significant interaction effect for *Murf1* mRNA gene expression (*P*=0.026); however, post-hoc testing showed no difference between groups from pre to post-intervention (NS - 61 ± 81%, CI [-114, -9%], *P*=0.253), (SR – 26 ± 33%, CI [-23, 75%], *P*=0.271), SR+EX (29 ± 35%, CI [-20, 28%], *P*=0.204) (**Figure 6.3**).

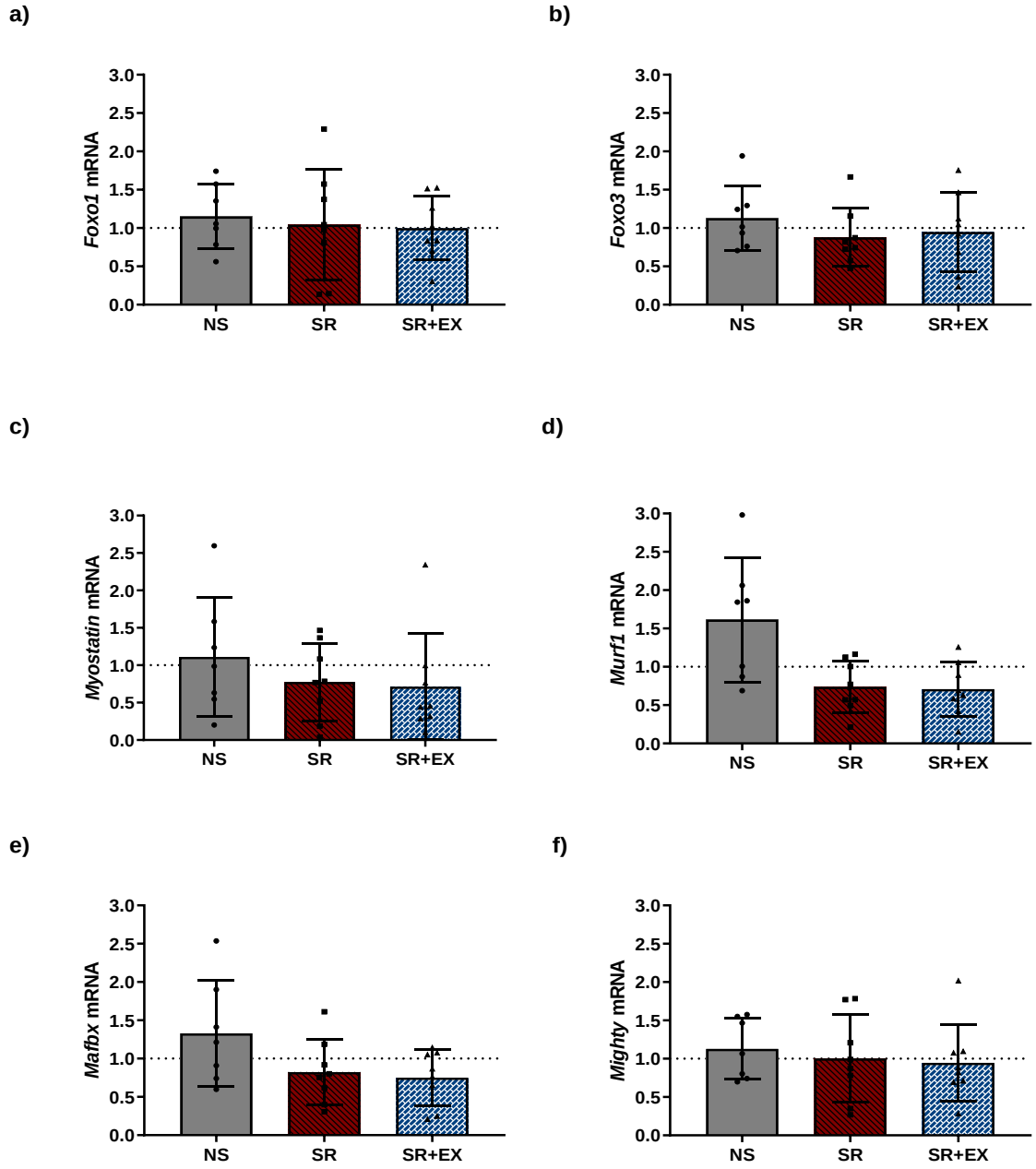

Figure 6.3 - Skeletal muscle mRNA expression of genes related to protein synthesis signalling pathways. Normalised to pre-intervention values (i.e., values shown are fold change from pre-intervention). Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX).

6.3.3 Protein synthesis related protein content

There was no significant interaction effect for mTOR (total) protein content (*P*=0.380), p-mTOR^{ser2448} (P=0.202), p-mTOR^{ser2448}/mTOR (Total) (*P*=0.148), p70S6K (*P*=0.280), Caspase 3 (*P*=0.185), p-4EBP1$^{thr37/46}$ (*P*=0.669), TSC2 (total) (*P*=0.663), p-TSC2^{thr1462} (*P*=0.687), p-TSC2^{thr1462}/TSC2 (Total) (*P*=0.287) or NFκB (*P*=0.724) protein content from pre- to post-intervention (**Figure 6.4**).

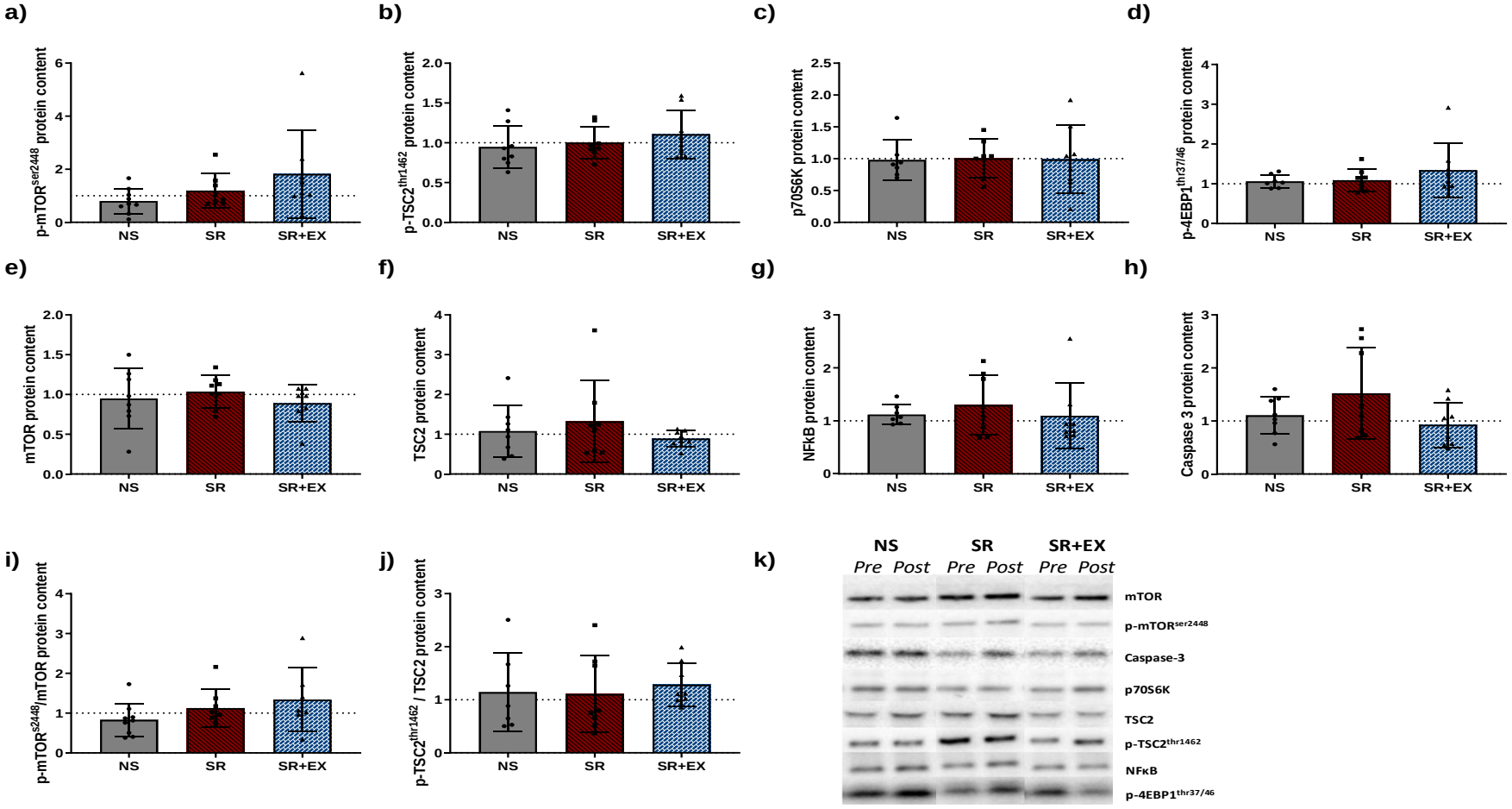

Figure 6.4 - Skeletal muscle protein content of protein synthesis, autophagy and protein degradation pathways.
a) p-mTOR^{ser2448}, b) p-TSC2^{thr462}, c) p70S6K (total), d) p-4EBP1$^{thr37/46}$, e) mTOR (total), f) TSC2 (total), g) NFkB, h) Caspase 3, i) p-mTOR^{ser2448}/mTOR (total), j) p-TSC2^{thr462}/TSC2 (total), and k) representative western blots. Normal Sleep (NS), Sleep Restriction (SR) and Sleep Restriction and Exercise (SR+EX).

6.4 Discussion

6.4.1 Main Findings

The aim of the study outlined in this chapter was to investigate the effect of sleep restriction on measures of skeletal muscle myofibrillar protein fractional synthetic rate (MyoPS) and some of the associated molecular mechanisms by which it is thought to be regulated. This study reports a significantly lower MyoPS for the SR group, compared to the NS and SR+EX groups, with no significant difference between the NS and SR+EX groups. Despite this finding, no changes were detected in molecular markers of the protein synthesis pathways. In addition, there were no changes in molecular markers of protein degradation pathways. These results provide some insights into the mechanisms underlying the previously described changes in muscle mass in the context of sleep loss. Importantly, they suggest that performing bouts of HIIE during periods of sleep loss can mitigate decreases in MyoPS.

6.4.2 Sleep restriction, exercise and myofibrillar protein synthesis

Rates of protein synthesis are considered the primary driving mechanism for changes in muscle mass (atrophy and hypertrophy) (Rennie 1985, Gibson, Halliday et al. 1987, de Boer, Selby et al. 2007). Despite previous reports of reductions in muscle mass with insufficient sleep (Nedeltcheva, Kilkus et al. 2010, Dattilo, Antunes et al. 2012, de Sa Souza, Antunes et al. 2016), the current study is the first to demonstrate in humans that sleep restriction of 4 h TIB for 5 nights reduces the rate of MyoPS. Reductions in MyoPS have been reported in a variety of interventions, such as step reduction, limb immobilisation and bed rest, designed to induce muscle atrophy (de Boer, Selby et al. 2007, Breen, Stokes et al. 2013). Likewise, exercise-based interventions that demonstrate muscle hypertrophy have been linked with increases in MyoPS (Brook, Wilkinson et al. 2015, Brook, Wilkinson et al. 2016). Together, the results of reduced MyoPS following sleep restriction are likely indicative of a less anabolic environment that may lend itself, if persistent, to the loss of muscle mass.

Exercise is well known as a potent stimulus for increasing rates of protein synthesis in skeletal muscle, with subsequent increases in muscle mass (Robinson, Dasari et al. 2017). The results of the current study demonstrate that performing three sessions of HIIE during a period of sleep restriction was able to mitigate the decrease in MyoPS and maintain it at similar rates to those observed in the NS group. Rodent models have previously shown that a prior 8-week period of resistance exercise training can mitigate the muscle atrophy observed following 96 h of sleep deprivation (Monico-Neto, Antunes et al. 2015). The higher MyoPS observed in the SR+EX group, compared to the SR group, may be explained by increases in MyoPS following aerobic exercise (Di Donato, West et al. 2014, Bell, Seguin et al. 2015). Indeed, Miller and colleagues

(2005) demonstrated that increases in MyoPS following 1 h of single-leg kicking (67% W_{max}), were apparent at 48 and 72 h post-exercise (Miller, Olesen et al. 2005). Although MyoPS in the SR+EX group was maintained at levels comparable to the NS group, the comparable effects of exercise with normal sleep on MyoPS is unknown. Therefore, an additional experimental group with normal sleep (8 h TIB) and exercise (i.e., NS+EX) would further help to demonstrate the detrimental effects that sleep restriction may impose. Nevertheless, previous interventions whereby MyoPS is reduced, in models such as step reduction have suggested the development of an 'anabolic resistance', whereby the MyoPS response to an amino acid infusion is blunted (Breen, Stokes et al. 2013). Given the decreased MyoPS in response to sleep restriction in the current study, it might be speculated that sleep restriction also develops a state of anabolic resistance. In summary, the three sessions of HIIE performed during the sleep restriction protocol appears to have been a sufficient stimulus to prevent the reduction in MyoPS that was observed in the SR group, in accordance with previous findings that demonstrate exercise as a potent stimulus for MyoPS.

6.4.3 Molecular markers of protein synthesis

The rates of protein synthesis are thought to be regulated by molecular signalling pathways that control the transcription and translation of new proteins. In the present study there were no changes in the mRNA expression or protein content of markers involved in these signalling pathways (i.e., p-mTOR^{ser2448}, p70S6K, p-4EBP1$^{thr37/46}$ or p-AKT^{ser473} (in Chapter 5)). Similar to the current research, previous studies have reported no change in key regulators of the protein synthesis pathway (i.e., p-mTOR ^{ser2448} or p-p70S6K^{thr389}) following 96 h of sleep deprivation in rats, despite reductions in muscle fibre CSA (Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016). However, while changes in protein synthesis signalling proteins and MyoPS are sometimes aligned (Brook, Wilkinson et al. 2015), this is not always the case. The dissociation between molecular markers of protein synthesis and MyoPS was demonstrated by De Boer et al. (2007), who showed a reduction in CSA, along with a reduction in MyoPS, but with no changes in the AKT-mTOR-p70S6K signalling pathway, following 10 days of muscle disuse (de Boer, Selby et al. 2007). This same dissociation has been reported by others (Greenhaff, Karagounis et al. 2008, Atherton, Etheridge et al. 2010), and suggests changes to mRNA and proteins may occur in the earlier stages of the sleep intervention (or accumulate throughout the day as the homeostatic drive for sleep increases) and in the hours immediately following HIIE (de Boer, Selby et al. 2007, Greenhaff, Karagounis et al. 2008, Atherton, Etheridge et al. 2010). Previously, a number of studies have reported increases in phosphorylation of AKT^{ser473}, mTOR^{ser2448}, and decreases in eEF2^{thr56} protein, immediately following an endurance exercise session, with levels returning to baseline within 3 to 4.5 h (Mascher, Andersson et al. 2007, Di Donato, West et al. 2014).

Therefore, while changes in the molecular signalling pathways for protein synthesis may occur in the hours immediately following exercise, or in the early stages of the sleep intervention, they do not necessarily persist for the duration that MyoPS is altered. As such, the timing of the muscle sample collection (i.e., 48 h post the final exercise sessions and three hours post waking following the final night of the sleep intervention) may have occurred when changes to mRNA gene expression and phosphorylation of proteins were no longer observable and may therefore, not reflect the changes to these signalling pathways throughout the intervention. It is also possible that an attenuated signalling response would have been observed in response to feeding, but we cannot ascertain if this occurred. To fully elucidate the mechanisms that underpin the decline in MyoPS observed in the SR group, additional research is required with additional muscle sampling throughout the intervention and in response to feeding.

6.4.4 Molecular markers of protein degradation

As direct measurements of MPB *in vivo* are technically challenging, the molecular markers of the signalling pathways involved in protein degradation are often used as surrogate measures (Tipton, Hamilton et al. 2018). In the current study, no change in *Foxo1/3, Mafbx,* or *Murf1* mRNA gene expression, and no change in LC3, p62/SQSTM (from Chapter 5), or Caspase-3 protein content, were observed from pre- to post-intervention. These observations are in contrast to previous work in rats, which demonstrated an increase in markers of different protein degradation pathways (including p-FOXO3, LC3 and p62/SQSTM protein content, from the UPS and autophagy signalling pathways) following 96 h of sleep deprivation (Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016). Again, the differences in findings between studies may be a result of the differences in the severity of interventions and species differences. Compared to the sleep deprivation interventions from these previous studies, the intervention in this study allowed 4 h TIB each night, which may have been a sufficient amount of sleep to prevent the increases in protein degradation markers (and possible increases in MPB) previously reported. How this changes at different time-points throughout the intervention is not known.

6.4.5 Limitations

While the rate of MyoPS is thought to be essential for the changes in muscle mass observed in atrophy and hypertrophy (Rennie 1985, Gibson, Halliday et al. 1987, de Boer, Selby et al. 2007), our study did not directly measure changes in muscle mass (via muscle CSA, MRI, CT or changes in fat-free soft tissue mass by DEXA). Although Nedeltcheva et al., (2010) used DEXA to assess changes in fat-free soft tissue mass with sleep restriction, this was across a 14-day period and the effects were likely enhanced in magnitude due to the calorie restricted diet used in the study (Nedeltcheva, Kilkus et al. 2010). The duration of the current study was likely too short to detect

changes in lean mass with DEXA given the variability of the measure (Bone, Ross et al. 2017). Further investigations should include measures of muscle CSA, and if possible magnetic resonance imaging (MRI), as validation of changes in muscle mass.

While it's suggested that changes in protein synthesis are more central to muscle atrophy, the rate of protein breakdown will also influence this (Gibson, Halliday et al. 1987). A direct measure of MPB was not included in this study and thus its influence can only be speculated. *In vivo* measures of MPB are technically challenging and uncommon, therefore, static markers of protein degradation pathways are often used concurrently with the assessment of protein synthesis to generate a more complete assessment of protein turnover (Tipton, Hamilton et al. 2018).

Finally, the measurement of p-p70S6K^{thr389} protein content is often used as a proxy measure of mTOR activity, because of its role in the regulation of protein translation (Ruvinsky and Meyuhas 2006). Unfortunately, it could not be included in this study due to issues in obtaining reliable results with the available antibody. However, a number of previous studies have demonstrated no change in phosphorylation of p70S6K^{thr389} in the hours immediately following HIIE (Coffey, Zhong et al. 2006, Mascher, Andersson et al. 2007, Di Donato, West et al. 2014).

6.4.6 Conclusions

Skeletal muscle is essential for physical strength and movement and also has a key role in metabolic function; therefore, reductions in muscle mass resulting from sleep loss may have a wide range of detrimental effects. The results of this study demonstrate a reduction in MyoPS following 5 nights of sleep restriction (4 h TIB, each night). Furthermore, 3 sessions of HIIE maintained MyoPS in the SR+EX group at the same rate as the NS group. However, there was a dissociation between the MyoPS results and the molecular signalling pathways that regulate protein synthesis and degradation, which has been observed elsewhere previously. While further investigation is needed, these findings suggest sleep loss can have detrimental effects on the processes that maintain muscle mass, via the suppression of MyoPS. These findings may, in part, help to explain previous reports of reduced muscle mass in those experiencing insufficient sleep. Therefore, our finding that HIIE was able to mitigate these changes has implications for those experiencing inadequate sleep and adds support to the concept of using HIIE as an intervention in the context of sleep loss.

Chapter 7. General Discussion, Conclusions and Future Research

This chapter presents an overview of the thesis' key findings in relation to the original aims and objectives set out in section **1.2**. A general discussion of these findings will be presented in the context of how the study has advanced the understanding of the topic, the implications of the findings, general limitations of the experimental design, and recommendations for future research.

7.1 Thesis aims and objectives

The overall aim of this thesis was to investigate the potential mechanisms underlying some of the detrimental metabolic effects that have been associated with sleep restriction, and to determine the potential role of exercise as a therapeutic intervention to mitigate these effects. This was achieved by investigating the following specific questions:

1. *Does sleep restriction disrupt circadian rhythms and can exercise mitigate this effect?*

This was addressed in Chapter 4. Indeed, the disruption of circadian rhythms with sleep restriction was demonstrated via measurements of peripheral skin temperature (amplitude and stability), as well as the measurement of key components of the molecular clock (i.e., clock gene expression and protein content). Peripheral skin temperature amplitude was reduced in both the SR and SR+EX group, while mRNA expression of *Bmal1* (a core clock gene) was significantly reduced in the SR group only. Together, these results indicate that sleep restriction caused a disruption to regular circadian rhythms and that by undertaking three sessions of HIIE the detrimental effects of sleep restriction on skeletal muscle *Bmal1* mRNA expression was alleviated.

2. *What are some of the potential mechanisms underlying the reductions in glucose tolerance that are induced by sleep restriction? Further, is exercise a potential therapeutic intervention to prevent these changes?*

This question was primarily addressed in Chapter 5, with a focus on the effect of sleep restriction on mitochondrial respiratory function, mitochondrial protein synthesis, and proteins of the mitochondrial biogenesis and insulin signalling pathways. A novel finding of this study was that skeletal muscle mitochondrial respiratory function was decreased following sleep restriction and that performing exercise prevented this decrease. Furthermore, sarcoplasmic protein fractional synthetic rate (used as a proxy measure for mitochondrial protein synthesis) was reduced in the SR group, compared to both the NS and SR+EX groups. This did not correspond to a decrease in mitochondrial content in the SR group, suggesting other mitochondrial regulatory processes, such as mitochondrial dynamics, may contribute to the decrease in respiratory function observed.

3. *Can changes in myofibrillar protein synthesis explain, in part, the mechanisms underpinning the previously demonstrated loss of muscle mass following sleep restriction? Further, can three sessions of HIIE prevent any potential changes?*

Myofibrillar protein synthesis (MyoPS) in response to sleep restriction with or without exercise was assessed in Chapter 6. Previous research indicates that sleep restriction leads to the loss of muscle mass; however, until now the effect of sleep restriction on the rate of MyoPS, a key determinant of changes to muscle mass, had not been assessed. Following five nights of sleep restriction MyoPS was significantly reduced, suggesting a potential mechanism underpinning changes in muscle mass observed with sleep restriction. Importantly, three sessions of high-intensity interval exercise provided a sufficient enough stimulus to maintain MyoPS at levels comparable to the NS group. Despite this, typical molecular markers of the myofibrillar protein synthesis pathway (Akt-mTOR-p70S6K) did not follow similar patterns of change.

7.2 General discussion of key findings

Each year, more than seven million Australians experience inadequate sleep (< 7 h per night on most nights), with the Sleep Health Foundation proclaiming a 'sleep loss epidemic' (Sleep Health Foundation 2017). This trend is common to many developed countries, with reports from the U.S.A suggesting 30% of the population are sleeping < 6 h per night (Centers for Disease Control and Prevention 2011). Through increased research, the consequences of this are becoming apparent and they may have significant implications for the health of the general population. Sleep loss has now been proposed as a major risk factor for a host of chronic diseases, including T2DM, cardiovascular disease, obesity, and depression (Sleep Health Foundation 2017). Despite this, current treatment approaches for increasing sleep duration, namely certain behavioural recommendations and pharmaceutical approaches, have thus far proven ineffective or have other innate risks involved (Irish, Kline et al. 2015, Sateia, Buysse et al. 2017). Therefore, research into additional therapeutic interventions, such as exercise, which are targeted at combating the underlying physiological mechanisms underpinning the effects of sleep loss are warranted and of significance to those experiencing inadequate sleep. This is something that this thesis has attempted to address.

Despite convincing experimental and epidemiological evidence that sleep loss causes a reduction in glucose tolerance (including the data presented in this thesis), the mechanisms that govern this response have scarcely been investigated (Spiegel, Leproult et al. 1999, Donga, van Dijk et al. 2010, Cedernaes, Osler et al. 2015). Closely linked to, and often occurring concurrently with sleep loss, circadian misalignment (as is often experienced with shift-work) has been recognised as a cause of reduced glucose tolerance (Bescos, Boden et al. 2018). The results of Chapter 4

specifically demonstrated that reducing sleep duration over five nights (to a total of 20 h), by extending waking hours (while minimising the effect of additional zeitgebers, such as light and food), can cause disruptions to circadian rhythms (assessed by wrist skin temperature amplitude and skeletal muscle clock gene expression). This may be a contributing factor for the decreases in glucose tolerance that were observed. While altered clock gene expression (*Bmal1* and *Cry1*) in response to a night of simulated shift-work has previously been identified (Cedernaes, Osler et al. 2015, Cedernaes, Schonke et al. 2018), this effect is reported in this thesis for the first time in response to a sleep restriction protocol coupled with additional measurements of circadian rhythm. These findings highlight the potential contribution of sleep loss in causing circadian disruption, which has considerable scope in the context of a range of metabolic pathologies.

The development of insulin resistance has often, but at times controversially, been associated with decreases in mitochondrial content and function (Kelley, He et al. 2002, Mogensen, Sahlin et al. 2007, Holloszy 2013, Fazakerley, Minard et al. 2018). This thesis provides an insight into the effect of sleep restriction on an array of skeletal muscle mitochondrial measurements, which included mitochondrial respiratory function in permeabilised muscle fibres, mitochondrial content (assessed via citrate synthase activity), sarcoplasmic protein synthesis (a proxy for mitochondrial protein synthesis), and a range of mitochondrial genes and proteins. For the first time it was reported that sleep restriction directly reduces human skeletal muscle mitochondrial respiratory function and sarcoplasmic protein synthesis, with a concomitant reduction in glucose tolerance. These effects, however, occurred independently of changes in mitochondrial content and raise intriguing questions regarding how the observed changes in mitochondrial respiration occur. One potential explanation is the inhibition of the processes that regulate the quality and efficiency of the mitochondrial reticulum and electron transport system, such as mitochondrial dynamics and mitophagy. While reductions in *Mfn2* mRNA were observed, no change in MFN2 protein content was detected and therefore the functional significance of this decrease in gene expression for changes in mitochondrial oxidative phosphorylation capacity can only be speculated. Future studies would benefit from additional analysis, such as transmission electron microscopy, to confirm the absence of changes in mitochondrial content and to assess changes in mitochondrial appearance and quality (i.e., mitochondrial elongation and changes to cristae density) (Picard, White et al. 2013). Considering the previously reported links between reduced mitochondrial function and insulin resistance/T2DM (Mogensen, Sahlin et al. 2007, Fazakerley, Minard et al. 2018), these changes in mitochondrial respiratory function provide a potential explanatory mechanism for the changes in glucose tolerance induced by sleep loss.

The existing literature supporting the detrimental metabolic effects of sleep loss has also alluded to a negative impact on the maintenance of muscle mass (Nedeltcheva, Kilkus et al. 2010, Dattilo,

Antunes et al. 2012, Monico-Neto, Antunes et al. 2015). The maintenance of muscle mass is regulated by the balance between protein synthesis and protein degradation (Tipton, Hamilton et al. 2018) and, therefore, the rate of myofibrillar protein fractional synthetic rate (MyoPS) during the sleep restriction protocol was assessed (and reported in **Chapter 6**). Sleep restriction caused a significant decrease in MyoPS, compared to the NS and SR+EX groups, which supports previous findings of decreases in muscle fibre CSA and losses of muscle mass following sleep deprivation in animals (Dattilo, Antunes et al. 2012, Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016) and following a period of sleep and calorie restriction in humans (Nedeltcheva, Kilkus et al. 2010). This is the first report in humans that suggests reduced MyoPS may be a potential mechanism within the muscle contributing to changes in skeletal muscle mass with sleep restriction. Indeed, it adds further support to previous findings in animal models that have demonstrated an increase in protein degradation pathways following sleep deprivation (Dattilo, Antunes et al. 2012, Monico-Neto, Antunes et al. 2015, de Sa Souza, Antunes et al. 2016). In the current study there was a dissociation between markers of the protein synthesis signalling pathway and the MyoPS results, which has also been reported previously, and is likely a result of the timing of sampling collection and consequently missing any potential changes in signalling processes (i.e., post exercise and as the drive for sleep increased) (de Boer, Selby et al. 2007, Greenhaff, Karagounis et al. 2008, Atherton, Etheridge et al. 2010). Overall, these findings are novel in that they provide the first measure in humans of skeletal muscle myofibrillar protein synthesis with sleep restriction and support previous findings of sleep-loss-induced reductions in muscle mass.

Aside from investigating the underlying mechanisms influencing the detrimental metabolic effects associated with sleep loss, an important component of this thesis was to explore the potential of high-intensity interval exercise (HIIE) as a therapeutic intervention to mitigate or prevent these changes. In recent times HIIE has become an increasingly popular form of endurance exercise, which has been shown to induce similar, if not greater, improvements in oxidative capacity, glucose tolerance, and cardio-metabolic health, when compared with traditional moderate-intensity exercise and often with a lower time demand (Jelleyman, Yates et al. 2015, Milanovic, Sporis et al. 2015, Gillen, Martin et al. 2016). It was hypothesised that the molecular mechanisms by which exercise coordinates the aforementioned benefits are intertwined with the molecular mechanisms that underpin sleep loss mediated changes in metabolic health (Saner, Bishop et al. 2018). Indeed, three sessions of high intensity-interval exercise performed during the sleep intervention prevented the decreases in glucose tolerance, aspects of circadian rhythm disruption, mitochondrial respiratory function, and both sarcoplasmic and myofibrillar protein synthesis (**Figure 7.1**). While the improvements of mitochondrial respiratory function and glucose tolerance are previously well documented (Little, Gillen et al. 2011, Granata, Oliveira et

al. 2016), the effect of HIIE on rates of MyoPS is less well characterised. Resistance exercise is classically regarded as the exercise mode responsible for the induction of myofibrillar protein synthesis; however, there are some reports of HIIE increasing MyoPS (Miller, Olesen et al. 2005, Di Donato, West et al. 2014, Bell, Seguin et al. 2015) and even inducing hypertrophy (Harber, Konopka et al. 2009, Harber, Konopka et al. 2012). However, aerobic exercise protocols with a lower intensity (i.e., 30 minutes of cycling at 55-60% heart rate maximum) have previously been shown not to induce increases in MyoPS (Bell, Seguin et al. 2015), which collectively indicates an intensity-dependent effect. Thus, coupled with the mechanistic results from the sleep restriction protocol and the improvements demonstrated with the exercise intervention, these findings provide an insight into the way in which the detrimental effects of sleep loss can be ameliorated in the future.

7.3 Contribution to literature

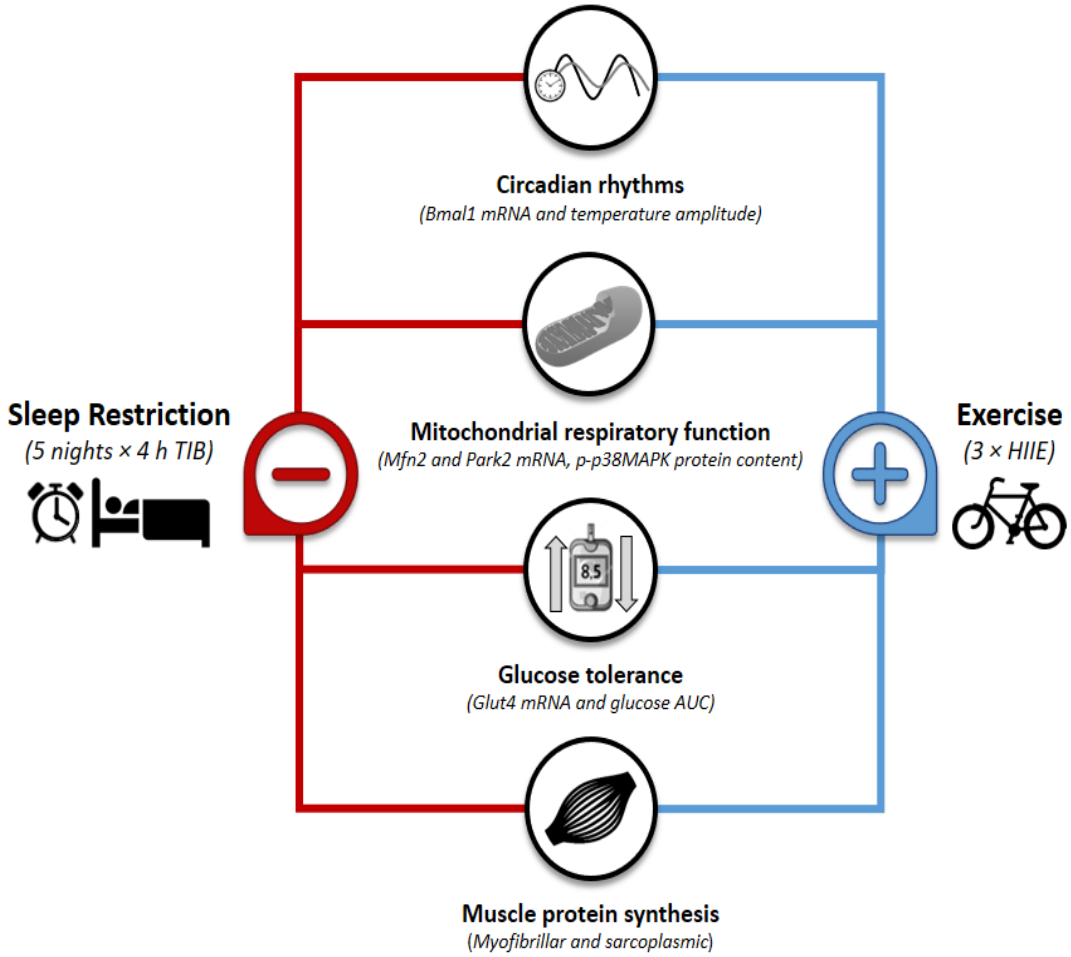

Figure 7.1 - Summary of key findings.
The figure highlights the detrimental metabolic effects induced by sleep restriction that were observed in this study and the beneficial influence of exercise in mitigating these effects. TIB, time in bed, HIIE, high-intensity interval exercise, *Bmal1*, brain and muscle ARNT-like protein-1 *Glut4*, glucose transporter isotope 4, AUC, area under the curve, *Mfn2*, Mitofusin-2, p-p38MAPK, phosphorylated-p38 mitogen activated protein kinase.

7.4 Research limitations and considerations

As with any study, the results must be considered in context with the inherent limitations that are associated with specific analytical techniques and methodological restrictions or choices. Whilst chapter-specific limitations have been discussed in the corresponding sections, the following provides a brief discussion of some of the limitations specific to the overall study design and how these limitations may influence the conclusions and key findings contained in this thesis.

Participant recruitment and training status:

Participant recruitment for this study was targeted at young, healthy, recreationally active males who met all the stringent inclusion criteria and who did not meet any exclusion criteria (as outlined in Chapter 3). 'Recreationally active' was defined as engaging in moderate physical activity two to four times per week and assessed via the International Physical Activity Questionnaire (IPAQ) and via self-reporting of physical activity conducted during the pre-study physical activity assessment (including habitual step count). As demonstrated in **Table 3.2**, extensive consideration was given to matching the experimental groups based on a range of physiological characteristics including the participants $\dot{V}O_{2max}$ result and, with pre-study mitochondrial respiratory function – which is correlated with aerobic fitness (Jacobs and Lundby 2013). However, it must be acknowledged that the training status of participants and their prior training history may influence individual responses to both the sleep and exercise interventions. For example, differential responses of molecular signalling pathways and the degree of skeletal muscle adaptation (e.g., improvements in oxidative capacity or strength) to the same exercise intervention, have previously been demonstrated between participants of different training statuses (Bouchard and Rankinen 2001, Coffey, Zhong et al. 2006).

It should also be acknowledged that due to the selective nature of participant recruitment (i.e., young, recreationally active, males), which is in itself a strength of the study, may mean the results from this study are not be entirely representative of other select populations, such as females, and older populations (i.e., > 40 years of age). Future studies should look to confirm these findings within different populations, especially considering sex-specific differences in exercise and metabolic responses have previously been reported and that older populations commonly report sleep complaints and insufficient sleep (Ancoli-Israel and Cooke 2005, Maher, Fu et al. 2009). Nevertheless, since changes have been observed in this healthy population, it is likely that the effects of sleep loss could even be more severe in clinical populations, such as obese and diabetic people. It is also likely that an exercise intervention would benefit these populations.

Influence of habitual physical activity and exercise:

Due to the NS and SR groups not performing any strenuous physical activity or exercise during the intervention (aside from meeting habitual step counts), this period of inactivity may be considered 'detraining' for participants who usually perform greater levels of physical activity. Previous studies have shown that periods of detraining, where physical activity is significantly reduced or stopped completely, can lead to reductions in mitochondrial content and respiratory function (Wibom, Hultman et al. 1992, Granata, Oliveira et al. 2016, Granata, Jamnick et al. 2018), although these changes have all been observed following longer periods of inactivity (i.e., a minimum of two weeks) than experienced during this study. Regardless, no change in mitochondrial content or respiratory function was observed in the NS group, suggesting that the one-week period during which the participants' habitual exercise routines were stopped, but habitual step counts were maintained (**Table 3.1**), was not sufficient to induce such changes.

Sample size:

Participant recruitment was particularly challenging due to the time-demanding, invasive, and restrictive nature of the study. Despite this, the sample size (n = 24) was sufficient to detect changes in our key outcome variables (glucose tolerance and mitochondrial respiratory function) and is of similar size to a number of comparable studies (Spiegel, Leproult et al. 1999, Reynolds, Dorrian et al. 2012, Rao, Neylan et al. 2015). Nevertheless, the relatively small sample size is likely a limiting factor in the context of the technical and biological variability associated with some of the analysis techniques (i.e., western blotting, RT-PCR) (Bass, Wilkinson et al. 2017, Cardinale, Gejl et al. 2018, Kuang, Yan et al. 2018) and the individual variation between responses (Bouchard and Rankinen 2001, Coffey, Zhong et al. 2006). Therefore, consideration was given to published recommendations for limiting the technical variation of the techniques used within this study. Given more time, the experimental design could also have been strengthened by the recruitment of additional participants as part of an additional experimental group that combined Normal Sleep (8 h TIB) and Exercise (i.e., a NS+EX group). This additional group would provide insight into the influence that sleep restriction may have had on the skeletal muscle adaptations to the exercise in the SR+EX group, highlighting any potential 'interference' of the sleep restriction on the beneficial effects of exercise.

Whole-muscle analysis:

The analysis of muscle samples was performed on whole-muscle homogenates obtained from the *vastus lateralis* muscle, which can contain a variable percentage of type I and type II fibres (Staron, Hagerman et al. 2000). These different fibres have been shown to respond differently following a variety of both sleep and exercise interventions (Kristensen, Albers et al. 2015, Monico-Neto, Giampa et al. 2015, de Sa Souza, Antunes et al. 2016). For example, reduced CSA

has been reported in type II, but not type I, fibres following 96 h of sleep deprivation in rats (Dattilo, Antunes et al. 2012, de Sa Souza, Antunes et al. 2016) and the protein content of myosin light-chain IIx is reduced following a night of sleep deprivation in humans (Cedernaes, Schonke et al. 2018), again suggesting a fibre-type-specific effect. Fibre-specific recruitment and adaptations have been reported in response to different exercise interventions (Kristensen, Albers et al. 2015). The HIIE protocol utilised in this study is likely to involve the recruitment of both type I and type II fibres, although the responses of these different fibre types to the stimulus is not known (Bishop, Botella et al. 2019). Therefore, the analyses in this thesis cannot distinguish potential fibre-type-specific changes to the interventions or disregard the potential for fibre-type specific changes being masked via the whole-muscle analyses performed.

The amount of muscle collected with each biopsy limits the amount of analysis that can be performed. To further specify the effects of the intervention, sub-fractionation of the homogenates (i.e., into mitochondrial, cytosolic and nuclear fractions) may provide insight and help to clarify the mechanisms underpinning the changes in mitochondrial respiratory function and protein synthesis. However, this process requires large amounts of sample which are not always available in human studies (e.g., the average muscle biopsy sample weighed approximately 120 mg, of which 60 mg was used for analysis of myofibrillar and sarcoplasmic protein synthesis, 20 mg, 15 mg and, 10 mg was used for western blotting, RT-PCR and mitochondrial respiratory function analysis respectively), which means there was insufficient sample to conduct additional analysis.

Future research direction

- *How do the findings of this study translate to the type of sleep loss commonly experienced by the general population?*

The current study design had a five-night sleep intervention, consisting of 4 h TIB each night, and was based on numerous previous studies that have demonstrated reductions in glucose tolerance in response to sleep restriction (Spiegel, Leproult et al. 1999, Buxton, Pavlova et al. 2010, Broussard, Ehrmann et al. 2012, Reynolds, Dorrian et al. 2012, Rao, Neylan et al. 2015). This is a severe sleep restriction protocol (20 h of sleep over five nights), which is designed as a 'proof of concept' intervention, and likely accentuated the mechanisms that underpin sleep loss induced metabolic dysfunction and facilitated their examination. How these findings translate to the general population, where it has been suggested that up to 20% of Australian adults commonly sleep less than 6 h per night, remains to be determined (Sleep Health Foundation 2017). To date, one study has investigated the effect of 3 nights of 'moderate' sleep restriction (i.e., 6 h TIB, each night) and reported decreases in insulin sensitivity (Wang, Greer et al. 2016). The mechanisms

underlying the changes in insulin sensitivity observed with 'moderate' sleep loss have not been investigated, nor has the potential of different interventions, such as exercise. Additionally, further research should explore the changes in glucose tolerance to different amounts of sleep (i.e., 4 h, 5 h, 6 h and 7 h), so that the full effects of reduced sleep can be identified.

- *What is the effect of long-term sleep loss on glucose tolerance and metabolic health?*

There is a lack of research that has investigated the effect of sleep loss over extended durations. How the findings from this thesis, and previous research investigating the detrimental effects of acute periods of sleep loss, translate in the long term remains to be seen. One previous study reported no effect of 8 weeks of reduced TIB (90 minutes less than habitual sleep times) on glucose tolerance; however, total sleep durations were still above recommended sleep guidelines (i.e., > 7 h) and therefore does not constitute 'sleep loss' in this context (Zielinski, Kline et al. 2008). Further studies that investigate the effect of chronic, long-term, sleep loss (< 7 h per night) and its effects on metabolic function such as glucose tolerance and the maintenance of skeletal muscle mass and function, are required.

- *Is the Insulin signalling pathway inhibited by sleep loss?*

While this study attempted to investigate the mechanisms regulating sleep-loss-induced reductions in glucose tolerance, further research is needed to fully elucidate this. One candidate for further exploration is the insulin signalling pathway, which should be investigated with the use of a hyperinsulinaemic euglycaemic clamp (HEC) protocol. By collecting muscle samples pre- and post-HEC the activity of the signalling pathway can be assessed in the context of sleep restriction and provide further mechanistic information regarding changes in glucose tolerance with sleep loss. Additionally, the HEC will allow for the contribution of hepatic glucose production to be assessed, which may give further insight into the changes in glucose metabolism observed with sleep loss.

- *How much 'recovery sleep' is needed to overcome the effects of a period of sleep loss and is exercise a time-effective method for counteracting moderate sleep loss?*

It is unclear at this point if a period of sleep restriction, such as that experienced in this study or commonly experienced by the wider population, can be overcome by extended periods of recovery or 'make up' sleep. A recent study reported that *ad libitum* weekend recovery sleep was insufficient to prevent the negative metabolic effects induced by a prior period of recurrent short sleep (< 5 h TIB per night, for 5 nights) (Depner, Melanson et al. 2019); however, another study indicated that two nights of recovery sleep (12 h and then 10 h TIB) following 4 nights of 4.5 h TIB mitigated the reductions in insulin sensitivity induced by the period of sleep restriction

(Eckel, Depner et al. 2015, Broussard, Wroblewski et al. 2016). More research is needed to clarify this matter and these findings further emphasise the potential importance of alternative solutions to recommendations of 'sleep more', especially considering that recommendations have generally been unsuccessful (Sleep Health Foundation 2017).

- *What is the optimal exercise stimulus to mitigate the negative effects of sleep loss?*

In the current study, the exercise protocol was from previous work that had reported improvements in glucose tolerance and oxidative capacity with a low-volume, high-intensity, cycling protocol. However, while this was an effective stimulus for the outcome measures of this study, future research may be able to establish an optimal exercise prescription to specifically mitigate the negative effects of sleep loss. For example, sprint-interval training (i.e., typically 4 to 6 intervals of 30-s 'all out' efforts), has previously been shown to increase mitochondrial respiration to a greater extent than lower-intensity endurance exercise (Granata, Oliveira et al. 2016). Accordingly, exercise protocols performed at high-intensity may also provide a strong stimulus for increasing MyoPS. Furthermore, the potential of resistance exercise to be used by itself, or in conjunction with aerobic exercise, to mitigate the detrimental effects of sleep loss should be investigated.

- *Which additional analysis techniques can help to elucidate the effects of sleep loss?*

The findings from the current study could be expanded with the use of additional analysis techniques, such as metabolomics, transcriptomics, and proteomics. While this thesis has selectively looked at genes and proteins directly related to molecular signalling pathways of interest, these additional techniques allow for changes of entire transcriptomes and proteomes to be mapped pre- and post-intervention. These types of analysis are becoming increasingly available and economical. These techniques, particularly within fields such as sleep in which there has been limited previous molecular research, may help to identify novel mechanisms and key regulators that may otherwise not be investigated.

7.5 Implications of the findings from this thesis

As highlighted throughout this thesis, sleep loss is becoming increasingly prevalent within modern society and the associated health implications are now beginning to emerge (Sleep Health Foundation 2017). Despite this, research into the mechanisms that underpin the detrimental effects associated with sleep loss is insufficient. Recently, the concept of 'weekend recovery sleep' has been investigated with results regarding its efficacy appearing to be inconclusive for mitigating periods of sleep loss whilst other behavioural and pharmacological interventions appear ineffective or inappropriate (Eckel, Depner et al. 2015, Irish, Kline et al. 2015, Broussard,

Wroblewski et al. 2016, Sateia, Buysse et al. 2017, Depner, Melanson et al. 2019). Therefore, additional interventions, such as exercise, which are designed and targeted at the underlying mechanisms of these detrimental effects need to be investigated. The improved understanding of the mechanisms presented in this thesis can provide the basis for future evidence-based strategic health recommendations and guidelines in those experiencing inadequate sleep.

This research has also highlighted the potential role of circadian misalignment in sleep-loss-induced changes to glucose tolerance, which adds to the body of evidence linking shift-work and an increased risk of T2DM (Bescos, Boden et al. 2018, Wefers, van Moorsel et al. 2018). Improving the understanding of the role that exercise might play in realigning or mitigating the effect of circadian misalignment in populations such as shift workers, who collectively make up 16% of the Australian work force (Australian Bureau of Statistics 2010), may help to prevent an increase in incidence of metabolic disease.

While sleep loss has been linked to reduced glucose tolerance for a number of years, the mechanisms underpinning this response have remained scarcely identified. The concurrent reductions (and concurrent mitigation of these reductions with exercise) in mitochondrial respiratory function and glucose tolerance documented in this study, represent important progress in our understanding of the pathophysiology underlying these changes. Again, this elucidates potential mechanisms that may be targeted to specifically combat the detrimental effects of sleep loss on glucose tolerance, which the results of this thesis demonstrate, with the effective use of HIIE. The efficacy of HIIE in this situation has been conclusively shown and therefore warrants further consideration for future health guidelines, especially considering its time efficiency and overall health benefits (Gibala, Little et al. 2006, Jelleyman, Yates et al. 2015).

The concept of sleep-loss-induced decreases in muscle mass has emerged in recent years but remains poorly understood. For the first time in humans, this study has provided insight into the potential mechanisms underlying these changes by demonstrating a reduced rate of protein synthesis following sleep restriction. Importantly, the data also provides support for the beneficial effect of aerobic exercise for mitigating these changes, under these conditions. While resistance training is perhaps a more potent stimulus for myofibrillar protein synthesis (Bell, Seguin et al. 2015), this data demonstrates HIIE may also be used in this capacity, especially considering its other additional cardio-metabolic benefits (Little, Gillen et al. 2011). This increased understanding of the mechanisms that regulate these changes provides potential options for tailoring specific interventions aimed at counteracting sleep loss and this should be reflected in future recommendations and health policy considerations.

Chapter 8. References

Akerstedt, T. (2003). "Shift work and disturbed sleep/wakefulness." Occup Med (Lond) **53**(2): 89-94.

Akimoto, T., S. C. Pohnert, P. Li, M. Zhang, C. Gumbs, P. B. Rosenberg, R. S. Williams and Z. Yan (2005). "Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway." J Biol Chem **280**(20): 19587-19593.

Amthor, H., R. Macharia, R. Navarrete, M. Schuelke, S. C. Brown, A. Otto, T. Voit, F. Muntoni, G. Vrbova, T. Partridge, P. Zammit, L. Bunger and K. Patel (2007). "Lack of myostatin results in excessive muscle growth but impaired force generation." Proc Natl Acad Sci U S A **104**(6): 1835-1840.

Ancoli-Israel, S. and J. R. Cooke (2005). "Prevalence and comorbidity of insomnia and effect on functioning in elderly populations." J Am Geriatr Soc **53**(7 Suppl): S264-271.

Andreazza, A. C., M. L. Andersen, T. A. Alvarenga, M. R. de-Oliveira, F. Armani, F. S. Ruiz, L. Giglio, J. C. Moreira, F. Kapczinski and S. Tufik (2010). "Impairment of the mitochondrial electron transport chain due to sleep deprivation in mice." J Psychiatr Res **44**(12): 775-780.

Andrews, J. L., X. Zhang, J. J. McCarthy, E. L. McDearmon, T. A. Hornberger, B. Russell, K. S. Campbell, S. Arbogast, M. B. Reid, J. R. Walker, J. B. Hogenesch, J. S. Takahashi and K. A. Esser (2010). "CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function." Proc Natl Acad Sci U S A **107**(44): 19090-19095.

Anothaisintawee, T., S. Reutrakul, E. Van Cauter and A. Thakkinstian (2015). "Sleep disturbances compared to traditional risk factors for diabetes development: Systematic review and meta-analysis." Sleep Med Rev **30**: 11-24.

Antle, M. C. and R. E. Mistlberger (2000). "Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster." J Neurosci **20**(24): 9326-9332.

Atherton, P. J., T. Etheridge, P. W. Watt, D. Wilkinson, A. Selby, D. Rankin, K. Smith and M. J. Rennie (2010). "Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling." Am J Clin Nutr **92**(5): 1080-1088.

Australian Bureau of Statistics (2010). Australian Labour Market Statistics. **6105.0**.

Bach, D., D. Naon, S. Pich, F. X. Soriano, N. Vega, J. Rieusset, M. Laville, C. Guillet, Y. Boirie, H. Wallberg-Henriksson, M. Manco, M. Calvani, M. Castagneto, M. Palacin, G. Mingrone, J. R. Zierath, H. Vidal and A. Zorzano (2005). "Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6." Diabetes **54**(9): 2685-2693.

Banks, S. and D. F. Dinges (2007). "Behavioral and physiological consequences of sleep restriction." J Clin Sleep Med **3**(5): 519-528.

Barnes, B. R., S. Marklund, T. L. Steiler, M. Walter, G. Hjalm, V. Amarger, M. Mahlapuu, Y. Leng, C. Johansson, D. Galuska, K. Lindgren, M. Abrink, D. Stapleton, J. R. Zierath and L. Andersson (2004). "The 5'-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle." J Biol Chem **279**(37): 38441-38447.

Baron, K. G., K. J. Reid and P. C. Zee (2013). "Exercise to improve sleep in insomnia: exploration of the bidirectional effects." J Clin Sleep Med **9**(8): 819-824.

Bartlett, J. D., C. Hwa Joo, T. S. Jeong, J. Louhelainen, A. J. Cochran, M. J. Gibala, W. Gregson, G. L. Close, B. Drust and J. P. Morton (2012). "Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle." J Appl Physiol (1985) **112**(7): 1135-1143.

Bass, J. J., D. J. Wilkinson, D. Rankin, B. E. Phillips, N. J. Szewczyk, K. Smith and P. J. Atherton (2017). "An overview of technical considerations for Western blotting applications to physiological research." Scand J Med Sci Sports **27**(1): 4-25.

Bell, K. E., C. Seguin, G. Parise, S. K. Baker and S. M. Phillips (2015). "Day-to-Day Changes in Muscle Protein Synthesis in Recovery From Resistance, Aerobic, and High-Intensity Interval Exercise in Older Men." J Gerontol A Biol Sci Med Sci **70**(8): 1024-1029.

Bell, L. N., J. M. Kilkus, J. N. Booth, 3rd, L. E. Bromley, J. G. Imperial and P. D. Penev (2013). "Effects of sleep restriction on the human plasma metabolome." Physiol Behav **122**: 25-31.

Benton, C. R., D. C. Wright and A. Bonen (2008). "PGC-1alpha-mediated regulation of gene expression and metabolism: implications for nutrition and exercise prescriptions." Appl Physiol Nutr Metab **33**(5): 843-862.

Berry, R. B., C. E. Gamaldo, S. M. Harding, R. Brooks, R. M. Lloyd, B. V. Vaughn and C. L. Marcus (2015). "AASM Scoring Manual Version 2.2 Updates: New Chapters for Scoring Infant Sleep Staging and Home Sleep Apnea Testing." J Clin Sleep Med **11**(11): 1253-1254.

Bescos, R., M. J. Boden, M. L. Jackson, A. J. Trewin, E. C. Marin, I. Levinger, A. Garnham, D. S. Hiam, F. Falcao-Tebas, F. Conte, J. A. Owens, D. J. Kennaway and G. K. McConell (2018). "Four days of simulated shift work reduces insulin sensitivity in humans." Acta Physiol (Oxf) **223**(2): e13039.

Birnbaum, M. J. (1989). "Identification of a novel gene encoding an insulin-responsive glucose transporter protein." Cell **57**(2): 305-315.

Bishop, D. J., J. Botella, A. J. Genders, M. J. Lee, N. J. Saner, J. Kuang, X. Yan and C. Granata (2019). "High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions." Physiology (Bethesda) **34**(1): 56-70.

Bishop, D. J., C. Granata and N. Eynon (2014). "Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content?" Biochim Biophys Acta **1840**(4): 1266-1275.

Bishop, D. J., C. Thomas, T. Moore-Morris, M. Tonkonogi, K. Sahlin and J. Mercier (2010). "Sodium bicarbonate ingestion prior to training improves mitochondrial adaptations in rats." Am J Physiol Endocrinol Metab **299**(2): E225-233.

Bodine, S. C., T. N. Stitt, M. Gonzalez, W. O. Kline, G. L. Stover, R. Bauerlein, E. Zlotchenko, A. Scrimgeour, J. C. Lawrence, D. J. Glass and G. D. Yancopoulos (2001). "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." Nat Cell Biol **3**(11): 1014-1019.

Bogan, J. S. (2012). "Regulation of glucose transporter translocation in health and diabetes." Annu Rev Biochem **81**: 507-532.

Bone, J. L., M. L. Ross, K. A. Tomcik, N. A. Jeacocke, W. G. Hopkins and L. M. Burke (2017). "Manipulation of Muscle Creatine and Glycogen Changes Dual X-ray Absorptiometry Estimates of Body Composition." Med Sci Sports Exerc **49**(5): 1029-1035.

Borg, G. (1970). "[Physical training. 3. Perceived exertion in physical work]." Lakartidningen **67**(40): 4548-4557.

Bouchard, C. and T. Rankinen (2001). "Individual differences in response to regular physical activity." Med Sci Sports Exerc **33**(6 Suppl): S446-451; discussion S452-443.

Breen, L., K. A. Stokes, T. A. Churchward-Venne, D. R. Moore, S. K. Baker, K. Smith, P. J. Atherton and S. M. Phillips (2013). "Two weeks of reduced activity decreases leg lean mass and induces "anabolic resistance" of myofibrillar protein synthesis in healthy elderly." J Clin Endocrinol Metab **98**(6): 2604-2612.

Brook, M. S., D. J. Wilkinson, W. K. Mitchell, J. N. Lund, N. J. Szewczyk, P. L. Greenhaff, K. Smith and P. J. Atherton (2015). "Skeletal muscle hypertrophy adaptations predominate in the early stages of resistance exercise training, matching deuterium oxide-derived measures of muscle protein synthesis and mechanistic target of rapamycin complex 1 signaling." FASEB J.

Brook, M. S., D. J. Wilkinson, K. Smith and P. J. Atherton (2016). "The metabolic and temporal basis of muscle hypertrophy in response to resistance exercise." Eur J Sport Sci **16**(6): 633-644.

Broussard, J. L., D. A. Ehrmann, E. Van Cauter, E. Tasali and M. J. Brady (2012). "Impaired insulin signaling in human adipocytes after experimental sleep restriction: a randomized, crossover study." Ann Intern Med **157**(8): 549-557.

Broussard, J. L., K. Wroblewski, J. M. Kilkus and E. Tasali (2016). "Two Nights of Recovery Sleep Reverses the Effects of Short-term Sleep Restriction on Diabetes Risk." Diabetes Care **39**(3): e40-41.

Buchmann, N., D. Spira, K. Norman, I. Demuth, R. Eckardt and E. Steinhagen-Thiessen (2016). "Sleep, Muscle Mass and Muscle Function in Older People." Dtsch Arztebl Int **113**(15): 253-260.

Bunger, M. K., L. D. Wilsbacher, S. M. Moran, C. Clendenin, L. A. Radcliffe, J. B. Hogenesch, M. C. Simon, J. S. Takahashi and C. A. Bradfield (2000). "Mop3 is an essential component of the master circadian pacemaker in mammals." Cell **103**(7): 1009-1017.

Burd, N. A., D. W. West, A. W. Staples, P. J. Atherton, J. M. Baker, D. R. Moore, A. M. Holwerda, G. Parise, M. J. Rennie, S. K. Baker and S. M. Phillips (2010). "Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men." PLoS One **5**(8): e12033.

Buxton, O. M., M. Pavlova, E. W. Reid, W. Wang, D. C. Simonson and G. K. Adler (2010). "Sleep restriction for 1 week reduces insulin sensitivity in healthy men." Diabetes **59**(9): 2126-2133.

Cajochen, C., M. E. Jewett and D. J. Dijk (2003). "Human circadian melatonin rhythm phase delay during a fixed sleep-wake schedule interspersed with nights of sleep deprivation." J Pineal Res **35**(3): 149-157.

Cajochen, C., K. Krauchi and A. Wirz-Justice (2003). "Role of melatonin in the regulation of human circadian rhythms and sleep." J Neuroendocrinol **15**(4): 432-437.

Cappuccio, F. P., L. D'Elia, P. Strazzullo and M. A. Miller (2010). "Quantity and quality of sleep and incidence of type 2 diabetes: a systematic review and meta-analysis." Diabetes Care **33**(2): 414-420.

Cardinale, D. A., K. D. Gejl, N. Ortenblad, B. Ekblom, E. Blomstrand and F. J. Larsen (2018). "Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibers from the vastus lateralis employing high-resolution respirometry." Physiol Rep **6**(4).

Cartoni, R., B. Leger, M. B. Hock, M. Praz, A. Crettenand, S. Pich, J. L. Ziltener, F. Luthi, O. Deriaz, A. Zorzano, C. Gobelet, A. Kralli and A. P. Russell (2005). "Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise." J Physiol **567**(Pt 1): 349-358.

Cedernaes, J., L. Lampola, E. K. Axelsson, L. Liethof, S. Hassanzadeh, A. Yeganeh, J. E. Broman, H. B. Schioth and C. Benedict (2015). "A single night of partial sleep loss impairs fasting insulin sensitivity but does not affect cephalic phase insulin release in young men." J Sleep Res.

Cedernaes, J., M. E. Osler, S. Voisin, J. E. Broman, H. Vogel, S. L. Dickson, J. R. Zierath, H. B. Schioth and C. Benedict (2015). "Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men." J Clin Endocrinol Metab: JC20152284.

Cedernaes, J., M. Schonke, J. O. Westholm, J. Mi, A. Chibalin, S. Voisin, M. Osler, H. Vogel, K. Hornaeus, S. L. Dickson, S. B. Lind, J. Bergquist, H. B. Schioth, J. R. Zierath and C. Benedict (2018). "Acute sleep loss results in tissue-specific alterations in genome-wide DNA methylation state and metabolic fuel utilization in humans." Sci Adv **4**(8): eaar8590.

Centers for Disease Control and Prevention (2011). "Effect of short sleep duration on daily activities - United States, 2005-2008." MMWR **60**(8): 233-268.

Centers for Disease Control and Prevention (2014). National Diabetes Statistics Report: Estimates of Diabetes and its Burden in the United States. Atlanta, GA, US Department of Health and Human Services.

Chien, M. Y., L. Y. Wang and H. C. Chen (2015). "The Relationship of Sleep Duration with Obesity and Sarcopenia in Community-Dwelling Older Adults." Gerontology **61**(5): 399-406.

Coffey, V. G., Z. Zhong, A. Shield, B. J. Canny, A. V. Chibalin, J. R. Zierath and J. A. Hawley (2006). "Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans." FASEB J **20**(1): 190-192.

Colten, H., R., Altevogt, B.M., (2006). Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem.

Corbalan-Tutau, M. D., J. A. Madrid, J. M. Ordovas, C. E. Smith, F. Nicolas and M. Garaulet (2011). "Differences in daily rhythms of wrist temperature between obese and normal-weight women: associations with metabolic syndrome features." Chronobiol Int **28**(5): 425-433.

Cuesta, M., N. Cermakian and D. B. Boivin (2015). "Glucocorticoids entrain molecular clock components in human peripheral cells." FASEB J **29**(4): 1360-1370.

Danne, T., R. Nimri, T. Battelino, R. M. Bergenstal, K. L. Close, J. H. DeVries, S. Garg, L. Heinemann, I. Hirsch, S. A. Amiel, R. Beck, E. Bosi, B. Buckingham, C. Cobelli, E. Dassau, F. J. Doyle, 3rd, S. Heller, R. Hovorka, W. Jia, T. Jones, O. Kordonouri, B. Kovatchev, A. Kowalski, L. Laffel, D. Maahs, H. R. Murphy, K. Norgaard, C. G. Parkin, E. Renard, B. Saboo, M. Scharf, W. V. Tamborlane, S. A. Weinzimer and M. Phillip (2017). "International Consensus on Use of Continuous Glucose Monitoring." Diabetes Care **40**(12): 1631-1640.

Dattilo, M., H. K. Antunes, A. Medeiros, M. Monico-Neto, S. Souza Hde, K. S. Lee, S. Tufik and M. T. de Mello (2012). "Paradoxical sleep deprivation induces muscle atrophy." Muscle Nerve **45**(3): 431-433.

Dattilo, M., H. K. Antunes, A. Medeiros, M. Monico Neto, H. S. Souza, S. Tufik and M. T. de Mello (2011). "Sleep and muscle recovery: endocrinological and molecular basis for a new and promising hypothesis." Med Hypotheses **77**(2): 220-222.

de Boer, M. D., A. Selby, P. Atherton, K. Smith, O. R. Seynnes, C. N. Maganaris, N. Maffulli, T. Movin, M. V. Narici and M. J. Rennie (2007). "The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse." J Physiol **585**(Pt 1): 241-251.

de Sa Souza, H., H. K. Antunes, M. Dattilo, K. S. Lee, M. Monico-Neto, S. Q. de Campos Giampa, S. M. Phillips, S. Tufik and M. T. de Mello (2016). "Leucine supplementation is anti-atrophic during paradoxical sleep deprivation in rats." Amino Acids **48**(4): 949-957.

de Souza, J. F. T., M. Dattilo, M. T. de Mello, S. Tufik and H. K. M. Antunes (2017). "High-Intensity Interval Training Attenuates Insulin Resistance Induced by Sleep Deprivation in Healthy Males." Front Physiol **8**: 992.

DeFronzo, R. A., J. D. Tobin and R. Andres (1979). "Glucose clamp technique: a method for quantifying insulin secretion and resistance." Am J Physiol **237**(3): E214-223.

Depner, C. M., E. L. Melanson, R. H. Eckel, J. K. Snell-Bergeon, L. Perreault, B. C. Bergman, J. A. Higgins, M. K. Guerin, E. R. Stothard, S. J. Morton and K. P. Wright, Jr. (2019). "Ad libitum Weekend Recovery Sleep Fails to Prevent Metabolic Dysregulation during a Repeating Pattern of Insufficient Sleep and Weekend Recovery Sleep." Curr Biol **29**(6): 957-967 e954.

Di Donato, D. M., D. W. West, T. A. Churchward-Venne, L. Breen, S. K. Baker and S. M. Phillips (2014). "Influence of aerobic exercise intensity on myofibrillar and mitochondrial protein synthesis in young men during early and late postexercise recovery." Am J Physiol Endocrinol Metab **306**(9): E1025-1032.

Dibner, C., U. Schibler and U. Albrecht (2010). "The mammalian circadian timing system: organization and coordination of central and peripheral clocks." Annu Rev Physiol **72**: 517-549.

Dolezal, B. A., E. V. Neufeld, D. M. Boland, J. L. Martin and C. B. Cooper (2017). "Interrelationship between Sleep and Exercise: A Systematic Review." Adv Prev Med **2017**: 1364387.

Donga, E., M. van Dijk, J. G. van Dijk, N. R. Biermasz, G. J. Lammers, K. W. van Kralingen, E. P. Corssmit and J. A. Romijn (2010). "A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects." J Clin Endocrinol Metab **95**(6): 2963-2968.

Drake, J. C., R. J. Wilson and Z. Yan (2016). "Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle." FASEB J **30**(1): 13-22.

Duez, H. and B. Staels (2009). "Rev-erb-alpha: an integrator of circadian rhythms and metabolism." J Appl Physiol (1985) **107**(6): 1972-1980.

Duffy, J. F. and K. P. Wright, Jr. (2005). "Entrainment of the human circadian system by light." J Biol Rhythms **20**(4): 326-338.

Dunlap, J. C., Loros, J.J., DeCoursey, P.J., (2004). Chronobiology: Biological timekeeping. Sunderland, MA, US, Sinauer Associates Chronobiology.

Dyar, K. A., S. Ciciliot, L. E. Wright, R. S. Bienso, G. M. Tagliazucchi, V. R. Patel, M. Forcato, M. I. Paz, A. Gudiksen, F. Solagna, M. Albiero, I. Moretti, K. L. Eckel-Mahan, P. Baldi, P. Sassone-Corsi, R. Rizzuto, S. Bicciato, H. Pilegaard, B. Blaauw and S. Schiaffino (2014). "Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock." Mol Metab **3**(1): 29-41.

Eckel, R. H., C. M. Depner, L. Perreault, R. R. Markwald, M. R. Smith, A. W. McHill, J. Higgins, E. L. Melanson and K. P. Wright, Jr. (2015). "Morning Circadian Misalignment during Short Sleep Duration Impacts Insulin Sensitivity." Curr Biol **25**(22): 3004-3010.

Edge, J., T. Mundel, H. Pilegaard, E. Hawke, M. Leikis, N. Lopez-Villalobos, R. S. Oliveira and D. J. Bishop (2015). "Ammonium Chloride Ingestion Attenuates Exercise-Induced mRNA Levels in Human Muscle." PLoS One **10**(12): e0141317.

Egan, B., B. P. Carson, P. M. Garcia-Roves, A. V. Chibalin, F. M. Sarsfield, N. Barron, N. McCaffrey, N. M. Moyna, J. R. Zierath and D. J. O'Gorman (2010). "Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle." J Physiol **588**(Pt 10): 1779-1790.

Fam, B. C., L. J. Rose, R. Sgambellone, Z. Ruan, J. Proietto and S. Andrikopoulos (2012). "Normal muscle glucose uptake in mice deficient in muscle GLUT4." J Endocrinol **214**(3): 313-327.

Faraut, B., K. Z. Boudjeltia, L. Vanhamme and M. Kerkhofs (2012). "Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery." Sleep Med Rev **16**(2): 137-149.

Fazakerley, D. J., A. Y. Minard, J. R. Krycer, K. C. Thomas, J. Stockli, D. J. Harney, J. G. Burchfield, G. J. Maghzal, S. T. Caldwell, R. C. Hartley, R. Stocker, M. P. Murphy and D. E. James (2018). "Mitochondrial oxidative stress causes insulin resistance without disrupting oxidative phosphorylation." J Biol Chem **293**(19): 7315-7328.

Fealy, C. E., A. Mulya, N. Lai and J. P. Kirwan (2014). "Exercise training decreases activation of the mitochondrial fission protein dynamin-related protein-1 in insulin-resistant human skeletal muscle." J Appl Physiol (1985) **117**(3): 239-245.

Febbraio, M. A., N. Hiscock, M. Sacchetti, C. P. Fischer and B. K. Pedersen (2004). "Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction." Diabetes **53**(7): 1643-1648.

Febbraio, M. A. and B. K. Pedersen (2002). "Muscle-derived interleukin-6: mechanisms for activation and possible biological roles." FASEB J **16**(11): 1335-1347.

Ford, E. S., T. J. Cunningham and J. B. Croft (2015). "Trends in Self-Reported Sleep Duration among US Adults from 1985 to 2012." Sleep **38**(5): 829-832.

Frosig, C. and E. A. Richter (2009). "Improved insulin sensitivity after exercise: focus on insulin signaling." Obesity (Silver Spring) **17 Suppl 3**: S15-20.

Gibala, M. J., J. P. Little, M. van Essen, G. P. Wilkin, K. A. Burgomaster, A. Safdar, S. Raha and M. A. Tarnopolsky (2006). "Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance." J Physiol **575**(Pt 3): 901-911.

Gibson, J. N., D. Halliday, W. L. Morrison, P. J. Stoward, G. A. Hornsby, P. W. Watt, G. Murdoch and M. J. Rennie (1987). "Decrease in human quadriceps muscle protein turnover consequent upon leg immobilization." Clin Sci (Lond) **72**(4): 503-509.

Gillen, J. B., B. J. Martin, M. J. MacInnis, L. E. Skelly, M. A. Tarnopolsky and M. J. Gibala (2016). "Twelve Weeks of Sprint Interval Training Improves Indices of Cardiometabolic Health Similar to Traditional Endurance Training despite a Five-Fold Lower Exercise Volume and Time Commitment." PLoS One **11**(4): e0154075.

Gordon, B. A., S. F. Fraser, S. R. Bird and A. C. Benson (2011). "Reproducibility of multiple repeated oral glucose tolerance tests." Diabetes Res Clin Pract **94**(3): e78-82.

Granata, C., N. A. Jamnick and D. J. Bishop (2018). "Training-Induced Changes in Mitochondrial Content and Respiratory Function in Human Skeletal Muscle." Sports Med **48**(8): 1809-1828.

Granata, C., N. A. Jamnick and D. J. Bishop (2018). "Training-Induced Changes in Mitochondrial Content and Respiratory Function in Human Skeletal Muscle." Sports Med.

Granata, C., R. S. Oliveira, J. P. Little, K. Renner and D. J. Bishop (2016). "Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle." FASEB J **30**(10): 3413-3423.

Granata, C., R. S. Oliveira, J. P. Little, K. Renner and D. J. Bishop (2016). "Training intensity modulates changes in PGC-1alpha and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle." FASEB J **30**(2): 959-970.

Greenhaff, P. L., L. G. Karagounis, N. Peirce, E. J. Simpson, M. Hazell, R. Layfield, H. Wackerhage, K. Smith, P. Atherton, A. Selby and M. J. Rennie (2008). "Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle." Am J Physiol Endocrinol Metab **295**(3): E595-604.

Groennebaek, T., N. R. Jespersen, J. E. Jakobsgaard, P. Sieljacks, J. Wang, E. Rindom, R. V. Musci, H. E. Botker, K. L. Hamilton, B. F. Miller, F. V. de Paoli and K. Vissing (2018). "Skeletal Muscle Mitochondrial Protein Synthesis and Respiration Increase With Low-Load Blood Flow Restricted as Well as High-Load Resistance Training." Front Physiol **9**: 1796.

Gurd, B. J. (2011). "Deacetylation of PGC-1alpha by SIRT1: importance for skeletal muscle function and exercise-induced mitochondrial biogenesis." Appl Physiol Nutr Metab **36**(5): 589-597.

Gurd, B. J., C. G. Perry, G. J. Heigenhauser, L. L. Spriet and A. Bonen (2010). "High-intensity interval training increases SIRT1 activity in human skeletal muscle." Appl Physiol Nutr Metab **35**(3): 350-357.

Haddad, F., F. Zaldivar, D. M. Cooper and G. R. Adams (2005). "IL-6-induced skeletal muscle atrophy." J Appl Physiol (1985) **98**(3): 911-917.

Harber, M. P., A. R. Konopka, M. D. Douglass, K. Minchev, L. A. Kaminsky, T. A. Trappe and S. Trappe (2009). "Aerobic exercise training improves whole muscle and single myofiber size and function in older women." Am J Physiol Regul Integr Comp Physiol **297**(5): R1452-1459.

Harber, M. P., A. R. Konopka, M. K. Undem, J. M. Hinkley, K. Minchev, L. A. Kaminsky, T. A. Trappe and S. Trappe (2012). "Aerobic exercise training induces skeletal muscle hypertrophy and age-dependent adaptations in myofiber function in young and older men." J Appl Physiol (1985) **113**(9): 1495-1504.

Harfmann, B. D., E. A. Schroder, J. H. England, N. J. Senn, P. M. Westgate, K. A. Esser and P. A. Kern (2017). "Temperature as a Circadian Marker in Older Human Subjects: Relationship to Metabolic Syndrome and Diabetes." J Endocr Soc **1**(7): 843-851.

Harfmann, B. D., E. A. Schroder and K. A. Esser (2015). "Circadian Rhythms, the Molecular Clock, and Skeletal Muscle." J Biol Rhythms **30**(2): 84-94.

Haun, C. T., C. G. Vann, B. M. Roberts, A. D. Vigotsky, B. J. Schoenfeld and M. D. Roberts (2019). "A Critical Evaluation of the Biological Construct Skeletal Muscle Hypertrophy: Size Matters but So Does the Measurement." Frontiers in Physiology **10**(247).

Hayashi, T., M. F. Hirshman, E. J. Kurth, W. W. Winder and L. J. Goodyear (1998). "Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport." Diabetes **47**(8): 1369-1373.

Henriksen, E. J. (2002). "Invited review: Effects of acute exercise and exercise training on insulin resistance." J Appl Physiol (1985) **93**(2): 788-796.

Herzog, N., K. Jauch-Chara, F. Hyzy, A. Richter, A. Friedrich, C. Benedict and K. M. Oltmanns (2013). "Selective slow wave sleep but not rapid eye movement sleep suppression impairs morning glucose tolerance in healthy men." Psychoneuroendocrinology **38**(10): 2075-2082.

Hirshkowitz, M., K. Whiton, S. M. Albert, C. Alessi, O. Bruni, L. DonCarlos, N. Hazen, J. Herman, P. J. Adams Hillard, E. S. Katz, L. Kheirandish-Gozal, D. N. Neubauer, A. E. O'Donnell, M. Ohayon, J. Peever, R. Rawding, R. C. Sachdeva, B. Setters, M. V. Vitiello and J. C. Ware (2015). "National Sleep Foundation's updated sleep duration recommendations: final report." Sleep Health **1**(4): 233-243.

Holloszy, J. O. (1967). "Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle." J Biol Chem **242**(9): 2278-2282.

Holloszy, J. O. (2013). ""Deficiency" of mitochondria in muscle does not cause insulin resistance." Diabetes **62**(4): 1036-1040.

Holloszy, J. O. and F. W. Booth (1976). "Biochemical adaptations to endurance exercise in muscle." Annu Rev Physiol **38**: 273-291.

Hoppeler, H., H. Howald, K. Conley, S. L. Lindstedt, H. Claassen, P. Vock and E. R. Weibel (1985). "Endurance training in humans: aerobic capacity and structure of skeletal muscle." J Appl Physiol (1985) **59**(2): 320-327.

Horne, J. A. and O. Ostberg (1976). "A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms." Int J Chronobiol **4**(2): 97-110.

Hu, X., J. Jiang, H. Wang, L. Zhang, B. Dong and M. Yang (2017). "Association between sleep duration and sarcopenia among community-dwelling older adults: A cross-sectional study." Medicine (Baltimore) **96**(10): e6268.

Hughes, M. E., J. B. Hogenesch and K. Kornacker (2010). "JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets." J Biol Rhythms **25**(5): 372-380.

Husek, P. and H. M. Liebich (1994). "Organic acid profiling by direct treatment of deproteinized plasma with ethyl chloroformate." J Chromatogr B Biomed Appl **656**(1): 37-43.

Iber, C. A.-I., S. Chesson, A. Quan, SF. (2007). The AASM Manual for the Scoring of Sleep and Associated Events: Rules, Terminology and Technical Specifications, American Academy of Sleep Medicine.

Irish, L. A., C. E. Kline, H. E. Gunn, D. J. Buysse and M. H. Hall (2015). "The role of sleep hygiene in promoting public health: A review of empirical evidence." Sleep Med Rev **22**: 23-36.

Jacobs, R. A. and C. Lundby (2013). "Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes." J Appl Physiol (1985) **114**(3): 344-350.

Jager, S., C. Handschin, J. St-Pierre and B. M. Spiegelman (2007). "AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha." Proc Natl Acad Sci U S A **104**(29): 12017-12022.

Jelleyman, C., T. Yates, G. O'Donovan, L. J. Gray, J. A. King, K. Khunti and M. J. Davies (2015). "The effects of high-intensity interval training on glucose regulation and insulin resistance: a meta-analysis." Obes Rev **16**(11): 942-961.

Jeon, C. Y., R. P. Lokken, F. B. Hu and R. M. van Dam (2007). "Physical activity of moderate intensity and risk of type 2 diabetes: a systematic review." Diabetes Care **30**(3): 744-752.

Kant, G. J., S. G. Genser, D. R. Thorne, J. L. Pfalser and E. H. Mougey (1984). "Effects of 72 hour sleep deprivation on urinary cortisol and indices of metabolism." Sleep **7**(2): 142-146.

Kelley, D. E., J. He, E. V. Menshikova and V. B. Ritov (2002). "Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes." Diabetes **51**(10): 2944-2950.

Kern, P. A., S. Ranganathan, C. Li, L. Wood and G. Ranganathan (2001). "Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance." Am J Physiol Endocrinol Metab **280**(5): E745-751.

Kim, J. H., R. A. Bachmann and J. Chen (2009). "Interleukin-6 and insulin resistance." Vitam Horm **80**: 613-633.

Kim, T. N., M. S. Park, K. I. Lim, H. Y. Choi, S. J. Yang, H. J. Yoo, H. J. Kang, W. Song, H. Choi, S. H. Baik, D. S. Choi and K. M. Choi (2013). "Relationships between sarcopenic obesity and insulin resistance, inflammation, and vitamin D status: the Korean Sarcopenic Obesity Study." Clin Endocrinol (Oxf) **78**(4): 525-532.

King, A. C., R. F. Oman, G. S. Brassington, D. L. Bliwise and W. L. Haskell (1997). "Moderate-intensity exercise and self-rated quality of sleep in older adults. A randomized controlled trial." JAMA **277**(1): 32-37.

Koliaki, C. and M. Roden (2016). "Alterations of Mitochondrial Function and Insulin Sensitivity in Human Obesity and Diabetes Mellitus." Annu Rev Nutr.

Kong, X., R. Wang, Y. Xue, X. Liu, H. Zhang, Y. Chen, F. Fang and Y. Chang (2010). "Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis." PLoS One **5**(7): e11707.

Konopka, A. R., M. K. Suer, C. A. Wolff and M. P. Harber (2014). "Markers of human skeletal muscle mitochondrial biogenesis and quality control: effects of age and aerobic exercise training." J Gerontol A Biol Sci Med Sci **69**(4): 371-378.

Kraniou, G. N., D. Cameron-Smith and M. Hargreaves (2004). "Effect of short-term training on GLUT-4 mRNA and protein expression in human skeletal muscle." Exp Physiol **89**(5): 559-563.

Kraniou, G. N., D. Cameron-Smith and M. Hargreaves (2006). "Acute exercise and GLUT4 expression in human skeletal muscle: influence of exercise intensity." J Appl Physiol (1985) **101**(3): 934-937.

Krauchi, K., C. Cajochen, E. Werth and A. Wirz-Justice (1999). "Warm feet promote the rapid onset of sleep." Nature **401**(6748): 36-37.

Kredlow, M. A., M. C. Capozzoli, B. A. Hearon, A. W. Calkins and M. W. Otto (2015). "The effects of physical activity on sleep: a meta-analytic review." J Behav Med **38**(3): 427-449.

Kristensen, D. E., P. H. Albers, C. Prats, O. Baba, J. B. Birk and J. F. Wojtaszewski (2015). "Human muscle fibre type-specific regulation of AMPK and downstream targets by exercise." J Physiol **593**(8): 2053-2069.

Krystal, A. D. and J. D. Edinger (2008). "Measuring sleep quality." Sleep Medicine **9**: S10-S17.

Kuang, J., X. Yan, A. J. Genders, C. Granata and D. J. Bishop (2018). "An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research." PLoS One **13**(5): e0196438.

Kubitz, K. A., D. M. Landers, S. J. Petruzzello and M. Han (1996). "The effects of acute and chronic exercise on sleep. A meta-analytic review." Sports Med **21**(4): 277-291.

Kumaki, Y., M. Ukai-Tadenuma, K. D. Uno, J. Nishio, K. H. Masumoto, M. Nagano, T. Komori, Y. Shigeyoshi, J. B. Hogenesch and H. R. Ueda (2008). "Analysis and synthesis of high-amplitude Cis-elements in the mammalian circadian clock." Proc Natl Acad Sci U S A **105**(39): 14946-14951.

Kung, C. P. and M. E. Murphy (2016). "The role of the p53 tumor suppressor in metabolism and diabetes." J Endocrinol.

Lamia, K. A., U. M. Sachdeva, L. DiTacchio, E. C. Williams, J. G. Alvarez, D. F. Egan, D. S. Vasquez, H. Juguilon, S. Panda, R. J. Shaw, C. B. Thompson and R. M. Evans (2009). "AMPK Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation." Science **326**(5951): 437-440.

Larsen, S., J. Nielsen, C. N. Hansen, L. B. Nielsen, F. Wibrand, N. Stride, H. D. Schroder, R. Boushel, J. W. Helge, F. Dela and M. Hey-Mogensen (2012). "Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects." J Physiol **590**(14): 3349-3360.

Lefta, M., G. Wolff and K. A. Esser (2011). "Circadian rhythms, the molecular clock, and skeletal muscle." Curr Top Dev Biol **96**: 231-271.

Leick, L., J. Fentz, R. S. Bienso, J. G. Knudsen, J. Jeppesen, B. Kiens, J. F. Wojtaszewski and H. Pilegaard (2010). "PGC-1{alpha} is required for AICAR-induced expression of GLUT4 and mitochondrial proteins in mouse skeletal muscle." Am J Physiol Endocrinol Metab **299**(3): E456-465.

Leproult, R., U. Holmback and E. Van Cauter (2014). "Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss." Diabetes **63**(6): 1860-1869.

Liang, H., B. Balas, P. Tantiwong, J. Dube, B. H. Goodpaster, R. M. O'Doherty, R. A. DeFronzo, A. Richardson, N. Musi and W. F. Ward (2009). "Whole body overexpression of PGC-1alpha has opposite effects on hepatic and muscle insulin sensitivity." Am J Physiol Endocrinol Metab **296**(4): E945-954.

Lim, S., J. H. Kim, J. W. Yoon, S. M. Kang, S. H. Choi, Y. J. Park, K. W. Kim, J. Y. Lim, K. S. Park and H. C. Jang (2010). "Sarcopenic obesity: prevalence and association with metabolic syndrome in the Korean Longitudinal Study on Health and Aging (KLoSHA)." Diabetes Care **33**(7): 1652-1654.

Little, J. P., J. B. Gillen, M. E. Percival, A. Safdar, M. A. Tarnopolsky, Z. Punthakee, M. E. Jung and M. J. Gibala (2011). "Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes." J Appl Physiol (1985) **111**(6): 1554-1560.

Little, J. P., A. Safdar, D. Bishop, M. A. Tarnopolsky and M. J. Gibala (2011). "An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle." Am J Physiol Regul Integr Comp Physiol **300**(6): R1303-1310.

Little, J. P., A. Safdar, G. P. Wilkin, M. A. Tarnopolsky and M. J. Gibala (2010). "A practical model of low-volume high-intensity interval training induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms." Journal of Physiology-London **588**(6): 1011-1022.

Liu, C., S. Li, T. Liu, J. Borjigin and J. D. Lin (2007). "Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism." Nature **447**(7143): 477-481.

Liu, J., B. Zhou, M. Yan, R. Huang, Y. Wang, Z. He, Y. Yang, C. Dai, Y. Wang, F. Zhang and Q. Zhai (2016). "CLOCK and BMAL1 Regulate Muscle Insulin Sensitivity via SIRT1 in Male Mice." Endocrinology **157**(6): 2259-2269.

Lowell, B. B. and G. I. Shulman (2005). "Mitochondrial dysfunction and type 2 diabetes." Science **307**(5708): 384-387.

MacInnis, M. J., E. Zacharewicz, B. J. Martin, M. E. Haikalis, L. E. Skelly, M. A. Tarnopolsky, R. M. Murphy and M. J. Gibala (2017). "Superior mitochondrial adaptations in human skeletal muscle after interval compared to continuous single-leg cycling matched for total work." J Physiol **595**(9): 2955-2968.

Maher, A. C., M. H. Fu, R. J. Isfort, A. R. Varbanov, X. A. Qu and M. A. Tarnopolsky (2009). "Sex differences in global mRNA content of human skeletal muscle." PLoS One **4**(7): e6335.

Mari, A., G. Pacini, E. Murphy, B. Ludvik and J. J. Nolan (2001). "A model-based method for assessing insulin sensitivity from the oral glucose tolerance test." Diabetes Care **24**(3): 539-548.

Mascher, H., H. Andersson, P. A. Nilsson, B. Ekblom and E. Blomstrand (2007). "Changes in signalling pathways regulating protein synthesis in human muscle in the recovery period after endurance exercise." Acta Physiol (Oxf) **191**(1): 67-75.

Mathur, N. and B. K. Pedersen (2008). "Exercise as a mean to control low-grade systemic inflammation." Mediators Inflamm **2008**: 109502.

Matsuda, M. and R. A. DeFronzo (1999). "Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp." Diabetes Care **22**(9): 1462-1470.

McCarthy, J. J., J. L. Andrews, E. L. McDearmon, K. S. Campbell, B. K. Barber, B. H. Miller, J. R. Walker, J. B. Hogenesch, J. S. Takahashi and K. A. Esser (2007). "Identification of the circadian transcriptome in adult mouse skeletal muscle." Physiol Genomics **31**(1): 86-95.

Meex, R. C., V. B. Schrauwen-Hinderling, E. Moonen-Kornips, G. Schaart, M. Mensink, E. Phielix, T. van de Weijer, J. P. Sels, P. Schrauwen and M. K. Hesselink (2010). "Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity." Diabetes **59**(3): 572-579.

Meinild Lundby, A. K., R. A. Jacobs, S. Gehrig, J. de Leur, M. Hauser, T. C. Bonne, D. Fluck, S. Dandanell, N. Kirk, A. Kaech, U. Ziegler, S. Larsen and C. Lundby (2018). "Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis." Acta Physiol (Oxf) **222**(1).

Milanovic, Z., G. Sporis and M. Weston (2015). "Effectiveness of High-Intensity Interval Training (HIT) and Continuous Endurance Training for VO2max Improvements: A Systematic Review and Meta-Analysis of Controlled Trials." Sports Med **45**(10): 1469-1481.

Miller, B. F. and K. L. Hamilton (2012). "A perspective on the determination of mitochondrial biogenesis." Am J Physiol Endocrinol Metab **302**(5): E496-499.

Miller, B. F., J. L. Olesen, M. Hansen, S. Dossing, R. M. Crameri, R. J. Welling, H. Langberg, A. Flyvbjerg, M. Kjaer, J. A. Babraj, K. Smith and M. J. Rennie (2005). "Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise." J Physiol **567**(Pt 3): 1021-1033.

Mitchell, W. K., J. Williams, P. Atherton, M. Larvin, J. Lund and M. Narici (2012). "Sarcopenia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review." Front Physiol **3**: 260.

Mogensen, M., K. Sahlin, M. Fernstrom, D. Glintborg, B. F. Vind, H. Beck-Nielsen and K. Hojlund (2007). "Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes." Diabetes **56**(6): 1592-1599.

Moller-Levet, C. S., S. N. Archer, G. Bucca, E. E. Laing, A. Slak, R. Kabiljo, J. C. Lo, N. Santhi, M. von Schantz, C. P. Smith and D. J. Dijk (2013). "Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome." Proc Natl Acad Sci U S A **110**(12): E1132-1141.

Monico-Neto, M., H. K. Antunes, K. S. Lee, S. M. Phillips, S. Q. Giampa, S. Souza Hde, M. Dattilo, A. Medeiros, W. M. de Moraes, S. Tufik and M. T. de Mello (2015). "Resistance training minimizes catabolic effects induced by sleep deprivation in rats." Appl Physiol Nutr Metab **40**(11): 1143-1150.

Monico-Neto, M., S. Q. Giampa, K. S. Lee, C. M. de Melo, S. Souza Hde, M. Dattilo, P. A. Minali, P. H. Santos Prado, S. Tufik, M. T. de Mello and H. K. Antunes (2015). "Negative energy balance induced by paradoxical sleep deprivation causes multicompartmental changes in adipose tissue and skeletal muscle." Int J Endocrinol **2015**: 908159.

Montgomery, M. K. and N. Turner (2015). "Mitochondrial dysfunction and insulin resistance: an update." Endocr Connect **4**(1): R1-R15.

Moser, D., P. Anderer, G. Gruber, S. Parapatics, E. Loretz, M. Boeck, G. Kloesch, E. Heller, A. Schmidt, H. Danker-Hopfe, B. Saletu, J. Zeitlhofer and G. Dorffner (2009). "Sleep classification according to AASM and Rechtschaffen & Kales: effects on sleep scoring parameters." Sleep **32**(2): 139-149.

Mullington, J. M., N. S. Simpson, H. K. Meier-Ewert and M. Haack (2010). "Sleep loss and inflammation." Best Pract Res Clin Endocrinol Metab **24**(5): 775-784.

Muniyappa, R., S. Lee, H. Chen and M. J. Quon (2008). "Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage." Am J Physiol Endocrinol Metab **294**(1): E15-26.

Murphy, R. M. and G. D. Lamb (2013). "Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes." Journal of Physiology-London **591**(23): 5823-5831.

Nagano, M., A. Adachi, K. Nakahama, T. Nakamura, M. Tamada, E. Meyer-Bernstein, A. Sehgal and Y. Shigeyoshi (2003). "An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center." J Neurosci **23**(14): 6141-6151.

National Collaborating Centre for Chronic Conditions (2008). Type 2 diabetes: national clinical guideline for management in primary and secondary care (update) London.

National Sleep Foundation. (2013). "International bedroom poll." from https://sleepfoundation.org/sites/default/files/RPT495a.pdf.

Nedeltcheva, A. V., J. M. Kilkus, J. Imperial, D. A. Schoeller and P. D. Penev (2010). "Insufficient sleep undermines dietary efforts to reduce adiposity." Ann Intern Med **153**(7): 435-441.

Neufeld-Cohen, A., M. S. Robles, R. Aviram, G. Manella, Y. Adamovich, B. Ladeuix, D. Nir, L. Rousso-Noori, Y. Kuperman, M. Golik, M. Mann and G. Asher (2016). "Circadian control of oscillations in mitochondrial rate-limiting enzymes and nutrient utilization by PERIOD proteins." Proc Natl Acad Sci U S A **113**(12): E1673-1682.

Nielsen, J., K. D. Gejl, M. Hey-Mogensen, H. C. Holmberg, C. Suetta, P. Krustrup, C. P. H. Elemans and N. Ortenblad (2017). "Plasticity in mitochondrial cristae density allows metabolic capacity modulation in human skeletal muscle." J Physiol **595**(9): 2839-2847.

Palacios, O. M., J. J. Carmona, S. Michan, K. Y. Chen, Y. Manabe, J. L. Ward, 3rd, L. J. Goodyear and Q. Tong (2009). "Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle." Aging (Albany NY) **1**(9): 771-783.

Pastore, S. and D. A. Hood (2013). "Endurance training ameliorates the metabolic and performance characteristics of circadian Clock mutant mice." J Appl Physiol (1985) **114**(8): 1076-1084.

Pedersen, B. K. and B. Saltin (2015). "Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases." Scand J Med Sci Sports **25 Suppl 3**: 1-72.

Peek, C. B., A. H. Affinati, K. M. Ramsey, H. Y. Kuo, W. Yu, L. A. Sena, O. Ilkayeva, B. Marcheva, Y. Kobayashi, C. Omura, D. C. Levine, D. J. Bacsik, D. Gius, C. B. Newgard, E.

Goetzman, N. S. Chandel, J. M. Denu, M. Mrksich and J. Bass (2013). "Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice." Science **342**(6158): 1243417.

Perlis, M. L., M. A. Grandner, S. Chakravorty, R. A. Bernert, G. K. Brown and M. E. Thase (2016). "Suicide and sleep: Is it a bad thing to be awake when reason sleeps?" Sleep Med Rev **29**: 101-107.

Perry, C. G. R., J. Lally, G. P. Holloway, G. J. F. Heigenhauser, A. Bonen and L. L. Spriet (2010). "Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle." Journal of Physiology-London **588**(23): 4795-4810.

Picard, M., K. White and D. M. Turnbull (2013). "Mitochondrial morphology, topology, and membrane interactions in skeletal muscle: a quantitative three-dimensional electron microscopy study." J Appl Physiol (1985) **114**(2): 161-171.

Pospisilik, J. A., C. Knauf, N. Joza, P. Benit, M. Orthofer, P. D. Cani, I. Ebersberger, T. Nakashima, R. Sarao, G. Neely, H. Esterbauer, A. Kozlov, C. R. Kahn, G. Kroemer, P. Rustin, R. Burcelin and J. M. Penninger (2007). "Targeted deletion of AIF decreases mitochondrial oxidative phosphorylation and protects from obesity and diabetes." Cell **131**(3): 476-491.

Puigserver, P., Z. Wu, C. W. Park, R. Graves, M. Wright and B. M. Spiegelman (1998). "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis." Cell **92**(6): 829-839.

Rao, M. N., T. C. Neylan, C. Grunfeld, K. Mulligan, M. Schambelan and J. M. Schwarz (2015). "Subchronic sleep restriction causes tissue-specific insulin resistance." J Clin Endocrinol Metab **100**(4): 1664-1671.

Rechtschaffen, A. K., A. (1968). A Manual of Standardised Terminology: Techniques nad Scoring System for Sleep Stages of Human Subjects. Los Angeles, CA, UCLA Brain Information Service/Brain Research Institute.

Ren, J. M., C. F. Semenkovich, E. A. Gulve, J. Gao and J. O. Holloszy (1994). "Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle." J Biol Chem **269**(20): 14396-14401.

Rennie, M. J. (1985). "Muscle protein turnover and the wasting due to injury and disease." Br Med Bull **41**(3): 257-264.

Rennie, M. J., R. H. Edwards, D. Halliday, D. E. Matthews, S. L. Wolman and D. J. Millward (1982). "Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting." Clin Sci (Lond) **63**(6): 519-523.

Reynolds, A. C., J. Dorrian, P. Y. Liu, H. P. Van Dongen, G. A. Wittert, L. J. Harmer and S. Banks (2012). "Impact of five nights of sleep restriction on glucose metabolism, leptin and testosterone in young adult men." PLoS One **7**(7): e41218.

Richardson, C. E., M. Gradisar, M. A. Short and C. Lang (2016). "Can Exercise Regulate the Circadian System of Adolescents? Novel Implications for the Treatment of Delayed Sleep-Wake Phase Disorder." Sleep Medicine Reviews.

Richter, E. A. and M. Hargreaves (2013). "Exercise, GLUT4, and skeletal muscle glucose uptake." Physiol Rev **93**(3): 993-1017.

Robertson, M. D., D. Russell-Jones, A. M. Umpleby and D. J. Dijk (2013). "Effects of three weeks of mild sleep restriction implemented in the home environment on multiple metabolic and endocrine markers in healthy young men." Metabolism **62**(2): 204-211.

Robinson, M. M., S. Dasari, A. R. Konopka, M. L. Johnson, S. Manjunatha, R. R. Esponda, R. E. Carter, I. R. Lanza and K. S. Nair (2017). "Enhanced Protein Translation Underlies Improved Metabolic and Physical Adaptations to Different Exercise Training Modes in Young and Old Humans." Cell Metab **25**(3): 581-592.

Rowe, G. C., I. S. Patten, Z. K. Zsengeller, R. El-Khoury, M. Okutsu, S. Bampoh, N. Koulisis, C. Farrell, M. F. Hirshman, Z. Yan, L. J. Goodyear, P. Rustin and Z. Arany (2013). "Disconnecting mitochondrial content from respiratory chain capacity in PGC-1-deficient skeletal muscle." Cell Rep **3**(5): 1449-1456.

Rudic, R. D., P. McNamara, A. M. Curtis, R. C. Boston, S. Panda, J. B. Hogenesch and G. A. Fitzgerald (2004). "BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis." PLoS Biol **2**(11): e377.

Rudrappa, S. S., D. J. Wilkinson, P. L. Greenhaff, K. Smith, I. Idris and P. J. Atherton (2016). "Human Skeletal Muscle Disuse Atrophy: Effects on Muscle Protein Synthesis, Breakdown, and Insulin Resistance-A Qualitative Review." Front Physiol **7**: 361.

Ruvinsky, I. and O. Meyuhas (2006). "Ribosomal protein S6 phosphorylation: from protein synthesis to cell size." Trends Biochem Sci **31**(6): 342-348.

Saleem, A., P. J. Adhihetty and D. A. Hood (2009). "Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle." Physiol Genomics **37**(1): 58-66.

Sandri, M. (2008). "Signaling in muscle atrophy and hypertrophy." Physiology (Bethesda) **23**: 160-170.

Saner, N. J., D. J. Bishop and J. D. Bartlett (2018). "Is exercise a viable therapeutic intervention to mitigate mitochondrial dysfunction and insulin resistance induced by sleep loss?" Sleep Med Rev **37**: 60-68.

Sarabia, J. A., M. A. Rol, P. Mendiola and J. A. Madrid (2008). "Circadian rhythm of wrist temperature in normal-living subjects A candidate of new index of the circadian system." Physiol Behav **95**(4): 570-580.

Sateia, M. J., D. J. Buysse, A. D. Krystal, D. N. Neubauer and J. L. Heald (2017). "Clinical Practice Guideline for the Pharmacologic Treatment of Chronic Insomnia in Adults: An American Academy of Sleep Medicine Clinical Practice Guideline." J Clin Sleep Med **13**(2): 307-349.

Sato, S., A. L. Basse, M. Schonke, S. Chen, M. Samad, A. Altintas, R. C. Laker, E. Dalbram, R. Barres, P. Baldi, J. T. Treebak, J. R. Zierath and P. Sassone-Corsi (2019). "Time of Exercise Specifies the Impact on Muscle Metabolic Pathways and Systemic Energy Homeostasis." Cell Metab.

Savikj, M., B. M. Gabriel, P. S. Alm, J. Smith, K. Caidahl, M. Bjornholm, T. Fritz, A. Krook, J. R. Zierath and H. Wallberg-Henriksson (2018). "Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial." Diabetologia.

Scalzo, R. L., G. L. Peltonen, S. E. Binns, M. Shankaran, G. R. Giordano, D. A. Hartley, A. L. Klochak, M. C. Lonac, H. L. Paris, S. E. Szallar, L. M. Wood, F. F. Peelor, 3rd, W. E. Holmes, M. K. Hellerstein, C. Bell, K. L. Hamilton and B. F. Miller (2014). "Greater muscle protein synthesis and mitochondrial biogenesis in males compared with females during sprint interval training." FASEB J **28**(6): 2705-2714.

Scheer, F. A. (2011). Fundamentals of the Circadian System. Basics of Sleep Guide Sleep Research Society.

Schiaffino, S., B. Blaauw and K. A. Dyar (2016). "The functional significance of the skeletal muscle clock: lessons from Bmal1 knockout models." Skelet Muscle **6**: 33.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C-T method." Nature Protocols **3**(6): 1101-1108.

Schroder, E. A., B. D. Harfmann, X. Zhang, R. Srikuea, J. H. England, B. A. Hodge, Y. Wen, L. A. Riley, Q. Yu, A. Christie, J. D. Smith, T. Seward, E. M. Wolf Horrell, J. Mula, C. A. Peterson, T. A. Butterfield and K. A. Esser (2015). "Intrinsic muscle clock is necessary for musculoskeletal health." J Physiol **593**(24): 5387-5404.

Sebastian, D., M. I. Hernandez-Alvarez, J. Segales, E. Sorianello, J. P. Munoz, D. Sala, A. Waget, M. Liesa, J. C. Paz, P. Gopalacharyulu, M. Oresic, S. Pich, R. Burcelin, M. Palacin and A. Zorzano (2012). "Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis." Proc Natl Acad Sci U S A **109**(14): 5523-5528.

Senn, J. J., P. J. Klover, I. A. Nowak and R. A. Mooney (2002). "Interleukin-6 induces cellular insulin resistance in hepatocytes." Diabetes **51**(12): 3391-3399.

Shad, B. J., J. L. Thompson, A. M. Holwerda, B. Stocks, Y. S. Elhassan, A. Philp, L. J. C. van Loon and G. A. Wallis (2019). "One Week of Step Reduction Lowers Myofibrillar Protein Synthesis Rates in Young Men." Med Sci Sports Exerc.

Shepherd, P. R. and B. B. Kahn (1999). "Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus." N Engl J Med **341**(4): 248-257.

Sleep Health Foundation. (2011). "Common Reasons Why People Don't Get Enough Sleep." Retrieved 13 Jan, 2016, from http://www.sleephealthfoundation.org.au/files/pdfs/Common-Causes-Inadequate-Sleep.pdf.

Sleep Health Foundation (2017). Asleep on the Job, Costs of Inadequate Sleep In Australia.

Spiegel, K., R. Leproult and E. Van Cauter (1999). "Impact of sleep debt on metabolic and endocrine function." Lancet **354**(9188): 1435-1439.

Staron, R. S., F. C. Hagerman, R. S. Hikida, T. F. Murray, D. P. Hostler, M. T. Crill, K. E. Ragg and K. Toma (2000). "Fiber type composition of the vastus lateralis muscle of young men and women." J Histochem Cytochem **48**(5): 623-629.

Stenvers, D. J., A. Jongejan, S. Atiqi, J. P. Vreijling, E. J. Limonard, E. Endert, F. Baas, P. D. Moerland, E. Fliers, A. Kalsbeek and P. H. Bisschop (2019). "Diurnal rhythms in the white

adipose tissue transcriptome are disturbed in obese individuals with type 2 diabetes compared with lean control individuals." Diabetologia **62**(4): 704-716.

Suwa, M., H. Nakano, Z. Radak and S. Kumagai (2008). "Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle." Metabolism **57**(7): 986-998.

Suwazono, Y., K. Sakata, Y. Okubo, H. Harada, M. Oishi, E. Kobayashi, M. Uetani, T. Kido and K. Nogawa (2006). "Long-term longitudinal study on the relationship between alternating shift work and the onset of diabetes mellitus in male Japanese workers." J Occup Environ Med **48**(5): 455-461.

Sweeney, E. L., S. Jeromson, D. L. Hamilton, N. E. Brooks and I. H. Walshe (2017). "Skeletal muscle insulin signaling and whole-body glucose metabolism following acute sleep restriction in healthy males." Physiol Rep **5**(23).

Symons, T. B., M. Sheffield-Moore, D. L. Chinkes, A. A. Ferrando and D. Paddon-Jones (2009). "Artificial gravity maintains skeletal muscle protein synthesis during 21 days of simulated microgravity." J Appl Physiol (1985) **107**(1): 34-38.

Tasali, E., R. Leproult, D. A. Ehrmann and E. Van Cauter (2008). "Slow-wave sleep and the risk of type 2 diabetes in humans." Proc Natl Acad Sci U S A **105**(3): 1044-1049.

Taylor, A. H., N. T. Cable, G. Faulkner, M. Hillsdon, M. Narici and A. K. Van Der Bij (2004). "Physical activity and older adults: a review of health benefits and the effectiveness of interventions." J Sports Sci **22**(8): 703-725.

Tipton, K. D., D. L. Hamilton and I. J. Gallagher (2018). "Assessing the Role of Muscle Protein Breakdown in Response to Nutrition and Exercise in Humans." Sports Med **48**(Suppl 1): 53-64.

Tjonna, A. E., S. J. Lee, O. Rognmo, T. O. Stolen, A. Bye, P. M. Haram, J. P. Loennechen, Q. Y. Al-Share, E. Skogvoll, S. A. Slordahl, O. J. Kemi, S. M. Najjar and U. Wisloff (2008). "Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study." Circulation **118**(4): 346-354.

Tranel, H. R., E. A. Schroder, J. England, W. S. Black, H. Bush, M. E. Hughes, K. A. Esser and J. L. Clasey (2015). "Physical activity, and not fat mass is a primary predictor of circadian parameters in young men." Chronobiol Int **32**(6): 832-841.

Trewin, A. J., B. J. Berry and A. P. Wojtovich (2018). "Exercise and Mitochondrial Dynamics: Keeping in Shape with ROS and AMPK." Antioxidants (Basel) **7**(1).

Trewin, A. J., L. Parker, C. S. Shaw, D. S. Hiam, A. Garnham, I. Levinger, G. K. McConell and N. K. Stepto (2018). "Acute HIIE elicits similar changes in human skeletal muscle mitochondrial H2O2 release, respiration, and cell signaling as endurance exercise even with less work." Am J Physiol Regul Integr Comp Physiol **315**(5): R1003-R1016.

Turek, F. W., C. Joshu, A. Kohsaka, E. Lin, G. Ivanova, E. McDearmon, A. Laposky, S. Losee-Olson, A. Easton, D. R. Jensen, R. H. Eckel, J. S. Takahashi and J. Bass (2005). "Obesity and metabolic syndrome in circadian Clock mutant mice." Science **308**(5724): 1043-1045.

van den Berg, R., D. O. Mook-Kanamori, E. Donga, M. van Dijk, J. G. van Dijk, G. J. Lammers, K. W. van Kralingen, C. Prehn, J. Adamski, J. A. Romijn, K. W. van Dijk, E. P. Corssmit, P. C. Rensen and N. R. Biermasz (2016). "A single night of sleep curtailment increases plasma acylcarnitines: Novel insights in the relationship between sleep and insulin resistance." Arch Biochem Biophys **589**: 145-151.

Van Dongen, H. P., G. Maislin, J. M. Mullington and D. F. Dinges (2003). "The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation." Sleep **26**(2): 117-126.

van Leeuwen, W. M., M. Lehto, P. Karisola, H. Lindholm, R. Luukkonen, M. Sallinen, M. Harma, T. Porkka-Heiskanen and H. Alenius (2009). "Sleep restriction increases the risk of developing cardiovascular diseases by augmenting proinflammatory responses through IL-17 and CRP." PLoS One **4**(2): e4589.

van Moorsel, D., J. Hansen, B. Havekes, F. A. J. L. Scheer, J. A. Jörgensen, J. Hoeks, V. B. Schrauwen-Hinderling, H. Duez, P. Lefebvre, N. C. Schaper, M. K. C. Hesselink, B. Staels and P. Schrauwen (2016). "Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity." Molecular Metabolism **5**(8): 635-645.

Van Reeth, O., J. Sturis, M. M. Byrne, J. D. Blackman, M. L'Hermite-Baleriaux, R. Leproult, C. Oliner, S. Refetoff, F. W. Turek and E. Van Cauter (1994). "Nocturnal exercise phase delays circadian rhythms of melatonin and thyrotropin secretion in normal men." Am J Physiol **266**(6 Pt 1): E964-974.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biology **3**(7).

VanHelder, T., J. D. Symons and M. W. Radomski (1993). "Effects of sleep deprivation and exercise on glucose tolerance." Aviat Space Environ Med **64**(6): 487-492.

Vieira, E., E. C. Nilsson, A. Nerstedt, M. Ormestad, Y. C. Long, P. M. Garcia-Roves, J. R. Zierath and M. Mahlapuu (2008). "Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle." Am J Physiol Endocrinol Metab **295**(5): E1032-1037.

Vondra, K., V. Brodan, A. Bass, E. Kuhn, J. Teisinger, M. Andel and A. Veselkova (1981). "Effects of Sleep-Deprivation on the Activity of Selected Metabolic Enzymes in Skeletal-Muscle." European Journal of Applied Physiology and Occupational Physiology **47**(1): 41-46.

Wang, X., J. Greer, R. R. Porter, K. Kaur and S. D. Youngstedt (2016). "Short-Term Moderate Sleep Restriction Decreases Insulin Sensitivity in Young Healthy Adults." Sleep Health **2**(1): 63-68.

Wefers, J., D. van Moorsel, J. Hansen, N. J. Connell, B. Havekes, J. Hoeks, W. D. van Marken Lichtenbelt, H. Duez, E. Phielix, A. Kalsbeek, M. V. Boekschoten, G. J. Hooiveld, M. K. C. Hesselink, S. Kersten, B. Staels, F. Scheer and P. Schrauwen (2018). "Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle." Proc Natl Acad Sci U S A **115**(30): 7789-7794.

Wehrens, S. M. T., S. Christou, C. Isherwood, B. Middleton, M. A. Gibbs, S. N. Archer, D. J. Skene and J. D. Johnston (2017). "Meal Timing Regulates the Human Circadian System." Curr Biol **27**(12): 1768-1775 e1763.

Wibom, R., E. Hultman, M. Johansson, K. Matherei, D. Constantin-Teodosiu and P. G. Schantz (1992). "Adaptation of mitochondrial ATP production in human skeletal muscle to endurance training and detraining." J Appl Physiol (1985) **73**(5): 2004-2010.

Wilkinson, D. J., M. V. Franchi, M. S. Brook, M. V. Narici, J. P. Williams, W. K. Mitchell, N. J. Szewczyk, P. L. Greenhaff, P. J. Atherton and K. Smith (2014). "A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans." Am J Physiol Endocrinol Metab **306**(5): E571-579.

Woldt, E., Y. Sebti, L. A. Solt, C. Duhem, S. Lancel, J. Eeckhoute, M. K. Hesselink, C. Paquet, S. Delhaye, Y. Shin, T. M. Kamenecka, G. Schaart, P. Lefebvre, R. Neviere, T. P. Burris, P. Schrauwen, B. Staels and H. Duez (2013). "Rev-erb-alpha modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy." Nat Med **19**(8): 1039-1046.

Wolff, G. and K. A. Esser (2012). "Scheduled exercise phase shifts the circadian clock in skeletal muscle." Med Sci Sports Exerc **44**(9): 1663-1670.

World Health Organization (1998). Life in the 21st Century - a Vision for All. Geneva, Switzerland.

Xie, F., P. Xiao, D. Chen, L. Xu and B. Zhang (2012). "miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs." Plant Mol Biol.

Yamazaki, S., R. Numano, M. Abe, A. Hida, R. Takahashi, M. Ueda, G. D. Block, Y. Sakaki, M. Menaker and H. Tei (2000). "Resetting central and peripheral circadian oscillators in transgenic rats." Science **288**(5466): 682-685.

Yang, P. Y., K. H. Ho, H. C. Chen and M. Y. Chien (2012). "Exercise training improves sleep quality in middle-aged and older adults with sleep problems: a systematic review." J Physiother **58**(3): 157-163.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T. L. Madden (2012). "Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction." BMC Bioinformatics **13**: 134.

Youngstedt, S. D., J. A. Elliott and D. F. Kripke (2019). "Human circadian phase-response curves for exercise." J Physiol **597**(8): 2253-2268.

Zambon, A. C., E. L. McDearmon, N. Salomonis, K. M. Vranizan, K. L. Johansen, D. Adey, J. S. Takahashi, M. Schambelan and B. R. Conklin (2003). "Time- and exercise-dependent gene regulation in human skeletal muscle." Genome Biol **4**(10): R61.

Zhang, R., N. F. Lahens, H. I. Ballance, M. E. Hughes and J. B. Hogenesch (2014). "A circadian gene expression atlas in mammals: implications for biology and medicine." Proc Natl Acad Sci U S A **111**(45): 16219-16224.

Zielinski, M. R., C. E. Kline, D. F. Kripke, R. K. Bogan and S. D. Youngstedt (2008). "No effect of 8-week time in bed restriction on glucose tolerance in older long sleepers." J Sleep Res **17**(4): 412-419.

Chapter 9. Appendices

9.1 Appendix A

9.1.1 Information to participants involved in research form

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled "The effect of sleep restriction and high intensity interval exercise on mitochondrial function, content and synthesis in males".

The project is being conducted by Mr Nicholas Saner (PhD candidate), Mr Matthew Lee, Mr Nathan Pitchford, Dr. Jon Bartlett, A/Prof Greg Roach and Prof. David Bishop from the College of Sport and Exercise Science at Victoria University.

Project explanation

Inadequate sleep has detrimental effects on short-term cognitive and physical performance. More recently, the effects of extended periods of sleep loss have been associated with a number of health concerns, which appear to be related to the role of sleep as a regulator of hormonal and metabolic processes within the body. It is possible that the dysregulation of these processes resulting from sleep loss may influence the function of mitochondria (the cellular structures responsible for producing energy for the body), however this has not been previously examined. Mitochondrial dysfunction is a common aspect of a number of disease states (such as muscle wasting (sarcopenia) and type 2 diabetes)

Exercise is a great way of staying fit and healthy and is often recommended as a preventative measure against cardiovascular and metabolic diseases (such as diabetes). Importantly, exercise is also known to induce a number of important processes within the cells of the body that lead to improved function of mitochondrial. Potentially, exercise may be able to counteract the metabolic problems associated with sleep loss.

.

The main aims of the project are:

1. To investigate the effect of 5 days sleep restriction on the function and integrity of mitochondrial and how cells within human skeletal muscle regulate these changes to mitochondria.
2. To investigate the ability of high-intensity interval exercise to counteract the negative effects of sleep loss

You are **eligible** to participate in this study if you are:

- Male and aged between 18 and 40, with a body mass index (BMI) between 19-30kg.m^2

You will be **excluded** from the study if you:

- Are currently performing shift work (overnight)
- Are currently taking any prescription medication known to affect sleep or blood glucose control
- Have recently returned from overseas travel
- Have irregular sleeping habits or previously diagnosed sleep disorders
- Have a VO_2 max above or below the study threshold
- Have a BMI of <19 or >30

- Are currently receiving treatment for a mental illness

Your participation is voluntary and you are free to withdraw from the study at any stage, without questioning or consequence.

What will I be asked to do?

- Initial meeting and medical history questionnaires
- Pre-trial aerobic fitness test (VO_2 max) on an exercise bike
- 1 week period of habitual physical activity, diet and sleep monitoring (via sleep watch and diary reporting)
- A sleep study protocol (8 nights in total, with a 5 night sleep intervention period) (see **Figure 1**). You will be asked to remain at the Victoria University Footscray Park campus for the majority of each protocol.
- Oral glucose tolerance testing (OGTT)
- The study involves the collection of muscle biopsies (3 over the course of the study), blood sampling and high-intensity interval exercise sessions.
- Ingestion of 150ml of deuterium oxide (D_2O) at the beginning of each protocol

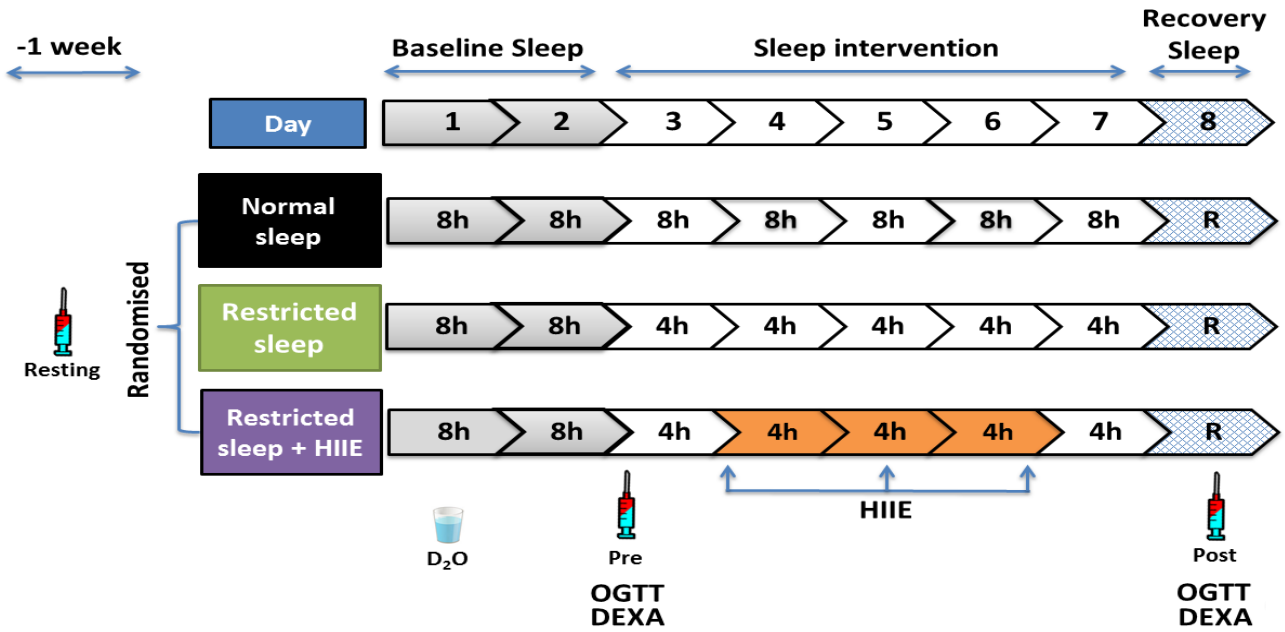

Figure 1: Protocol overview 8h hours in bed each night 4h hours in bed each night, 4h HIIE session, R recovery sleep, D_2O ingestion, OGTT – oral glucose tolerance test, muscle biopsy and blood sample

How will this project be conducted?

Initial interview:
You will firstly meet with the student researcher to discuss the project, before being asked to fill out several short questionnaires about your family medical history and exercise habits, to assess your eligibility to participate in this study.

Pre-trial fitness assessment:
After confirming your eligibility for this study and giving informed consent, you will be asked to perform a pre-trial maximal oxygen uptake test (VO_2 max) via an incremental ramp test on a cycle ergometer (exercise bike) to assess your aerobic fitness status. Participants may be excluded from the study if their VO_2 max exceeds the designated limits.

Preliminary monitoring:
Over the course of 2 weeks you will be asked to wear a sleep watch and fill out a diary detailing your habitual sleep times, food intake and physical activity details. You will also complete a small questionnaire regarding your physical activity habits.

Sleep Study:
The sleep study involves an 8 night stay within the Victoria University altitude hotel (used as a sleep facility only). You will be allocated into 1 of 3 protocol groups. All protocols consist of an initial 2 nights of baseline sleep, before beginning a 5 night sleep intervention and finishing with a final night of recovery sleep. The 3 sleep interventions (you will only be allocated to one of these) are:

- **Normal sleep** (8 hours time in bed (TIB) each night)
- **Restricted sleep** (4 hours TIB)
- **Restricted sleep + HIIE** (4 hours TIB and 3 HIIE sessions)

The HIIE sessions consist of 10 x 60 second intervals (at peak power output) on a cycle ergometer. Each interval will be interspersed by 75 seconds of active recovery (at 30W). HIIE sessions will be conducted on Days 4, 5 and 6 of the restricted sleep + HIIE protocol.

During the protocols, participants will be asked to remain within the Victoria University Footscray campus. **All meals will be provided** for the duration of the stay and the participant will have their own room to sleep in each night.

An overview of the study protocols is provided in **Figure 1**.

Analysis and sampling:

Prior to starting the sleep protocol, a resting **muscle biopsy** (taken from the thigh muscle) and blood sample (from forearm vein) is required. Once arriving at the Victoria University facilities, participants will be asked to ingest 150ml of **D_2O** (heavy water). This allows us to measure the production of new proteins throughout the study and is a safe and validated method. A muscle biopsy and blood sample will be collected following the initial 2 days of baseline sleep and then again following the 5 nights of sleep intervention (contributing to a total of 3 muscle biopsies).

An oral glucose tolerance test (**OGTT**) will be performed before and after the 5 night sleep intervention. The OGTT involves drinking a sugary drink, followed by the collection of blood samples for a period of 2 hours. Blood samples will be collected at 0, 10, 20, 30, 60, 90 and 120 minute time points, via a plastic cannula in a forearm vein.

For the duration of the sleep protocols, sleep will be monitored with sleep watches (actigraphy watches), that enable the amount of sleep the participant gets each night to be monitored. On 2 of the nights, sleep will also be monitored using specialised sleep monitoring equipment known as polysomnography (PSG). This is a painless procedure that involves the connection of electrodes to your body while you sleep.

Each morning, participants will be asked to complete a profile of mood states **(POMS)** questionnaire. This is a simple document and helps to assess the participants wellness and mind set throughout the protocols.

DEXA scans will be performed on Days 3 and 8 (pre and post the sleep intervention stage) (i.e. 2 DEXA scans in total). This technique will allow us to analyse any potential changes in body composition (i.e. lean

mass vs fat mass), resulting from the sleep loss or the sleep loss and HIIE protocols. This involves lying still on the DEXA scanner for approximately 7 minutes while a scanner passes over you.

Taxi vouchers will be supplied at the completion of each protocol so that the participant doesn't have to drive home.

What will I gain from participating?

Whilst we cannot guarantee that you will gain any benefits from your participation in this study, you will gain a better understanding of your fitness level, general health and how exercise can improve your health. In addition, this study will allow you the opportunity to have your sleeping habits examined as well as receiving feedback and information regarding current best practices in sleep hygiene. As a part of the protocols, food will be provided for the duration of the stay so that your habitual diet can be maintained at the same level as the monitoring period.

How will the information I give be used?

Any information obtained in this research project that can identify you will remain confidential and will only be used for research purposes and as part of a PhD thesis. It will only be disclosed with your permission, except as required by law. Data collected will be used in scientific publications and/or presentations, information will be provided in a way that you cannot be identified, except with your permission. The data will be reported as averages of the entire group and as individuals. With your written consent, photographs or videos may be taken during experimental trials for use in presentations or to assist in future experimental set-ups. Any images will only be taken with your written consent.

What are the potential risks of participating in this project?

The procedures involved in participating in this study are of low risk. Nevertheless, as in any invasive or exercise procedure, there are small risks and some discomfort that may be experienced:

Fitness testing and high-intensity interval exercise:

All exercise activity carries a risk of injury and in extreme cases, risks of suffering a heart attack or stroke. All protocols within this study are commonly performed in exercise physiology laboratories and potential risks to participants have been minimised by employing appropriate warm-up procedures and researcher supervision (with adequate first-aid training). Although not expected, cardiac arrest, stroke and sudden death have been reported with exercise.

Dual energy X-ray absorptiometry (DEXA) scans:

This research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is less than 0.06 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal.

Deuterium Oxide (D_2O) ingestion:

The ingestion of D_2O is necessary to allow us to monitor the processes occurring within your muscle. There are no long term effects of drinking D_2O, however on very rare occasions a period of dizziness has been reported following the ingestion of large volumes of D_2O (almost 4 x the volume we require).

Intravenous cannulation/venepuncture:

Needle insertion into a vein is required for placement of a cannula into a forearm vein and for venepuncture. During the needle insertion you will feel minor to moderate discomfort. However, for the intravenous cannulation (oral glucose tolerance test only) the needle is quickly removed and only a flexible plastic tube remains in your vein for the duration of blood sampling (approximately 2 hours for muscle biopsy and OGTT). When the cannula is removed, direct pressure will be applied to the area to reduce the chances of bruising. Cannulas are routinely placed into veins of participants in clinical research studies and in hospital patients.

The risks of IV cannulation are low, but very occasionally infection and bruising can occur. Cannulation and venepuncture will only be conducted by qualified and experienced researchers.

Muscle biopsy:

The muscle biopsy will be performed by a qualified medical doctor who is experienced in taking muscle biopsies. Xylocaine, a local anaesthetic, will be injected at the site of the muscle biopsy (vastus lateralis – mid outer thigh). The anaesthetic may burn or sting when injected before the area becomes numb. A slight pressure or a "pulling" sensation will be felt during the procedure and this can be painful. To extract the muscle biopsy a small incision needs to be made where the muscle biopsy needle will be inserted. After the incision has been made a Bergstrom needle will then be inserted to extract a small muscle sample (approx. 2 rice grains in size). Once the local anaesthetic has worn off and for the next day or two the area will likely feel like you have been "corked" and this can be painful. You should not feel any discomfort after 2-3 days. In rare cases haematomas have been reported, although these symptoms typically disappear within a week. On very rare occasions, altered sensation (numbness or tingling) on the skin near the site of the biopsy has been reported; however this sensation disappears over a period of a few weeks to months.

Sleep restriction:

The sleep restriction protocols will have a varied effect on participants. Some participants may not be strongly affected by the restricted sleep times and will function as you would regularly, however others may find it difficult to concentrate and function to their usual standards. It is likely that you will be very tired by the conclusion of the study and have a poor mood state. You will be required to spend a night of recovery sleep at the facility as part of the protocol to ensure you are fully recuperated before leaving the facility. You will be monitored by a member of the research team to ensure you are not put at any risk associated with the sleep loss. It is possible that the sleep restriction protocols in combination with exercise may cause participants to feel "run down" and they may potentially develop symptoms of the common cold.

Participation in this study is voluntary. If at any point during the study you change your mind, you're able to withdraw without any further consequences or questioning. In addition to this, if you experience any adverse effects (outside of the expected and normal effects of the sleep intervention or exercise) you will be removed from the study by the chief investigator. In the event that you experience any psychological distress resulting from any aspect of the study, you will be invited to discuss this with a registered psychologist who is not in any way connected to the research. This will be free of charge to you.

Who is conducting the study?

The project is being conducted by Mr Nicholas Saner (PhD candidate), Dr. Jon Bartlett, Prof. Greg Roach and Prof. David Bishop from the College of Sport and Exercise Science at Victoria University.

For more information or to organise a meeting, please contact:

Dr. Jon Bartlett
Chief investigator
Mob: 0424 980 643
Email: jon.bartlett@vu.edu.au

Mr Nicholas Saner
PhD candidate
Mob: 0418 376 762
Email: nicholas.saner@live.vu.edu.au

Any queries about your participation in this project may be directed to the Chief Investigator listed above.
If you have any queries or complaints about the way you have been treated, you may contact the Ethics Secretary, Victoria University Human Research Ethics Committee, Office for Research, Victoria University, PO Box 14428, Melbourne, VIC, 8001, email researchethics@vu.edu.au or phone (03) 9919 4781 or 4461.

9.1.2 Participant consent form

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:
You are invited to participate in research investigating the effect of sleep and high-intensity interval exercise on mitochondrial function content and synthesis.

The project is being conducted by Mr Nicholas Saner (PhD student), Mr Matthew Lee, Mr Nathan Pitchford, Dr. Jon Bartlett, A/Prof Greg Roach and Prof. David Bishop from the College of Sport and Exercise Science at Victoria University.

Project explanation

Inadequate sleep has detrimental effects on short-term cognitive and physical performance. More recently, the effects of extended periods of sleep loss have been associated with a number of health concerns, which appear to be related to the role of sleep as a regulator of hormonal and metabolic processes within the body. It is possible that the dysregulation of these processes resulting from sleep loss may influence the function of mitochondria (the cellular structures responsible for producing energy for the body), however this has not been previously examined. Mitochondrial dysfunction is a common aspect of a number of disease states (such as sarcopenia and type 2 diabetes)

Exercise is a great way of staying fit and healthy and it's often recommended as a preventative measure against cardiovascular and metabolic diseases (such as diabetes). Importantly, exercise is also known to induce a number of important adaptations to mitochondria and can improve mitochondrial function. Potentially, exercise may be able to counteract the metabolic dysregulation associated with sleep loss.

The main aims of the project are:

3. To investigate the effect of 5 days sleep restriction on mitochondrial content, function, protein synthesis and the molecular regulators of mitochondrial adaptations in human skeletal muscle.
4. To investigate the ability of high-intensity interval exercise to counteract the negative effects of sleep loss

What will I be asked to do?

- An initial meeting to discuss your participation and complete medical history/risk factor questionnaires.
- Pre-trial aerobic fitness test (VO_2 max) on a cycle ergometer (gym bike) at the Victoria University Footscray Park, exercise laboratory
- A 2 week period of habitual physical activity, diet and sleep monitoring (to be assessed by filling out a food/ physical activity/ sleep diary using a sleep watch).
- A sleep study protocol (detailed in 'information to participants' form), consisting of an 8 night stay at the Victoria University altitude hotel, during which you will remain at the Footscray Park campus.
- Oral glucose tolerance testing (OGTT) at the start and finish of the sleep intervention to assess glucose tolerance.
- The study involves collection of muscle biopsies (3 in total)), blood sampling and high-intensity interval exercise sessions.

- Ingestion of 150ml of deuterium oxide (D_2O, heavy water) at the beginning of each protocol.
- Dual energy X-ray absorptiometry (DEXA) scans pre and post each protocol (3 in total).

Risks involved

The procedures involved in participating in this study are of low risk. Nevertheless, as in any invasive and exercise procedure, there are small risks and some discomfort that may be experienced. All potential risks associated with participation in this study are **fully explained in the 'Information to Participants' form**.

CERTIFICATION BY SUBJECT

I, ……. (full name)

of …….. (street address)

……..…. (suburb)…….(postcode)

Phone: …….

Email: ……

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study: the effect of sleep and high-intensity interval exercise on mitochondrial function being conducted at Victoria University by: Dr. Jon Bartlett from the College of Sport and Exercise Science.

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

Mr Nicholas Saner

and that I freely consent to participation involving the below mentioned procedures:

- Maximal oxygen uptake test (VO_2 max)
- Pre-trial sleep, physical activity and dietary monitoring
- Blood and muscle sampling
- Oral glucose tolerance test
- Sleep study protocols
- Ingestion of the deuterium oxide (D_2O, heavy water)
- Polysomnography sleep monitoring
- DEXA scans

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

ADDITIONAL CONSENT

I also agree to allow photographs or video of me to be used in publications or conference presentations. I understand that I am free to withdraw my consent for this at any time without prejudice.

☐ Yes ☐ No

I have been informed that the information I provide will be kept confidential.

Signed: _____

Date: _____

Any queries about your participation in this project may be directed to the researcher
Dr Jon Bartlett
Chief Investigator
Mob: 0424 980 643
Email: jon.bartlett@vu.edu.au

Mr Nicholas Saner
PhD researcher
Mob: 0418 376 762
Email: nicholas.saner@live.vu.edu.au

If you have any queries or complaints about the way you have been treated, you may contact the Ethics Secretary, Victoria University Human Research Ethics Committee, Office for Research, Victoria University, PO Box 14428, Melbourne, VIC, 8001, email Researchethics@vu.edu.au or phone (03) 9919 4781 or 4461.

9.1.3 Cardiovascular and other risk factor questionnaire

CARDIOVASCULAR AND OTHER RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating: "The effect of sleep and high-intensity interval exercise on mitochondrial function", you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: _____ **Date of birth:** _____

Address: _____ **Phone:** _____

Age: _____ years **Weight:** _____ kg **Height:** _____ cm **Gender: M F**

Give a brief description of your average activity pattern in the past 2 months:

_____
_____
_____

1.	Are you overweight?	Yes	No
	Don't know		
2.	Do you smoke?	Yes	No
	Social		
3.	Are you an asthmatic?	Yes	No
	Don't Know		
4.	Are you a diabetic?	Yes	No
	Don't Know		
5.	Does your family have a history of diabetes?	Yes	No
	Don't Know		
6.	Do you have a thyroid disorder?	Yes	No
	Don't Know		
7.	Does your family have a history of thyroid disorders?	Yes	No
	Don't Know		

8. Do you have a pituitary disorder? Yes No
   Don't Know
9. Does your family have a history of pituitary disorders? Yes No
   Don't Know
10. Do you have a heart rhythm disturbance? Yes No
    Don't Know
11. Do you have a high blood cholesterol level? Yes No
    Don't Know
12. Do you have elevated blood pressure? Yes No
    Don't Know
13. Are you being treated with diuretics? Yes No
14. Are you on any other medications? Yes No

    List all medications:

_____

_____

15. Do you think you have any medical complaint or any other reason which you know of which you think may prevent you from participating in strenuous exercise? Yes No
    If Yes, please elaborate

_____

16. Have you had any musculoskeletal problems that have required medical treatment (eg, broken bones, joint reconstruction etc)? Yes No
    If Yes, please provide details (including dates)

_____

17. Does your family have a history of premature cardiovascular problems
    (e.g. heart attack, stroke)? Yes No
    Don't Know

9.2 Appendix B

9.2.1 Sleep diary

Sleep Diary

Date	Sleep Location	Caffeine Intake Amount & Time	Did you watch TV (or computer) in bed before sleep? Yes/No & Time	Pre-Sleep Arousal Circle	Bed Time hh:mm	Get-up Time hh:mm	Sleep Quality Circle
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor
				very high | high | moderate | low | none			very good | good | average | poor | very poor

9.2.2 Example daily diet

			Macronutrient Goals:			Actual:						Daily Intake Goals:	
Code:			Carbs	Protein	Fats	Carbs	Protein	Fats		Day 2		Carbs:	310.5 g
Group:	SR		4.5	1.5	1.0	4.5	1.5	1.0				Protein:	103.5 g
Body Mas	69		g.kg.d			g.kg.d						Fat:	69.0 g
												kcal:	2,277
			Macros/100g			Serving	Per Serving					kJ:	9,532
Meal	Food		Carbs	Protein	Fat	(g) or (ml)	Carbs	Protein	Fat				
Meal 1 - Breakfast	Lowan Original Muesli (Coles)		57	12	12	78	44.3	9.0	9.7			Goals per meal:	
	Whole Milk (Coles)		5	3	2	170	8.2	5.8	3.9			Carbs:	84.5 g
												Protein:	33.8 g
												Fat:	22.8 g
										Meal 1 Energy:		kcal:	678
		Meal 1 Total:					52.5	14.8	13.6	391 kcal	1,638 kJ	kJ:	2,839
		Meal 1 Macros Remaining:					32.0	18.9	9.2				
Snack	Banana		23.0	1.1	0.3	150	34.5	1.7	0.5				
										Protein Shake Energy:			
		Total:					34.5	1.7	0.5	149 kcal	622 kJ		
Meal 2 - Lunch	Mustard chicken			38.46	5.9		56	17.2	6.3				
	Apple		14	0.3	0.2	100	14.0	0.3	0.2				
	Coles Nut Bar		42.5	11.5	37.2	35	17.0	4.6	14.9				
										Meal 2 Energy:			
		Meal 2 Total:					87.4	22.1	21.4	630 kcal	2,150 kJ		
		Meal 2 Macros Remaining:					-2.9	11.7	1.4				
Snack	Pear		15.0	0.4	0.1	150	22.5	0.6	0.2				
										Protein Shake Energy:			
		Total:					22.5	0.6	0.2	94 kcal	392 kJ		
Meal 3 - Dinner	1 Serve 195-04 Slow Cooked Lamb		34	41.8	8.0		34	41.8	8.0				
	Coles Nut Bar (choc)		42.5	11.5	37.2	35	17.0	4.6	14.9				
										Meal 3 Energy:			
										594 kcal	2,486 kJ		
						40				Total Energy:			
		Meal 3 Total:					50.7	46.4	22.9	2,296 kcal	9,609 kJ		
		Meal 3 Macros Remaining:					33.8	-12.7	-0.1				
Snack	Yoghurt		22.1	8.2	3.3	175	38.7	14.4	5.8				
	Rice Crackers (shapes)		68.5	7.4	14.4	40	27.4	3.0	5.8				
		Daily Total:					313.6	102.9	69.9				
		Daily Macros Remaining:					-3.1	0.6	-0.9				

9.2.3 Morning-eveningness questionnaire

MORNINGNESS-EVENINGNESS QUESTIONNAIRE
Self-Assessment Version (MEQ-SA)[1]

Name: _____ Date: _____

For each question, please select the answer that best describes you by circling the point value that best indicates how you have felt in recent weeks.

1. *Approximately* what time would you get up if you were entirely free to plan your day?
   [5] 5:00 AM–6:30 AM *(05:00–06:30 h)*
   [4] 6:30 AM–7:45 AM *(06:30–07:45 h)*
   [3] 7:45 AM–9:45 AM *(07:45–09:45 h)*
   [2] 9:45 AM–11:00 AM *(09:45–11:00 h)*
   [1] 11:00 AM–12 noon *(11:00–12:00 h)*
2. *Approximately* what time would you go to bed if you were entirely free to plan your evening?
   [5] 8:00 PM–9:00 PM *(20:00–21:00 h)* [4]
   9:00 PM–10:15 PM *(21:00–22:15 h)* [3]
   10:15 PM–12:30 AM *(22:15–00:30 h)* [2]
   12:30 AM–1:45 AM *(00:30–01:45 h)* [1]
   1:45 AM–3:00 AM *(01:45–03:00 h)*
3. If you usually have to get up at a specific time in the morning, how much do you depend on an alarm clock?
   [4] Not at all
   [3] Slightly
   [2] Somewhat
   [1] Very much

[1]Some stem questions and item choices have been rephrased from the original instrument (Horne and Östberg, 1976) to conform with spoken American English. Discrete item choices have been substituted for continuous graphic scales. Prepared by Terman M, Rifkin JB, Jacobs J, White TM (2001), New York State Psychiatric Institute, 1051 Riverside Drive, Unit 50, New York, NY, 10032. January 2008 version. Supported by NIH Grant MH42931. *See also:* automated version (AutoMEQ) at www.cet.org.

Horne JA and Östberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. International Journal of Chronobiology, 1976: 4, 97-100.

4. How easy do you find it to get up in the morning (when you are not awakened unexpectedly)?

   [1] Very difficult

   [2] Somewhat difficult

   [3] Fairly easy
   [4] Very easy

5. How alert do you feel during the first half hour after you wake up in the morning?

   [1] Not at all alert
   [2] Slightly alert
   [3] Fairly alert

   [4] Very alert

6. How hungry do you feel during the first half hour after you wake up?

   [1] Not at all hungry
   [2] Slightly hungry
   [3] Fairly hungry
   [4] Very hungry

7. During the first half hour after you wake up in the morning, how do you feel?

   [1] Very tired
   [2] Fairly tired
   [3] Fairly refreshed

   [4] Very refreshed

8. If you had no commitments the next day, what time would you go to bed compared to your usual bedtime?
   [4] Seldom or never later

   [3] Less that 1 hour later

   [2] 1-2 hours later

   [1] More than 2 hours later

9. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week, and the best time for him is between 7-8 AM *(07-08 h).* Bearing in mind nothing but your own internal "clock," how do you think you would perform?

   [4] Would be in good form

   [3] Would be in reasonable form

   [2] Would find it difficult

   [1] Would find it very difficult

10. At *approximately* what time in the evening do you feel tired, and, as a result, in need of sleep?

    [5] 8:00 PM–9:00 PM *(20:00–21:00 h)*

    [4] 9:00 PM–10:15 PM *(21:00–22:15 h)*

    [3] 10:15 PM–12:45 AM *(22:15–00:45 h)*

    [2] 12:45 AM–2:00 AM *(00:45–02:00 h)*

    [1] 2:00 AM–3:00 AM *(02:00–03:00 h)*

11. You want to be at your peak performance for a test that you know is going to be mentally exhausting and will last two hours. You are entirely free to plan your day. Considering only your "internal clock," which one of the four testing times would you choose?

    [6] 8 AM–10 AM *(08–10 h)*

    [4] 11 AM–1 PM *(11–13 h)*

    [2] 3 PM–5 PM *(15–17 h)*

    [0] 7 PM–9 PM *(19–21 h)*

12. If you got into bed at 11 PM *(23 h)*, how tired would you be?

[0] Not at all tired
[2] A little tired
[3] Fairly tired
[5] Very tired

13. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which one of the following are you most likely to do?

[4] Will wake up at usual time, but will not fall back asleep

[3] Will wake up at usual time and will doze thereafter
[2] Will wake up at usual time, but will fall asleep again

[1] Will not wake up until later than usual

14. One night you have to remain awake between 4-6 AM *(04-06 h)* in order to carry out a night watch. You have no time commitments the next day. Which one of the alternatives would suit you best?

[1] Would not go to bed until the watch is over

[2] Would take a nap before and sleep after
[3] Would take a good sleep before and nap after

[4] Would sleep only before the watch

15. You have two hours of hard physical work. You are entirely free to plan your day.

Considering only your internal "clock," which of the following times would you choose?

[4] 8 AM–10 AM *(08–10 h)*

[3] 11 AM–1 PM *(11–13 h)*

[2] 3 PM–5 PM *(15–17 h)* [1]

7 PM–9 PM *(19–21 h)*

16. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week. The best time for her is between 10-11 PM *(22-23 h).* Bearing in mind only your internal "clock," how well do you think you would perform?

[1] Would be in good form

[2] Would be in reasonable form
[3] Would find it difficult
[4] Would find it very difficult

17. Suppose you can choose your own work hours. Assume that you work a five-hour day (including breaks), your job is interesting, and you are paid based on your performance. At *approximately* what time would you choose to begin?

    [5] 5 hours starting between 4–8 AM *(05–08 h)*

    [4] 5 hours starting between 8–9 AM *(08–09 h)*
    [3] 5 hours starting between 9 AM–2 PM *(09–14 h)*

    [2] 5 hours starting between 2–5 PM *(14–17 h)*
    [1] 5 hours starting between 5 PM–4 AM *(17–04 h)*

18. At *approximately* what time of day do you usually feel your best?

    [5] 5–8 AM *(05–08 h)*
    [4] 8–10 AM *(08–10 h)*
    [3] 10 AM–5 PM *(10–17 h)*
    [2] 5–10 PM *(17–22 h)*

    [1] 10 PM–5 AM *(22–05 h)*

19. One hears about "morning types" and "evening types." Which one of these types do you consider yourself to be?

    [6] Definitely a morning type

    [4] Rather more a morning type than an evening type
    [2] Rather more an evening type than a morning type

    [1] Definitely an evening type

____ Total points for all 19 questions

INTERPRETING AND USING YOUR MORNINGNESS-EVENINGNESS SCORE

This questionnaire has 19 questions, each with a number of points. First, add up the points you circled and enter your total morningness-eveningness score here:

Scores can range from 16-86. Scores of 41 and below indicate "evening types." Scores of 59 and above indicate "morning types." Scores between 42-58 indicate "intermediate types."

16-30	31-41	42-58	59-69	70-86
definite evening	moderate evening	intermediate	moderate morning	definite morning

Occasionally a person has trouble with the questionnaire. For example, some of the questions are difficult to answer if you have been on a shift work schedule, if you don't work, or if your bedtime is unusually late. Your answers may be influenced by an illness or medications you may be taking. If you are not confident about your answers, you should also not be confident about the advice that follows.

One way to check this is to ask whether your morningness-eveningness score approximately matches the sleep onset and wake-up times listed below:

Score	16-30	31-41	42-58	59-69	70-86
Sleep onset	2:00-3:00 AM (02:00-03:00 h)	12:45-2:00 AM (00:45-02:00 h)	10:45 PM-12:45 AM (22:45-00:45 h)	9:30-10:45 PM (21:30-22:45 h)	9:00-9:30 PM (21:00-21:30 h
Wake-up	10:00-11:30 AM	8:30-10:00 AM	6:30-8:30 AM	5:00-6:30 AM	4:00-5:00 AM

If your usual sleep onset is earlier than 9:00 PM (21:00 h) or later than 3:00 AM (03:00 h), or your wake-up is earlier than 4:00 AM (04:00 h) or later than 11:30 AM (11:30 h), you should seek the advice of a light therapy clinician in order to proceed effectively with treatment.

We use the morningness-eveningness score to improve the antidepressant effect of light therapy. Although most people experience good antidepressant response to light therapy when they take a
regular morning session using a 10,000 lux white light device (see www.cet.org for recommendations) for 30 minutes, often this will not give the best possible response. If your internal clock is shifted relative to external time (as indirectly measured by your morningness-eveningness score), the timing of light therapy needs to be adjusted.

The table at the top of the next page shows the recommended start time for light therapy for a wide range of morningness-eveningness scores. If your score falls beyond this range (either very low or very high), you should seek the advice of a light therapy clinician in order to proceed effectively with treatment.
.

Morningness-Eveningness Score	Start time for light therapy
23-26	8:15 AM
27-30	8:00 AM
31-34	7:45 AM
35-38	7:30 AM
39-41	7:15 AM
42-45	7:00 AM
46-49	6:45 AM
50-53	6:30 AM
54-57	6:15 AM
58-61	6:00 AM
62-65	5:45 AM
66-68	5:30 AM
69-72	5:15 AM 5:00 AM

If you usually sleep longer than 7 hours per night, you will need to wake up somewhat earlier than normal to achieve the effect – but you should feel better for doing that. Some people compensate by going to bed earlier, while others feel fine with shorter sleep. If you usually sleep less than 7 hours per night you will be able to maintain your current wake-up time. If you find yourself automatically waking up more than 30 minutes before your session start time, you should try moving the session later. Avoid taking sessions earlier than recommended, but if you happen to oversleep your alarm clock, it is better to take the session late than to skip it.

Our recommended light schedule for evening types – say, 8:00 AM (08:00 h) for a morningness- eveningness score of 30 – may make it difficult to get to work on time, yet taking the light earlier may not be helpful. Once you have noted improvement at the recommended hour, however, you can begin inching the light therapy session earlier by 15 minutes per day, enabling your internal clock to synchronize with your desired sleep-wake cycle and work schedule.

The personalized advice we give you here is based on a large clinical trial of patients with seasonal affective disorder (SAD) at Columbia University Medical Center in New York. Patients who took the light too late in the morning experienced only half the improvement of those who took it approximately at the times indicated. These guidelines are not only for SAD, but are also helpful in treatment of nonseasonal depression, for reducing insomnia at bedtime, and for reducing the urge to oversleep in the morning.

Our advice serves only as a general guideline for new users of light therapy. There are many individual factors that might call for a different schedule or dose (intensity, duration) of light. Any person with clinical depression should proceed with light therapy only under clinical guidance.

Reference: Terman M, Terman JS. Light therapy for seasonal and nonseasonal depression: efficacy, protocol, safety, and side effects. CNS Spectrums, 2005;10:647-663. (Downloadable at www.cet.org)

9.2.4 International physical activity questionnaire (IPAQ)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(October 2002)

http://www.ipaq.ki.se/ipaq.htm

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

☐ Yes

☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous**

physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

    _____ **hours per day**
    _____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

    _____ **hours per day**
    _____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

9.3 Appendix C

9.3.1 RNA sample quantification

Table 9.1 - Quantification of RNA samples using the Nanodrop spectrophotometer

Participant	Sample	RNA concentration (ng/µL)	A260/A280	A260/A230
1	Pre	784.4	1.52	0.24
	Post	841.9	1.91	1.55
2	Pre	501.0	1.57	0.40
	Post	781.5	1.55	0.18
3	Pre	734.6	1.55	0.21
	Post	280.1	1.71	0.27
4	Pre	551.9	1.55	0.43
	Post	682.7	1.53	0.22
5	Pre	348.0	1.91	0.69
	Post	583.9	1.52	0.20
6	Pre	445.8	1.90	0.57
	Post	632.5	1.53	0.17
7	Pre	294.0	1.84	0.40
	Post	232.4	1.95	0.43
8	Pre	348.5	1.80	0.40
	Post	419.5	1.83	0.44
9	Pre	339.7	1.88	0.42
	Post	556.9	1.62	0.26
10	Pre	323.0	1.87	0.45
	Post	340.4	1.88	0.61
11	Pre	331.9	1.77	0.33
	Post	243.3	1.65	0.25
12	Pre	325.0	1.82	0.46
	Post	294.1	1.86	0.52
13	Pre	320.3	1.79	0.38
	Post	552.6	1.62	0.32
14	Pre	582.5	1.57	0.26
	Post	256.7	1.93	0.86
15	Pre	239.9	1.92	0.86
	Post	229.3	1.91	0.88
16	Pre	382.6	1.79	0.38
	Post	1238.9	1.73	0.31
17	Pre	1377.9	1.74	0.30
	Post	645.5	1.62	0.24
19	Pre	538.3	1.65	0.24
	Post	456.6	1.87	0.46
20	Pre	536.1	1.61	0.27
	Post	454.6	1.86	0.43
22	Pre	774.7	1.64	0.25
	Post	1730.6	1.83	0.40
23	Pre	309.4	1.86	0.44
	Post	697.4	1.69	0.33
24	Pre	1173.6	1.72	0.32
	Post	260.9	1.89	0.46
26	Pre	364.3	1.79	0.43
	Post	331.6	1.69	0.32

9.4 **Appendix D**

9.4.1 Sleep Medicine Reviews 37 (2018)

Contents lists available at ScienceDirect

Sleep Medicine Reviews

journal homepage: www.elsevier.com/locate/smrv

THEORETICAL REVIEW

Is exercise a viable therapeutic intervention to mitigate mitochondrial dysfunction and insulin resistance induced by sleep loss?

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SUMMARY

Sleep loss has emerged as a risk factor comparable to that of physical inactivity for the development of insulin resistance, impaired glucose tolerance and type 2 diabetes mellitus. This is a concern as it was estimated in 2012 that approximately 70 million adults in the United States are sleeping less than 6 h each night, and the average nightly sleep duration of a representative sample of the U.S. adult population is reported to be significantly less than in previous decades. The underlying mechanisms responsible for chronic sleep loss induced insulin resistance include modifications in the regulation of hormone secretion, peripheral clock gene regulation, and the cellular signaling processes associated with regulating mitochondrial respiratory function. Emerging evidence shows these mechanisms share similar biochemical signaling pathways to those underpinning exercise-induced adaptations, which together suggest exercise might be a viable, suitable, and potent treatment alternative to alleviate sleep loss induced insulin resistance and glucose intolerance. In this theoretical review, we provide a summary of the impact of reduced sleep duration and quality on mitochondrial function and insulin resistance, before detailing the possible underlying mechanisms. Finally, we propose how and why regular exercise may be a therapeutic intervention to mitigate sleep loss induced mitochondrial dysfunction and insulin resistance.

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) continues to rise in modern society. The World Health Organization (WHO) estimates that by the year 2025, 300 million people across the world will be diagnosed with diabetes [1]. To give this additional context, T2DM was the 7th leading cause of death in the United States in 2012 and costs their economy an estimated
$245 billion each year [2]. Typically, the onset of T2DM coincides with impaired glucose tolerance (characterized by a fasting blood glucose level >7 mmol/L) and increased insulin resistance (reduced efficiency of insulin-stimulated glucose uptake) [3]. Furthermore, the health consequences arising from diabetic complications (such as heart disease, stroke and kidney damage) add to the already extraordinary burden of this metabolic disease on health services [2].

The most common risk factors associated with the onset of insulin resistance and glucose intolerance include genetic pre- disposition and lifestyle factors such as diet and physical inactivity [4]. However, in the last 15 y sleep has emerged as a prominent factor influencing the development of T2DM. In fact, sleep loss and reduced sleep quality are now reported to be comparable to physical inactivity [4] in terms of their relative contribution to the risk of developing T2DM. The percentage of sleep deficient people who develop insulin resistance and T2DM is difficult to determine with the available data. Nonetheless, studies investigating shift workers (a cohort often associated with insufficient sleep and reduced sleep quality [5]) indicate a significantly increased risk (40%) of developing diabetes compared to those performing day work [4]. Considering the significant reduction in average nightly sleep duration compared to previous decades [6], and that it was recently estimated via self-reported measures that ~40% of the population are sleeping less than the recommended 7 h per night [7], the role of sleep loss in contributing to the already high prevalence of T2DM is an important area for further investigation.

Potential mechanisms by which sleep loss may contribute to insulin resistance and glucose intolerance have been identified, and

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1087-0792/

Abbreviations

AMPK	AMP-activated protein kinase
ACSM	American College of Sports Medicine
ATF2	activating transcription factor 2
Bmal1	brain and muscle Arnt-like protein-1
CAMK	calmodulin-dependent protein kinase
CCG	clock controlled genes
Clock	circadian locomotor output cycles kaput
CREB	cyclic-AMP response element binding protein
CS	citrate synthase
COX-1	cytochrome c oxidase subunit 1
Cry 1,2	cryptochrome 1 and 2
GLUT4	glucose transporter 4
HbA1c	glycated hemoglobin
HIIE	high-intensity interval exercise
LKB-1	liver kinase B1
MEF2	myocyte enhancer factor 2
mRNA	messenger ribonucleic acid
$NAD^þ$	nicotinamide adenine dinucleotide
nREM	non-rapid eye movement
NRF1/2	nuclear respiratory factor 1/2
OGTT	oral glucose tolerance test
OSA	obstructive sleep apnea
p38MAPK	p38 mitogen-activated protein kinase
PPAR	peroxisome proliferator-activated receptor
Per 1,2,3	period 1,2 and 3
PGC-1a	peroxisome proliferator-activated receptor g co-activator 1a
REM	rapid eye movement
Rev-erba	NR1D1; Nuclear receptor subfamily 1, group D, member 1
ROR-a	RAR-related orphan receptor a
SCN	suprachiasmatic nucleus
SIRT1/3	sirtuin 1/3
SWS	slow wave sleep
T2DM	type 2 diabetes mellitus
TIB	time in bed
Tfam	mitochondrial transcription factor A

include modifications to the regulation of hormone secretion [8], misalignment of the molecular clock (i.e., circadian misalignment) [9], and disruptions to cellular signaling processes (such as those involved in the insulin signaling pathway and the pathways associated with mitochondrial respiratory function) [10,11]. If sleep loss affects signaling pathways related to mitochondrial respiratory function, then this has important implications as mitochondrial dysfunction has been associated with increased insulin resistance and T2DM [12,13]. The gold standard for the assessment of mitochondrial respiratory function is the measurement of oxygen utilization in isolated mitochondria or permeabilized tissue, and indicates the ability of mitochondria to generate adenosine triphosphate (ATP) via oxidative phosphorylation [14]. Using this method, it has been reported that mitochondrial respiratory function is significantly reduced in the skeletal muscle of T2DM patients compared with obese non-diabetic subjects [13]. Furthermore, this decrease in mitochondrial respiratory function was negatively associated with HbA1c results (glycated hemoglobin e a test that reflects blood glucose levels of the previous 3 mo) [13]. However, the effects of sleep loss on mitochondrial function are not well characterized.

Many of the aforementioned mechanisms that are potentially disrupted with sleep loss, particularly insulin signaling and mitochondrial function, share similar biochemical signaling pathways to those connected with exercise (an area of research that is comparably well studied). This suggests exercise as a potential intervention to counteract some of the negative effects associated with sleep loss. For example, single [15] and multiple [16] bouts of exercise induce a cascade of cellular signaling events that lead to improvements in mitochondrial function and insulin sensitivity [17]. Exercise has long been considered the cornerstone for the treatment and prevention of T2DM, with reports that those who regularly engage in moderate physical activity (even brisk walking) have approximately a 30% lower risk of developing T2DM compared to sedentary individuals [18]. However, limited research has investigated the ability of exercise to counteract the negative effects of sleep loss on glucose tolerance and insulin resistance [19]. Nevertheless, it is plausible that by increasing basal levels of exercise some of the detrimental effects associated with sleep loss might be mitigated.

The aim of this theoretical review is to draw on the available literature and propose exercise as a viable strategy to alleviate the negative effects of sleep loss. In doing so we contextualize the effect of reduced sleep duration and quality on insulin resistance, glucose tolerance, and T2DM, along with the potential underlying mechanisms, while demonstrating how and why engaging in regular exercise may act as a therapeutic intervention to mitigate sleep loss induced insulin resistance and T2DM.

Sleep loss and health

The U.S.-based National Sleep Foundation recommends adults between the age of 18 and 64 y sleep 7e9 h each night [7]. Despite this approximately 70 million Americans (30% of the population) sleep less than 6 h per night e significantly less per night than that reported in 1985 (22%), based on a large representative sample of the U.S. adult population. This reduction in sleep has been attributed to a range of lifestyle factors and work pressures, including shift work, increased work demands, changing social pressures and roles, increased travel and the consequent jet lag, sleep disorders, and stress [20]. Changes in sleep habits have important consequences and have been linked to increased absenteeism, disability, industrial and motor vehicle accidents, increased alcohol consumption, risk of cardiovascular disease, and possibly increased mental health issues [21,22].

Recent epidemiological and laboratory-based studies have also associated sleep loss with a range of chronic health concerns, particularly in relation to insulin resistance and T2DM [4,21,23]. In humans, a 40% reduction in glucose tolerance and a 30% reduction in the acute insulin response following a glucose tolerance test (measurement of glucose and insulin levels following oral glucose administration) has been reported after six nights of 4 h of time in bed (TIB) per night [8]. More recently, it was reported that just one night of 4 h of sleep time reduces insulin sensitivity [24e26], with many other studies consistently reporting sleep loss induced insulin resistance [10,24e28]. Thus, evidence from both epidemiological studies and intervention studies support a key role for sleep in the regulation of glucose tolerance and insulin resistance.

Sleep loss, circadian misalignment, and mitochondrial function

One proposed mechanism contributing to sleep loss induced changes in glucose tolerance and insulin resistance is circadian misalignment, a condition in which the exogenous behavioral sleep/wake schedule and feeding schedule are not aligned with endogenously generated circadian rhythms [29]. These circadian rhythms include changes in hormone secretion, body temperature, heart rate, muscle tone, and substrate utilization/metabolism, which persist throughout a 24-h period under constant conditions (i.e., without environmental/exogenous time cues) [30,31]. Circadian rhythms are regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, and the molecular clocks of peripheral tissues (such as skeletal muscle) acting synchronously as biological time- keepers. Altering the sleep/wake cycle (i.e., reduced sleep duration) may also independently alter the central and peripheral molecular clocks of human skeletal muscle [9] such that they become desynchronized with exogenous stimuli e a notion supported by rodent studies [32,33]. As over 800 genes are regulated in a circa- dian manner within skeletal muscle of mice [34], sleep loss induced disruption of the molecular clocks that regulate the expression of these genes may have far-reaching effects, particularly in regard to normal cellular function and health. As such, the misalignment of both the central and peripheral clocks by exogenous stimuli has been proposed as another potential mechanism contributing to sleep loss induced insulin resistance and impaired glucose tolerance [9,29,35,36].

Shift workers who undertake variable rotating day and night shifts have an increased risk of circadian misalignment [37]. These workers experience reduced sleep durations, a constantly changing light/dark cycle, altered sleep/wake patterns, and mixed feeding patterns, which together can contribute to circadian misalignment [37,38]. Of significance, these changes in daily behaviors are also associated with a 40% increase in the risk of developing diabetes [4]. Indeed, a longitudinal study (conducted between 1991 and 2001) of Japanese alternating shift workers concluded that, compared to day shift workers, alternating shift work was an independent risk factor for the onset of T2DM [39]. Importantly, in both human and mouse models, exercise has been shown to be capable of causing a phase shift (i.e., advance or delay the circadian phase depending on the time of day that the exercise occurs) [38,40], and has thus been suggested as a potential intervention to help realign circadian and diurnal rhythms in those with sleep issues [40]. Linked to phase shifts of endogenously-regulated circadian rhythms, exercise has also been shown to cause shifts of the peripheral molecular clock in skeletal muscle (both rodent and human), thus highlighting the potential role for exercise to realign disrupted metabolic rhythms to their optimal state [38,41].

Emerging evidence demonstrates that skeletal muscle function, and specifically that of mitochondrial respiration, also fluctuates rhythmically throughout the day. When mitochondrial respiratory function was measured in human skeletal muscle across a 24-h period, ADP-stimulated mitochondrial respiration oscillated in a "robust day/night rhythm", with a difference of approximately 20% between the highest (11 PM) and lowest (1 PM) values [42]. In mice, the expression of key rate-limiting mitochondrial enzymes (i.e., pyruvate dehydrogenase and carnitine palmitoyl transferase) and ~38% of the mitochondrial proteome, which are linked to the regulation of glucose tolerance and insulin resistance, also display a diurnal rhythm [43]. Similar to circadian rhythms, a diurnal rhythm follows a distinct 24-h cycle, but is synchronized to exogenous stimuli such as day and night and other factors such as the timing of meals [44]. Therefore, it is possible that misalignment of these diurnal rhythms is detrimental to mitochondrial function, which would have further implications for glucose tolerance and insulin resistance.

As mitochondrial function is regulated in a diurnal manner, and sleep loss can lead to circadian misalignment, it is plausible that sleep loss may alter mitochondrial function e even if this remains to be fully elucidated in well-controlled laboratory studies. It has been reported that 120 h of sustained wakefulness (sleep loss) reduces the activity of citrate synthase (CS) (24%), malate dehydrogenase (35%), and glycerol-3-phosphate dehydrogenase (17%) in human skeletal muscle [11], collectively suggesting a decreased functional capacity of the mitochondria. More recently, 72-h of sleep deprivation was associated with reduced mitochondrial respiratory function in the hypothalamus of rats [45]. Whilst these studies indicate that mitochondrial function may be directly reduced as a consequence of severe sleep loss, these initial findings have not been characterized in models that replicate the sleep loss encountered by humans. Nonetheless, the implications are significant considering the proposed contribution of mitochondrial dysfunction to the development of insulin resistance and T2DM [12,13]. It may therefore be hypothesized that chronic sleep loss, such as that experienced by at least 30% of the American population, leads to defects in mitochondrial function and a consequent increase in the risk of developing insulin resistance and T2DM [46]. Strategies to improve mitochondrial function, such as exercise [14], might be useful to combat some of the negative consequences of sleep loss.

Mechanistic pathways connecting sleep loss, misalignment of circadian rhythms, and the potential benefits of exercise

At a molecular level, circadian rhythms are controlled by a number of genes e collectively known as Clock genes. A tran-scriptional:translational feedback loop, which includes the core Clock genes brain and muscle Arnt-like protein-1 (Bmal1), Clock and RAR-related orphan receptor a (Ror-a) (often referred to as the activators of the feedback loop), and period 1,2 and 3 (Per 1e3), cryptochrome 1/2 (Cry 1/2) and NR1D1; Nuclear receptor subfamily 1, group D, member 1 (Rev-erb-a) (considered the repressors of the feedback loop), together coordinate circadian rhythmicity and metabolism at a cellular level via their ability to regulate the transcriptional activity of a host of other genes (clock controlled genes e CCGs) (see [47] for review). Of significant note, the messenger ribonucleic acid (mRNA) expression in human skeletal muscle of two of these clock genes (Bmal1 and Cry1) is decreased following a night of sleep loss [9].

Central to metabolic health and circadian regulation is the energy sensor, AMP-activated protein kinase (AMPK). AMPK is activated upon changes in the AMP:ATP ratio, is important in the regulation of mitochondrial biogenesis, and interacts closely with clock genes. Activation of AMPK causes the phosphorylation and destabilization of Cry1, leading to de-repression of the BMAL1:- CLOCK complex [48]. Moreover, Rev-erb-a (a regulator of the clock gene transcriptional feedback loop and mitochondrial biogenesis) [49] regulates AMPK [50] via liver kinase B1 (LKB1) e a protein responsible for upstream regulation of AMPK. It has also been suggested that clock genes within skeletal muscle (i.e., Per1, Cry2) are regulated by AMPKg3 [51] e an AMPK isoform specific to skeletal muscle and essential for the regulation of glucose tolerance and insulin sensitivity [52]. Exercise also activates and phosphor- ylates AMPK [15], which subsequently plays a role in GLUT4 (glucose transporter 4) mediated skeletal muscle glucose uptake [53]. Furthermore, muscle-specific Bmal1 knockout mice have decreased skeletal muscle glucose uptake through decreased GLUT4 translocation [36], which may be due to reduced expression of Tbc1d1 e a Rab-GTPase member involved in the GLUT4

translocation process and a potential target of the CLOCK:BMAL1 complex [54]. Together, this suggests exercise-induced AMPK activation might help counteract impairments in insulin resistance and glucose tolerance that may be associated with abnormalities in clock gene expression.

Downstream of AMPK is the transcriptional co-activator peroxisome proliferator-activated receptor g (PGC-1a) [55], which is also upregulated in response to exercise [15]. PGC-1a is often referred to as the 'master regulator' of mitochondrial biogenesis, due to its role in the transcriptional co-activation of a number of transcription factors (nuclear respiratory factor 1/2 (NRF 1/2), mitochondrial transcription factor A (Tfam) and peroxisome proliferator-activated receptor (PPARs)) involved in the regulation of mitochondrial respiratory function and content [16]. Importantly, PGC-1a also regulates the expression of, and is in itself regulated by interactions with, members of the clock gene family (as demonstrated in a number of rodent studies) [35,56,57]. For example, PGC-1a knockout mice models demonstrate reduced clock gene expression, while Bmal1 knockout mice models and mice with a mutated CLOCK protein (truncated $CLOCK^{D19}$ protein) display reduced PGC-1a protein content [35,56,57]. Decreases in PGC-1a protein content may contribute to the reductions in mitochondrial content and respiration that are observed in Bmal1 knockout mice and $CLOCK^{D19}$ mutant mice [35,57]. These same clock gene mutant mice have also been shown to have reduced insulin sensitivity, and the early development of T2DM [47,58e60], which may be explained in part by the close association between mitochondrial dysfunction and the development of insulin resistance and T2DM [12,13,46,61]. These findings suggest that reductions in the protein content and expression of clock genes and PGC-1a may help to explain the reported effects of sleep loss on insulin sensitivity and T2DM. Importantly, both single and multiple bouts of exercise increase PGC-1a expression [15,62] and mitochondrial function [63,64], with subsequent improvements in insulin sensitivity [17]. Thus, there is emerging evidence suggesting exercise might be a viable strategy to counteract the effects of sleep loss on clock gene expression, PGC-1a expression, reduced mitochondrial function, and subsequently increased insulin resistance.

While the role of PGC-1a as a key metabolic regulator is well established, it is also important to note its role may be determined by factors such as tissue specificity and its level of expression [65,66]. Hepatic overexpression of PGC-1a in mice induces hepatic insulin resistance, potentially via increased stimulation of gluconeogenesis, leading to hyperglycemia [66]. However, increased expression of PGC-1a within physiological levels (as seen with exercise), improves mitochondrial content, GLUT4 expression, and insulin sensitivity in skeletal muscle [65].

Another key regulator of mitochondrial function, which can also be classified as a clock-controlled gene, is SIRT3 (a member of the sirtuin family) [67]. SIRT3 is a mitochondrial protein that controls the acetylation levels of key functional oxidative metabolism and fatty acid oxidation proteins (i.e., long-chain acyl dehydrogenase), and thus can influence the overall respiratory function of the mitochondria [67]. Knockout of SIRT3 in mice causes a reduction in the expression of mitochondria related genes that are induced by PGC-1a [68]. Using liver-specific Bmal1 knockout mice, it was re- ported that mitochondrial respiration in the liver could be influenced in a circadian manner via clock gene dependent regulation of nicotinamide adenine dinucleotide ($NAD^{þ}$ e a mitochondrial co-enzyme), which in turn controls the deacetylase activity of SIRT3 [67]. Another member of the sirtuin family, SIRT1 e a regulator of insulin sensitivity in mouse skeletal muscle, also appears to be activated by CLOCK and BMAL1, with mutation and knockout of these clock proteins, respectively, leading to the induction of insulin resistance [69]. This indicates the dysregulation of clock genes, suggested to occur in response to sleep loss [9], may also lead to increased insulin resistance via a reduced content of SIRT proteins.

Of note, and similar to both AMPK and PGC-1a, it has been shown that SIRT1 and SIRT3 can be upregulated by exercise in the skeletal muscle of both animals [70,71] and humans [72]. Elevated SIRT1 activity has been shown to be important in the subsequent deacetylation and induction of PGC-1a transcriptional activity [73]. Together, this demonstrates that both clock genes and exercise influence many regulators of mitochondrial biogenesis. This might help to explain why there is a diurnal rhythm to mitochondrial function and enzyme activity, why these may be affected by sleep loss, and equally why exercise may play a role in mitigating sleep loss induced insulin resistance. The connection between clock genes, AMPK, PGC-1a, SIRT1 and SIRT3 is summarized in Fig. 1.

Exercise increases mitochondrial signaling and clock gene expression

Exercise induced increases in the activation of AMPK, the protein content of PGC-1a, and activity of SIRT1 and SIRT3, are important signaling events that coordinate the expression of mitochondria-related genes (leading to increased mitochondrial content and function) as well as improvements in insulin sensitivity [74,75]. Among the genes that PGC-1a increases the expression of, are the mitochondrial enzymes that have been reported to have diminished activity following sleep loss in humans. For example, CS activity (a key enzyme of the citric acid cycle and a marker of mitochondrial content) is significantly reduced in human skeletal muscle following 120 h of sustained wakefulness [11]. Importantly, skeletal muscle CS mRNA and PGC-1a mRNA is increased following a single bout of high-intensity interval exercise (HIIE), with subsequent increases in CS activity and PGC-1a content [16]. Moreover, activity of the same complexes in the electron transport system that are reduced following sleep deprivation in rats [45] can be improved via exercise training [76]. Therefore, exercise can promote increased activity and content of mitochondrial enzymes that are reduced following sleep loss and which may lead to insulin resistance and T2DM.

Despite our increased understanding of mitochondrial signaling, and models of sleep disruption and circadian misalignment, there are a number of additional exercise-induced signaling pathways associated with improved mitochondrial function that have yet to be investigated in response to sleep loss. In humans muscle contraction leads to the activation of kinases such as calmodulin- dependent protein kinase (CAMK) and p38 mitogen activated protein kinase (p38MAPK) (in addition to AMPK), via changes in calcium and reactive oxygen species, respectively, which converge on myocyte enhancer factor 2 (MEF-2), activating transcription factor 2 (ATF2) and cyclic-AMP response element binding protein (CREB), and exert their influence on enhancing PGC-1a promoter activity [77]. In addition, Perry et al. [16] demonstrated that exercise training modulates mitochondrial fission and fusion proteins important for regulating mitochondrial integrity and turnover. Moreover, we have recently reported in human skeletal muscle that single [15] and multiple [62] bouts of exercise increase phosphorylation and protein levels of the tumor suppressor protein, p53. Ablation of p53 content in mice results in reduced mitochondrial respiration, lowered PGC-1a content and decreased exercise capacity [78], whilst p53 has also been shown to regulate insulin resistance [79]. Collectively, these pathways represent varying avenues for future research in the context of sleep loss, exercise, mitochondrial function and insulin resistance.

The detrimental mitochondrial characteristics previously demonstrated in clock gene knockout mice can also be somewhat ameliorated by exercise [57]. Following an 8-wk exercise training

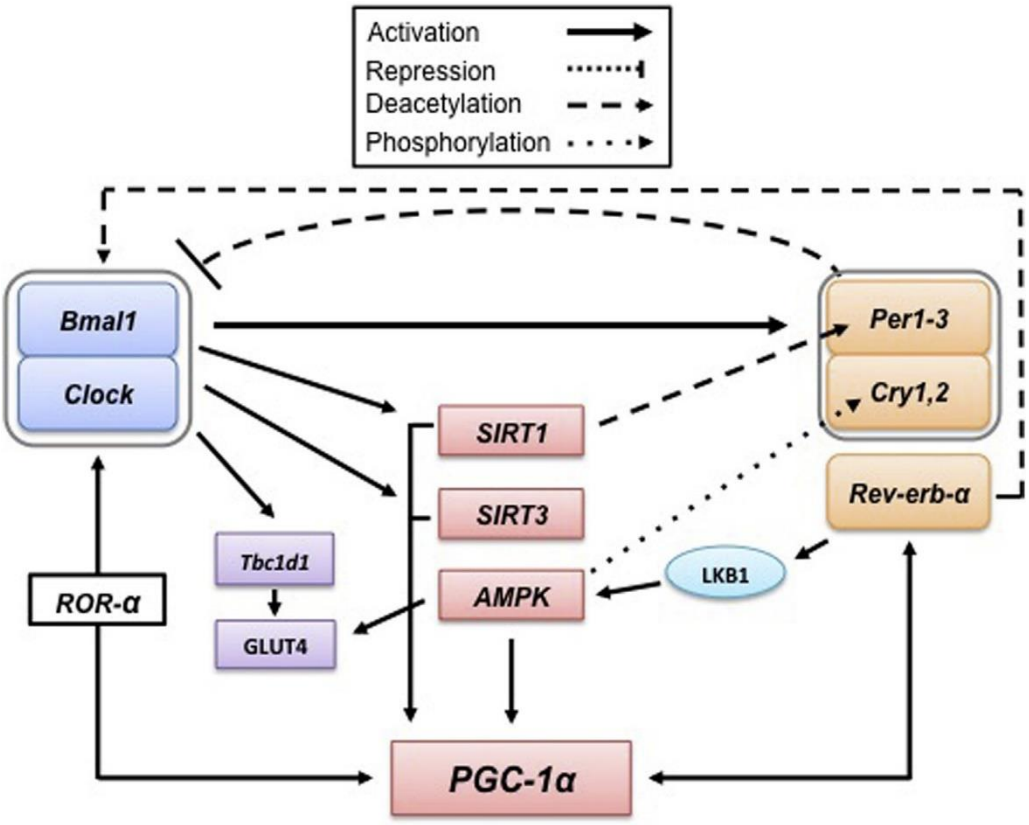

Fig. 1. Interplay between peripheral molecular clock genes, AMPK, SIRT1, SIRT3 and PGC-1a. BMAL1 and CLOCK activate Per1e3 and Cry1,2. PER1-3 and CRY1,2 transcriptionally repress the action of Bmal1 and Clock. AMPK phosphorylates CRY1,2 leading to less repression of Bmal1 and Clock, whilst PGC-1a transcriptionally regulates and is itself regulated by Rev-erb-a. Rev-erb-a also represses the effects of BMAL1. PGC-1a regulates Clock through a ROR-a dependent pathway and is regulated also by the CLOCK:BMAL1 complex. CLOCK:BMAL1 can activate both SIRT1 and SIRT3 whereby SIRT1 deacetylates Per2, whilst SIRT1 and SIRT3 upregulates transcription of PGC-1a. Furthermore, BMAL1:CLOCK directly exerts its influence on Tbc1d1 which is required for GLUT4 translocation. AMPK also regulates GLUT4 translocation to the plasma membrane. Rev-erb-a activates AMPK via LKB-1, which directly upregulates transcription of PGC-1a. Abbreviations: AMPK, AMP-activated protein kinase; BMAL1, brain and muscle Arnt-like protein-1; CLOCK, circadian locomotor output cycles kaput; CRY 1,2, crytochrome 1 and 2; GLUT4, glucose transporter 4; LKB-1, liver kinase B1; Per1 -3, period 1,2 and 3; PGC-1a, peroxisome proliferator- activated receptor g co-activator 1a; ROR-a, RAR-related orphan receptor a; SIRT11/3, sirtuin 1/3.

program, mice with mutated CLOCK^{D19} proteins were able to restore PGC-1a protein content to that of trained wild-type mice [57]. This was accompanied by similar increases in cytochrome c oxidase subunit 1 (COX-I) activity (another marker of mitochondrial content [80]). Moreover, a single bout of resistance exercise is associated with increased expression of Per2, Cry1 and Bmal1 in human skeletal muscle, compared with resting muscle [41]. Together, these associations between clock controlled genes, PGC-
1a, and mitochondrial dysfunction [35,56] provide a theoretical mechanism by which exercise-induced contractile activity may play a role in ameliorating sleep loss induced disruptions of clock genes following a night of sleep loss [9] and hence mitigate consequent impairments in mitochondrial function and insulin sensitivity. That said, future research should focus on uncovering the specific relationships and underpinning mechanisms between sleep loss, changes in skeletal muscle mitochondrial function, and exercise. A schematic overview summarizing the hypothesized connection between sleep, exercise and insulin resistance is shown in Fig. 2.

The role of sleep on muscle mass and insulin resistance

As skeletal muscle is the major site for glucose disposal, it is conceivable that maintenance of muscle mass and function is also important for maintaining glucose tolerance and insulin sensitivity. In this context, it has been reported that sleep loss can lead to a decrease in muscle mass e likely due to an increased catabolic and reduced anabolic hormone profile [81e83]. Moreover, while on a calorie restricted diet, curtailing sleep opportunities to 5.5 h a night for 14 consecutive nights increased the fraction of muscle mass lost compared to participants who had an 8.5 h sleep opportunity each night [84]. Together, this marks another possible convergence point at which sleep and exercise may interact.

While inconclusive thus far, evidence from animal models used to investigate muscle atrophy and sarcopenia (a progressive loss of muscle mass, quality and function, associated with aging [85]) point towards the disruption of clock genes as a contributing mechanism. For example, Bmal1 knockout mice display features associated with advanced aging and sarcopenia, including significantly reduced strength and altered myofilament structure [35,86]. This suggests that the molecular clock is necessary for the maintenance of skeletal muscle function and phenotype. Another mechanism thought important in the regulation of muscle mass is inflammation. Indeed, lifestyle factors such as sedentary behaviors and sleep loss, as well as obesity and T2DM, induce dramatic in- creases in pro-inflammatory signaling [87], which may also be implicated in muscle atrophy [88].

Strategies that can regulate the molecular clock, such as resistance-based exercise interventions, have also been shown to increase muscle mass and to protect against sarcopenia [89]. Furthermore, exercise has been shown to have anti-inflammatory properties [90], providing a potential additional mechanism to

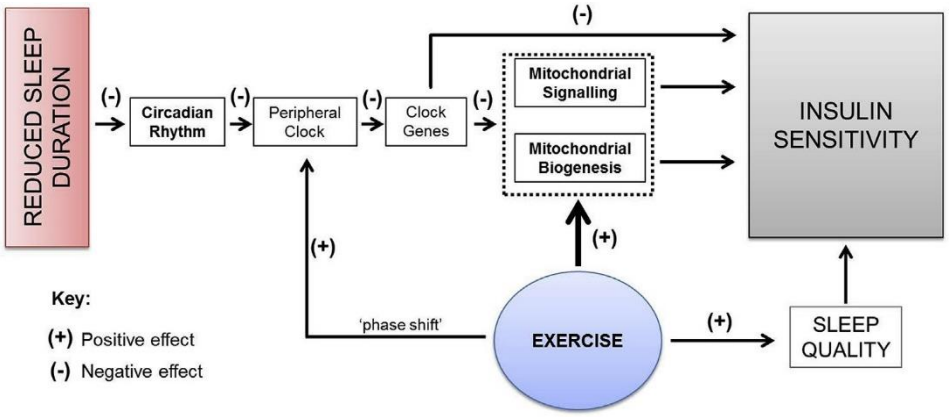

Fig. 2. One of the main proposed models, based on current literature, of the impact of sleep loss with or without exercise on mitochondrial signaling, mitochondrial biogenesis, and insulin sensitivity. Reduced sleep duration disrupts circadian rhythm and alters molecular clock gene expression, which subsequently impacts on mitochondrial signaling events associated with mitochondrial biogenesis and results in a reduction in insulin sensitivity. Conversely, exercise activates the mitochondrial signaling events associated with mitochondrial biogenesis and, therefore, may be a viable strategy to mitigate sleep loss induced reductions in insulin sensitivity. Furthermore, exercise may induce a phase shift and thus a 'resyncing' of the peripheral clock. While reduced sleep quality also induces reductions in insulin sensitivity, exercise induced improvements in sleep quality may mitigate these responses.

counteract sleep loss induced muscle loss. Importantly, it has been shown in rats that resistance exercise training performed prior to sleep loss attenuates muscle atrophy [82], providing preliminary evidence of the role contractile activity can play in protecting against sleep loss induced muscle mass loss. Whether it also helps reduce the development of insulin resistance, by maintaining skeletal muscle functionality, remains to be determined. Nonetheless, considering the increased prevalence of sarcopenia in T2DM patients [91,92], this appears an important area of emerging research.

Sleep quality, insulin sensitivity and exercise

In addition to sleep loss, sleep quality may also be a critical factor influencing the regulation of insulin sensitivity [93]. It was recently reported that, like sleep loss, reduced sleep quality is associated with a similar increase in the risk of T2DM as physical inactivity (~40%) [4]. Additionally, patients with sleep disorders, such as obstructive sleep apnea (OSA), in which sleep quality is significantly reduced, have an increased risk of insulin resistance [4]. Sleep quality can be determined via polysomnography, which analyzes multiple physiological parameters relevant to sleep. Specifically, the time spent in different stages of sleep can be quantified (i.e., non-rapid eye movement (nREM, stages 1e3) and rapid eye movement (REM)) [94]. A reduction in the time spent in slow wave sleep (SWS, nREM stage 3) (i.e., restorative sleep) is also associated with increased insulin resistance [93,95]. Indeed, when SWS is disturbed, but the overall time spent asleep is not reduced, there are increases in insulin resistance of between 20 and 25%. However, a night of REM sleep disturbance did not produce similar re- ductions in insulin sensitivity, suggesting that SWS rather than REM sleep may play a role in the regulation of insulin sensitivity [95]. Taken together with the fact that there is no association be- tween sleep stages and fasting insulin resistance following one night of sleep restricted sleep [25], more research is warranted to determine the role of REM and SWS on the regulation of glucose tolerance and insulin resistance.

Given that sleep quality is an important determinant of glucose tolerance and insulin sensitivity [23,93], improving sleep quality by means of regular engagement in exercise could also prove beneficial in the treatment and prevention of insulin resistance and T2DM. There are a number of strategies for improving sleep quality (e.g., sleep hygiene practices such as caffeine restriction and reducing the use of electronic devices prior to bed). However, engaging in physical activity and exercise (both acutely and chronically), as alternative or complementary approaches, may be beneficial [96,97]. In a comparison of older adults who were assigned to either a moderate-intensity exercise program or a health education control program over a 12-month period, an exercise program was shown to improve aspects of sleep quality [98]. Indeed, decreases in nREM stage 1 sleep (considered a transitional state from wake to sleep), increases in nREM stage 2 sleep (considered more stable sleep than stage 1) and reduced sleep disturbances (as assessed via polysomnography) were reported, which coincided with improved self-reported parameters of sleep quality following the exercise program. Moreover, exercise is currently prescribed, and has been shown to be effective in improving sleep quality, for a variety of clinical sleep disorders, including insomnia and obstructive sleep apnea, in which sleep quality is typically reduced [99,100]. However, further research is required to determine whether, in addition to metabolic function, exercise-induced improvements in sleep quality (regardless of duration) can help to maintain glucose tolerance and insulin resistance.

Conclusion

To date, sleep and exercise in the context of human health have remained relatively independent lines of research. How- ever, with the emergence of sleep loss as a significant risk factor for the development of insulin resistance and T2DM, engagement in exercise to mitigate these risks may offer an alternative solution. Indeed, this review presents and uncovers a framework and mechanistic underpinning of how exercise can be of significant benefit to counteract the mechanisms by which sleep loss might increase the risk of impaired mitochondrial function and the subsequent development of insulin resistance and T2DM.

The critical next step is to perform well controlled experiments that reveal the specific mechanisms by which sleep loss contributes to the development of insulin resistance and reductions in glucose tolerance. Such findings will allow for more targeted interventions aimed at improving human health, whether that is obtaining more and a better quality of sleep and/or increasing basal levels of exercise. Furthermore, this research may help to

Practice points

Reduced sleep duration and quality is associated with an increased risk for the development of insulin resistance and T2DM.

Sleep loss leads to disruptions in circadian rhythm and the expression of skeletal muscle clock genes, which negatively influences mitochondrial content and function.

Mitochondrial dysfunction is associated with the development of insulin resistance and T2DM, suggesting a possible relationship between sleep loss, changes in mitochondrial function, and the development of insulin resistance.

Exercise induces a cascade of signaling events associated with mitochondrial biogenesis, and may thus alleviate sleep loss induced mitochondrial dysfunction, insulin resistance and impaired glucose tolerance.

Exercise may improve sleep quality, which could improve insulin sensitivity and reduce the risk of development of T2DM.

Research agenda

To uncover the relationships and underpinning mechanisms associated with sleep loss, skeletal muscle mitochondrial function, and insulin sensitivity.

The role of exercise (single bouts and chronic exercise training) in mitigating sleep loss induced reductions in whole-body metabolic health and skeletal muscle function remains to be determined.

To investigate how sleep and exercise influence the synchronicity between central and peripheral clocks and the subsequent effects on metabolic health.

Further research is needed to determine the most time efficient approach to improve insulin sensitivity and glucose tolerance in the context of sleep and exercise.

inform which exercise modalities might be best to counteract the detrimental effects of sleep loss. To this end, it is particularly important for future research to elucidate whether an emphasis should be placed on improving sleep duration and quality, or whether it would be more time efficient (in the context of improving human health) to focus attention on increasing habitual exercise in the face of reduced sleep, so as to counteract the detrimental impact of sleep loss.

Conflict of interest

The authors do not have any conflicts of interest to disclose.

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* The most important references are denoted by an asterisk.

References

[1] World Health Organization. Life in the 21st century e a vision for all. 1998. Geneva, Switzerland.

[2] Centers for Disease Control and Prevention. National diabetes statistics report: estimates of diabetes and its burden in the United States. Atlanta, GA: US Department of Health and Human Services; 2014. Report No.

[3] National Collaborating Centre for Chronic Conditions. Type 2 diabetes: national clinical guideline for management in primary and secondary care (update) London. 2008.

*[4] Anothaisintawee T, Reutrakul S, Van Cauter E, Thakkinstian A. Sleep disturbances compared to traditional risk factors for diabetes development: systematic review and meta-analysis. Sleep Med Rev 2015;30:11e24.

[5] Akerstedt T. Shift work and disturbed sleep/wakefulness. Occup Med Lond 2003;53(2):89e94.

[6] Ford ES, Cunningham TJ, Croft JB. Trends in self-reported sleep duration among US adults from 1985 to 2012. Sleep 2015;38(5):829e32.

[7] National sleep foundation. International bedroom poll. 2013 [cited 2016 13 January]. Available from: https://sleepfoundation.org/sites/default/files/RPT495a.pdf.

*[8] Spiegel K, Leproult R, Van Cauter E. Impact of sleep debt on metabolic and endocrine function. Lancet 1999;354(9188):1435e9.

*[9] Cedernaes J, Osler ME, Voisin S, Broman JE, Vogel H, Dickson SL, et al.Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men. J Clin Endocrinol Metab 2015;100(9):E1255e61. JC20152284.

[10] Broussard JL, Ehrmann DA, Van Cauter E, Tasali E, Brady MJ. Impaired insulin signaling in human adipocytes after experimental sleep restric- tion: a randomized, crossover study. Ann Intern Med 2012;157(8): 549e57.

[11] Vondra K, Brodan V, Bass A, Kuhn E, Teisinger J, Andel M, et al. Effects of sleep-deprivation on the activity of selected metabolic enzymes in skeletal-muscle. Eur J Appl Physiol Occup Physiol 1981;47(1):41e6.

[12] Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. Science 2005;307(5708):384e7.

*[13] Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H, et al. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 2007;56(6):1592e9.

[14] Bishop DJ, Granata C, Eynon N. Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content? Biochim Biophys Acta 2014;1840(4):1266e75.

[15] Bartlett JD, Hwa Joo C, Jeong TS, Louhelainen J, Cochran AJ, Gibala MJ, et al. Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphory- lation in human skeletal muscle. J Appl Physiol (1985) 2012;112(7): 1135e43.

[16] Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol-Lond 2010;588(23):4795e810.

[17] Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paral- leled by increased myocellular fat storage and improved insulin sensi- tivity. Diabetes 2010;59(3):572e9.

[18] Jeon CY, Lokken RP, Hu FB, van Dam RM. Physical activity of moderate intensity and risk of type 2 diabetes: a systematic review. Diabetes Care 2007;30(3):744e52.

[19] VanHelder T, Symons JD, Radomski MW. Effects of sleep deprivation and exercise on glucose tolerance. Aviat Space Environ Med 1993;64(6): 487e92.

[20] Sleep Health Foundation. Common reasons why people don't get enough sleep. 2011. Available from: http://www.sleephealthfoundation.org.au/files/pdfs/Common-Causes-Inadequate-Sleep.pdf.

[21] Banks S, Dinges DF. Behavioral and physiological consequences of sleep restriction. J Clin Sleep Med 2007;3(5):519e28.

[22] Perlis ML, Grandner MA, Chakravorty S, Bernert RA, Brown GK, Thase ME. Suicide and sleep: is it a bad thing to be awake when reason sleeps? Sleep Med Rev 2016;29:101e7.

[23] Cappuccio FP, D'Elia L, Strazzullo P, Miller MA. Quantity and quality of sleep and incidence of type 2 diabetes: a systematic review and meta-analysis. Diabetes Care 2010;33(2):414e20.

[24] Donga E, van Dijk M, van Dijk JG, Biermasz NR, Lammers GJ, van Kralingen KW, et al. A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects. J Clin Endocrinol Metab 2010;95(6):2963e8.

[25] Cedernaes J, Lampola L, Axelsson EK, Liethof L, Hassanzadeh S, Yeganeh A, et al. A single night of partial sleep loss impairs fasting insulin sensitivity but does not affect cephalic phase insulin release in young men. J Sleep Res 2015;25(1):5e10.

[26] van den Berg R, Mook-Kanamori DO, Donga E, van Dijk M, van Dijk JG, Lammers GJ, et al. A single night of sleep curtailment increases plasma acylcarnitines: novel insights in the relationship between sleep and in- sulin resistance. Arch Biochem Biophys 2016;589:145e51.

[27] Rao MN, Neylan TC, Grunfeld C, Mulligan K, Schambelan M, Schwarz JM. Subchronic sleep restriction causes tissue-specific insulin resistance. J Clin Endocrinol Metab 2015;100(4):1664e71.

[28] Robertson MD, Russell-Jones D, Umpleby AM, Dijk DJ. Effects of three weeks of mild sleep restriction implemented in the home environment on multiple metabolic and endocrine markers in healthy young men. Meta- bolism 2013;62(2):204e11.

[29] Leproult R, Holmback U, Van Cauter E. Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. Diabetes 2014;63(6):1860e9.

[30] Colten HR, Altevogt BM. Sleep disorders and sleep deprivation: an unmet public health problem. 2006.

[31] Dunlap JC, Loros JJ, DeCoursey PJ. Chronobiology: biological timekeeping. Sunderland, MA, US: Sinauer Associates Chronobiology; 2004.

[32] Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, et al. Resetting central and peripheral circadian oscillators in transgenic rats. Science 2000;288(5466):682e5.

[33] Antle MC, Mistlberger RE. Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster. J Neurosci 2000;20(24): 9326e32.

[34] McCarthy JJ, Andrews JL, McDearmon EL, Campbell KS, Barber BK, Miller BH, et al. Identification of the circadian transcriptome in adult mouse skeletal muscle. Physiol Genomics 2007;31(1):86e95.

*[35] Andrews JL, Zhang X, McCarthy JJ, McDearmon EL, Hornberger TA, Russell B, et al. CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. Proc Natl Acad Sci U. S. A 2010;107(44):19090e5.

*[36] Dyar KA, Ciciliot S, Wright LE, Bienso RS, Tagliazucchi GM, Patel VR, et al. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. Mol Metab 2014;3(1):29e41.

[37] Nagano M, Adachi A, Nakahama K, Nakamura T, Tamada M, Meyer-Bernstein E, et al. An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center. J Neurosci 2003;23(14):6141e51.

*[38] Wolff G, Esser KA. Scheduled exercise phase shifts the circadian clock in skeletal muscle. Med Sci Sports Exerc 2012;44(9):1663e70.

[39] Suwazono Y, Sakata K, Okubo Y, Harada H, Oishi M, Kobayashi E, et al. Long-term longitudinal study on the relationship between alternating shift work and the onset of diabetes mellitus in male Japanese workers. J Occup Environ Med 2006;48(5):455e61.

[40] Richardson CE, Gradisar M, Short MA, Lang C. Can exercise regulate the circadian system of adolescents? Novel implications for the treatment of delayed sleep-wake phase disorder. Sleep Med Rev 2016. pii: S1087-0792(16)30062-4.

*[41] Zambon AC, McDearmon EL, Salomonis N, Vranizan KM, Johansen KL, Adey D, et al. Time- and exercise-dependent gene regulation in human skeletal muscle. Genome Biol 2003;4(10):R61.

[42] van Moorsel D, Hansen J, Havekes B, Scheer FAJL, Joϵrgensen JA, Hoeks J, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. Mol Metab 2016;5(8):635e45.

[43] Neufeld-Cohen A, Robles MS, Aviram R, Manella G, Adamovich Y, Ladeuix B, et al. Circadian control of oscillations in mitochondrial rate-limiting enzymes and nutrient utilization by PERIOD proteins. Proc Natl Acad Sci U. S. A 2016;113(12):E1673e82.

[44] Scheer FA. Fundamentals of the circadian system. In: Basics of sleep guide [Internet]. Sleep Research Society; 2011.

[45] Andreazza AC, Andersen ML, Alvarenga TA, de-Oliveira MR, Armani F, Ruiz FS, et al. Impairment of the mitochondrial electron transport chain due to sleep deprivation in mice. J Psychiatr Res 2010;44(12):775e80.

[46] Koliaki C, Roden M. Alterations of mitochondrial function and insulin sensitivity in human obesity and diabetes mellitus. Annu Rev Nutr 2016;36:337e67.

*[47] Harfmann BD, Schroder EA, Esser KA. Circadian rhythms, the molecular clock, and skeletal muscle. J Biol Rhythms 2015;30(2):84e94.

[48] Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, Egan DF, et al. AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science 2009;326(5951):437e40.

[49] Woldt E, Sebti Y, Solt LA, Duhem C, Lancel S, Eeckhoute J, et al. Rev-erb-alpha modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. Nat Med 2013;19(8):1039e46.

[50] Duez H, Staels B. Rev-erb-alpha: an integrator of circadian rhythms and metabolism. J Appl Physiol (1985) 2009;107(6):1972e80.

[51] Vieira E, Nilsson EC, Nerstedt A, Ormestad M, Long YC, Garcia-Roves PM, et al. Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle. Am J Physiol Endocrinol Metab 2008;295(5):E1032e7.

[52] Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, et al. The 5'-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. J Biol Chem 2004;279(37):38441e7.

[53] Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 1998;47(8):1369e73.

[54] Kumaki Y, Ukai-Tadenuma M, Uno KD, Nishio J, Masumoto KH, Nagano M, et al. Analysis and synthesis of high-amplitude Cis-elements in the mammalian circadian clock. Proc Natl Acad Sci U. S. A 2008;105(39): 14946e51.

[55] Jager S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U. S. A 2007;104(29):12017e22.

[56] Liu C, Li S, Liu T, Borjigin J, Lin JD. Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. Nature 2007;447(7143):477e81.

*[57] Pastore S, Hood DA. Endurance training ameliorates the metabolic and performance characteristics of circadian Clock mutant mice. J Appl Physiol (1985) 2013;114(8):1076e84.

[58] Lefta M, Wolff G, Esser KA. Circadian rhythms, the molecular clock, and skeletal muscle. Curr Top Dev Biol 2011;96:231e71.

[59] Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, et al. Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005;308(5724):1043e5.

[60] Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, et al. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2004;2(11):e377.

[61] Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an update. Endocr Connect 2015;4(1):R1e15.

[62] Edge J, Mundel T, Pilegaard H, Hawke E, Leikis M, Lopez-Villalobos N, et al. Ammonium chloride ingestion attenuates exercise-induced mRNA levels in human muscle. PLoS One 2015;10(12):e0141317.

[63] Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ. Training intensity modulates changes in PGC-1alpha and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. FASEB J 2016;30(2):959e70.

[64] Bishop DJ, Thomas C, Moore-Morris T, Tonkonogi M, Sahlin K, Mercier J. Sodium bicarbonate ingestion prior to training improves mitochondrial adaptations in rats. Am J Physiol Endocrinol Metab 2010;299(2): E225e33.

[65] Benton CR, Wright DC, Bonen A. PGC-1alpha-mediated regulation of gene expression and metabolism: implications for nutrition and exercise prescriptions. Appl Physiol Nutr Metab 2008;33(5):843e62.

[66] Liang H, Balas B, Tantiwong P, Dube J, Goodpaster BH, O'Doherty RM, et al. Whole body overexpression of PGC-1alpha has opposite effects on hepatic and muscle insulin sensitivity. Am J Physiol Endocrinol Metab 2009;296(4):E945e54.

[67] Peek CB, Affinati AH, Ramsey KM, Kuo HY, Yu W, Sena LA, et al. Circadian clock NADþ cycle drives mitochondrial oxidative metabolism in mice. Science 2013;342(6158):1243417.

[68] Kong X, Wang R, Xue Y, Liu X, Zhang H, Chen Y, et al. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS One 2010;5(7):e11707.

[69] Liu J, Zhou B, Yan M, Huang R, Wang Y, He Z, et al. CLOCK and BMAL1 regulate muscle insulin sensitivity via SIRT1 in male mice. Endocrinology 2016;157(6):2259e69.

[70] Suwa M, Nakano H, Radak Z, Kumagai S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. Metabolism 2008;57(7): 986e98.

[71] Palacios OM, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward 3rd JL, et al. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. Aging Albany NY 2009;1(9):771e83.

[72] Gurd BJ, Perry CG, Heigenhauser GJ, Spriet LL, Bonen A. High-intensity interval training increases SIRT1 activity in human skeletal muscle. Appl Physiol Nutr Metab 2010;35(3):350e7.

[73] Gurd BJ. Deacetylation of PGC-1alpha by SIRT1: importance for skeletal muscle function and exercise-induced mitochondrial biogenesis. Appl Physiol Nutr Metab 2011;36(5):589e97.

[74] Leick L, Fentz J, Bienso RS, Knudsen JG, Jeppesen J, Kiens B, et al. PGC-1 {alpha} is required for AICAR-induced expression of GLUT4 and mitochondrial proteins in mouse skeletal muscle. Am J Physiol Endocrinol Metab 2010;299(3):E456e65.

[75] Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogen- esis. Cell 1998;92(6):829e39.

[76] Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ. Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. FASEB J 2016;30(10):3413e23.

[77] Egan B, Carson BP, Garcia-Roves PM, Chibalin AV, Sarsfield FM, Barron N, et al. Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with dif-

ferential activation of upstream signalling kinases in human skeletal muscle. J Physiol 2010;588(Pt 10):1779e90.

[78] Saleem A, Adhihetty PJ, Hood DA. Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. Physiol Genomics 2009;37(1):58e66.

[79] Kung CP, Murphy ME. The role of the p53 tumor suppressor in metabolism and diabetes. J Endocrinol 2016;231(2):R61e75.

[80] Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 2012;590(14):3349e60.

[81] Dattilo M, Antunes HK, Medeiros A, Monico-Neto M, Souza Hde S, Lee KS, et al. Paradoxical sleep deprivation induces muscle atrophy. Muscle Nerve 2012;45(3):431e3.

[82] Monico-Neto M, Antunes HK, Lee KS, Phillips SM, Giampa SQ, Souza Hde S, et al. Resistance training minimizes catabolic effects induced by sleep deprivation in rats. Appl Physiol Nutr Metab 2015;40(11):1143e50.

[83] Bell LN, Kilkus JM, Booth 3rd JN, Bromley LE, Imperial JG, Penev PD. Effects of sleep restriction on the human plasma metabolome. Physiol Behav 2013;122:25e31.

[84] Nedeltcheva AV, Kilkus JM, Imperial J, Schoeller DA, Penev PD. Insufficient sleep undermines dietary efforts to reduce adiposity. Ann Intern Med 2010;153(7):435e41.

[85] Mitchell WK, Williams J, Atherton P, Larvin M, Lund J, Narici M. Sarco- penia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review. Front Physiol 2012;3:260.

[86] Schroder EA, Harfmann BD, Zhang X, Srikuea R, England JH, Hodge BA, et al. Intrinsic muscle clock is necessary for musculoskeletal health. J Physiol 2015;593(24):5387e404.

[87] Mullington JM, Simpson NS, Meier-Ewert HK, Haack M. Sleep loss and inflammation. Best Pract Res Clin Endocrinol Metab 2010;24(5): 775e84.

[88] Haddad F, Zaldivar F, Cooper DM, Adams GR. IL-6-induced skeletal muscle atrophy. J Appl Physiol (1985) 2005;98(3):911e7.

[89] Taylor AH, Cable NT, Faulkner G, Hillsdon M, Narici M, Van Der Bij AK.Physical activity and older adults: a review of health benefits and the effectiveness of interventions. J Sports Sci 2004;22(8):703e25.

[90] Mathur N, Pedersen BK. Exercise as a mean to control low-grade systemic inflammation. Mediat Inflamm 2008;2008:109502.

[91] Kim TN, Park MS, Lim KI, Choi HY, Yang SJ, Yoo HJ, et al. Relationships between sarcopenic obesity and insulin resistance, inflammation, and vitamin D status: the Korean Sarcopenic Obesity Study. Clin EndocrinolOxf 2013;78(4):525e32.

[92] Lim S, Kim JH, Yoon JW, Kang SM, Choi SH, Park YJ, et al. Sarcopenic obesity: prevalence and association with metabolic syndrome in the Korean longitudinal study on health and aging (KLoSHA). Diabetes Care 2010;33(7):1652e4.

[93] Tasali E, Leproult R, Ehrmann DA, Van Cauter E. Slow-wave sleep and the risk of type 2 diabetes in humans. Proc Natl Acad Sci U. S. A 2008;105(3):1044e9.

[94] Moser D, Anderer P, Gruber G, Parapatics S, Loretz E, Boeck M, et al. Sleep classification according to AASM and Rechtschaffen & Kales: effects on sleep scoring parameters. Sleep 2009;32(2):139e49.

*[95] Herzog N, Jauch-Chara K, Hyzy F, Richter A, Friedrich A, Benedict C, et al. Selective slow wave sleep but not rapid eye movement sleep suppression impairs morning glucose tolerance in healthy men. Psychoneur- oendocrinology 2013;38(10):2075e82.

[96] Kredlow MA, Capozzoli MC, Hearon BA, Calkins AW, Otto MW. The effects of physical activity on sleep: a meta-analytic review. J Behav Med 2015;38(3):427e49.

[97] Yang PY, Ho KH, Chen HC, Chien MY. Exercise training improves sleep quality in middle-aged and older adults with sleep problems: a systematic review. J Physiother 2012;58(3):157e63.

[98] King AC, Oman RF, Brassington GS, Bliwise DL, Haskell WL. Moderate- intensity exercise and self-rated quality of sleep in older adults. A ran- domized controlled trial. JAMA 1997;277(1):32e7.

[99] Kubitz KA, Landers DM, Petruzzello SJ, Han M. The effects of acute and chronic exercise on sleep. A meta-analytic review. Sports Med 1996;21(4):277e91.

[100] Baron KG, Reid KJ, Zee PC. Exercise to improve sleep in insomnia: explo- ration of the bidirectional effects. J Clin Sleep Med 2013;9(8):819e24.