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Nutrient timing, metabolism, and health in humans

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The material presented here for examination for the award of a higher degree by research has not been incorporated into a submission for another degree.

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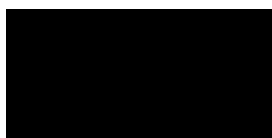
Declaration of authorship:

I am the author of this thesis, and the work described therein was carried out by myself personally, with the exception Chapter 4 in which I performed biochemical and statistical analysis of existing samples and unpublished data from a study conducted in 2014 (Chapters 4). Melatonin analysis was performed by Dr Jonathan Johnston (University of Surrey) (Chapters 4 & 5).

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Acknowledgements

Abstract

Biological rhythms in physiological and behavioural processes anticipate regular environmental changes and therefore adjust physiology and behaviour accordingly. These biological rhythms appear to facilitate the response of skeletal muscle to variable nutrient supply. The timing and/or distribution of nutrients across the day could conceivably alter biological rhythms in this tissue, which could be associated with metabolic health outcomes. However no human research to date has characterised temporal rhythms in *in vivo* human muscle alongside systemic markers of metabolism. Furthermore, recent studies have revealed links between the first meal of the day and human health, so it is now important to examine the potential underlying mechanisms of these relationships. The aim of this thesis was to take a multi-faceted approach to studying the metabolic and behavioural effects of meal timing, with focus on both absolute and relative timing. Initially, Chapter 4 provided 24-h characterisation of diurnal rhythms in human skeletal muscle gene expression alongside circulating metabolic and endocrine markers. Chapter 5 then built on this by demonstrating the ability of enteral feeding pattern (i.e., continuous versus bolus nutrient delivery) to affect rhythms in skeletal muscle and circulating metabolites – for example, revealing that systemic glucose and NEFA are primarily driven by 24 h profiles of insulin. Chapter 6 confirmed that next morning metabolic control is not affected by hourly sleep fragmentation but is affected when coffee is consumed to remedy a poor night of sleep. The acute experiment described in Chapter 7 demonstrated that enrichment of a typical carbohydrate-rich breakfast with whey protein effectively elicited the second-meal effect but did not differentially alter diet-induced thermogenesis or appetite above that of a carbohydrate-rich breakfast. Finally, Chapter 8 showed that regular daily consumption of that protein-enriched breakfast did not meaningfully alter the major components of energy balance, nor did it change postprandial metabolism or appetite relative either to regular daily consumption of a typical carbohydrate-rich breakfast or daily extended morning fasting. Collectively the research in this thesis highlights the effects of nutrient timing in both absolute terms (i.e., in relation to clock time) and relative terms (i.e., in relation to other daily events) – with implications for how the scheduling of daily meals and nutrients can affect the acute and chronic regulation of metabolism and behaviour.

Publications

Aspects of this thesis have been published in the following works:

Smith, HA. & Betts, JA. (2022). Nutrient Timing and Metabolic Regulation in Humans: Symposium Review from "Novel dietary approaches to appetite regulation, health and performance (2021)". *Journal of Physiology.* DOI: 10.1113/JP280756.

Smith, H.A. et al (2020). Glucose control upon waking is unaffected by hourly sleep fragmentation during the night but is impaired by morning caffeinated coffee. *British Journal of Nutrition.* DOI: 10.1017/S0007114520001865.

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List of Abbreviations

Abbreviation	Full Term
AgGRP	Agouti-related peptide
ADP	Adenosine Diphosphate
AEE	Activity Energy Expenditure
Akt	Serine/threonine kinase
AMPK	Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis of Variance
ARC	Arcuate nucleus
ATG	Autophagy related protein
ATP	Adenosine Triphosphate
AUC	Area Under Curve
BMAL1	Brain and Muscle ARNT-Like 1
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine and amphetamine regulated transcript
CCK	Cholecystokinin
CD36	Cluster of Differentiation 36
CHO	Carbohydrate
CI	Confidence Interval
CLOCK	Circadian Locomotor Output Cycles Kaput
CO_2	Carbon Dioxide
CPT1	Carnitine Palmitoyltransferase 1
CRB	Carbohydrate rich breakfast
CREB	cAMP Response Element Binding Protein
CR	Constant routine
CRY	Cryptochrome
CV	Coefficient of variation
DBP	D-Box Binding PAR BZIP Transcription Factor
DIT	Dietary induced thermogenesis
DLMO	Dim Light Melatonin Onset
DNA	Deoxyribonucleic Acid

EB	Energy balance
E-Box	Enhancer-box
EDTA	Ethyldiaminetetraacetic acid
EE	Energy expenditure
EI	Energy intake
EMF	Extended morning fast
FATPs	Fatty acid binding proteins
FD	Forced desynchrony
G6Pase	Glucose-6-phosphotase
GOAT	Ghrelin o acyl transferase
GLP-1	Glucagon-like peptide 1
GLUT4	Glucose transporter 4
HOMA-IR	Homeostatic model assessment for insulin resistance
HSL	Hormone sensitive lipase
LC3	Microtubule-associated protein 1A/1B-light chain 3
LPL	Lipoprotein lipase
mTORC1	Mammalian target of rapamycin 1
MYOD1	Myogenic differentiation 1
NcOR1-HDAC3	Nuclear receptor corepressor 1- histone deacetylase 3
NEFA	Non-esterified fatty acids
NPAS2	Neuronal PAS domain 2
NPY	Neuropeptide Y
NR1D1/2	Nuclear receptor subfamily 1 group D 1/2
OXM	Oxyntomodulin
PEPCK	Phosphoenolpyruvate carboxykinase
PER	Period
PGC1-a	Pparg coactivator 1 alpha
P13P	Phosphatidylinositol-3-phosphate
PKA	Protein Kinase A
PP	Pancreatic polypeptide
PPARG	Peroxisome proliferator-activated receptor gamma
PEB	Protein enriched breakfast
POMC	Pro-opiomelanocortin

PTMs	Post translational modifications
PYY	Peptide YY
REV-ERB	Nuclear receptor subfamily 1 group D member 1
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
ROR	Retinoic acid-related orphan receptor
SCN	Suprachiasmatic nucleus
SD	Standard deviation
TAG	Triacylglycerol
TBC1D	TBC1 domain family member
TCA	Tricarboxylic acid
TEE	Total energy expenditure
TRF	Time restricted feeding
TTFL	Transcriptional-Translational Feedback Loop
$\dot{V}CO_2$	Volume of carbon dioxide production
$\dot{V}O_2$	Volume of oxygen consumption
WAT	White adipose tissue

Chapter 1 – Introduction

Life on earth has evolved under a 24-hour cycle whereby factors such as light exposure predictably fluctuate within each solar day. In response, human beings have evolved biological rhythms to anticipate these regular environmental changes and therefore adjust physiology and behaviour accordingly (Jagannath *et al.*, 2017).

Daily variations in physiology are driven by an endogenous timing system, which is comprised of a "master" clock in the brain, as well as a network of clocks in major tissues throughout the body (Albrecht, 2017). Whereas the master clock is primarily regulated by light exposure, clocks in peripheral tissues respond and adapt to other external factors such as exercise, and sleep (Vollmers *et al.*, 2009; Tuvia *et al.*, 2021). Considering the role of peripheral tissues in facilitating the coordinated disposal, degradation, synthesis, and recycling of metabolic substrates it is highly plausible that nutrient timing is an important regulator of metabolism.

Nutrient timing refers to both the frequency and regularity of daily eating occasions, along with their timing both in absolute terms (i.e., time-of-day) and relative to when other inter-related daily events occur - or usually occur (e.g., light exposure, meals, sleep, exercise, *etc.*). Research in rodents has been invaluable in establishing the links between circadian rhythms, meal timing, and metabolism - yet fundamental physiological and behavioural differences between those animals and humans limits our ability to confidently translate those findings to public health guidelines regarding diet (Suarez *et al.*, 2004; Ellacott *et al.*, 2010; Gonzalez & Betts, 2018). Despite recent advances in our knowledge of meal timing, this area remains relatively under-studied compared to quantity and quality of diet. Consequently, there is an urgent need for more studies in this field, especially considering that current nutrition guidelines are disproportionately informed by epidemiological evidence, as well-controlled free-living RCTs are often difficult to complete so there are few available (Fabry *et al.*, 1964b; Betts *et al.*, 2014; Chowdhury *et al.*, 2016a).

Rhythms in skeletal muscle physiology represents a vital avenue of research as this tissue is responsible for a significant proportion (~40-85 %) of dietary glucose and fat disposal (DeFronzo *et al.*, 1981a; Ferrannini *et al.*, 1985; Meyer *et al.*, 2002; Ruge *et al.*, 2009a). Insulin is a vital hormone in regulating nutrient metabolism in both the

fasted- and fed-state, yet also holds the ability to entrain circadian clocks in peripheral tissues (Tuvia *et al.*, 2021). Likewise, the cellular recycling process of autophagy is another critical component of the skeletal muscle response to variable nutrient supply that varies across the day (Kim & Lee, 2014; Yin *et al.*, 2016). Despite this, it is remarkable that no studies have directly studied 24-h skeletal muscle metabolism, circulating markers of nutrient metabolism or tissue turnover in response to varying nutrient delivery pattern.

Within the last decade a series of randomised control trials have begun to provide insight regarding the relationship between breakfast and health (Betts *et al.*, 2016; Ruddick-Collins *et al.*, 2018; Ruddick-Collins *et al.*, 2022). Breakfast ingestion, relative to omission, acutely suppresses appetite and elicits the second-meal effect later the same day (Chowdhury *et al.*, 2015). More chronically, consumption of regular daily breakfast can result in higher morning physical activity levels than those who omit breakfast completely, which may be driven by a higher morning glycaemia after breakfast than when fasting. Moreover, of the majority of adults consuming breakfast on a daily basis, the morning meal in most human cultures is typically limited to a narrow range of carbohydrate-rich "breakfast foods". In particular, the first meal of the day tends to have the lowest protein content (NHANES, 2016). Increasing the protein content of breakfast takes advantage of the insulinogenic, satiating, and thermogenic properties of this macronutrient. Consequently, this provides a possible means through which the underlying biological mechanisms that link breakfast and metabolism may be targeted and exploited.

The studies described in this thesis attempt to further our knowledge of how nutrient timing influences metabolic regulation at the molecular, tissue, systemic, and whole-body level. This series of investigations begins with characterisation of hormonal and metabolite rhythms in the context of time series skeletal muscle gene expression under controlled diurnal conditions (Chapter 4). Specifically oral nutrition will be limited to waking hours only, such that participants undergo a typical cycle of fasting-feeding. This study will provide the first concurrent serial 24 h measures of human skeletal muscle metabolism alongside systemic markers of nutrient metabolism and tissue turnover. Chapter 5 will establish how feeding patterns influence the rhythms observed in Chapter 4 by continuous enteral feeding throughout a 24-h cycle (including during

the night/sleep phase). The additional contrast of *Bolus* feeding (i.e., two nutrient deliveries at 12-h intervals) further contributes to the novelty of Chapter 5, allowing for examination of how feeding patterns that more closely reflect daily human behaviour (i.e., meals) may influence temporal metabolic regulation. Furthermore, direct assessment of autophagy in whole blood and muscle will provide further novel insight into the role of cellular recycling in facilitating the divergent responses between fasted and fed states. Following the studies in Chapters 4 and 5 an interest in understanding the effects of sleep fragmentation on next day metabolic control arose. As such, Chapter 6 aimed to determine whether hourly sleep disruption through the night influences next morning metabolic control. An additional ecologically valid contrast was added to Chapter 6 by also providing participants with a strong caffeinated coffee, to ascertain any additive effects of caffeine on metabolic control. Considering that the aforementioned studies necessarily required manipulation of fasted- and fed-states, the studies outlined in Chapters 7 and 8 sought to explore how macronutrient composition of the first meal of the day alters metabolism and behaviour relative to extension of the overnight fast to midday. Specifically, these studies aimed to establish the acute (hours; day) and short/mid-term (days; weeks) metabolic and behavioural responses to sequential feedings when enriching a typical breakfast with whey protein – both relative to a typical carbohydrate-rich breakfast and no breakfast. Finally, the General Discussion reviews how the cumulative information gained from these studies can be considered collectively to further understanding of how nutrient timing influences metabolic regulation and associated health outcomes.

Chapter 2 – Review of the Literature

2.1 Circadian rhythms and metabolism

Life on earth has evolved within the context of a repetitive cycle of *circa* 24 hours, whereby environmental variables such as light exposure predictably oscillate during each daily period. As such, natural selection has provided almost all organisms on this planet with endogenous circadian rhythms to help anticipate impending environmental challenges and thus pre-emptively adjust our physiology, metabolism and/or behaviour accordingly (Jagannath *et al.*, 2017). The mammalian circadian timing system comprises both a central 'master' clock located in the suprachiasmatic nuclei of the hypothalamus, along with an integrated network of peripheral clocks located throughout various organs, tissues and cell-types (Albrecht, 2017). Collectively, these molecular clocks facilitate the coordinated disposal, degradation, synthesis, and recycling of metabolic substrates in order that our periodic delivery of dietary nutrients (i.e., mealtimes) can appropriately meet our ongoing physiological requirements (Frayn, 2019).

2.1.1 The Circadian Molecular Clock

At the molecular level, circadian clocks are regulated by inter-locking transcriptional-translational feedback loops (TTFLs) which maintain approximate 24 hour rhythmicity, therefore broadly coordinating with a single solar period (i.e., the full rotation of our planet on its axis relative to the sun) (Mazzoccoli *et al.*, 2012; McGinnis & Young, 2016). Within the primary TTFL there are both positive and negative limbs. The positive limb is primarily characterised by the proteins CLOCK (Circadian Locomotor Output Cycles Kaput), its paralog NPAS2 (neuronal PAS domain protein 2), and BMAL1 (Brain and Muscle ARNT-Like 1), which can be typically found in the nucleus (Kwon *et al.*, 2006). Dimerisation of these proteins forms a multimer capable of binding to the Enhancer-box sequence (E-Box) of target genes to initiate transcription prior to dissociation in order for a new cycle of transactivation to begin (Figure 2.1) (Hao *et al.*, 1997; Hirano *et al.*, 2016). Importantly this positive loop targets clock-controlled genes, which are involved in a wide variety of physiological process, but also activates rhythmic transcription of genes in the negative loop, including the *Period* (*PER*) and *Cryptochrome* (*CRY*) genes (Mohawk *et al.*, 2012).

The primary function of the feedback loop is to inhibit the activity of CLOCK: BMAL1 activity, through dimerisation of PER and CRY proteins (Ye *et al.*, 2014). This is achieved through translocation of the PER and CRY proteins to the nucleus, which can only be achieved once a sufficient quantity of the complex has accumulated (Schmutz *et al.*, 2010; St John *et al.*, 2014; Hirano *et al.*, 2016). Once nuclear localisation occurs, CRY and PER act through a dual mechanism whereby binding of the CRY protein to the CLOCK:BMAL1 complex inhibits binding to E-box elements, thereby stopping transcription of proteins involved in the negative loop (Ko & Takahashi, 2006). On the other hand, PER displaces the CLOCK: BMAL1 heterodimer from the promoter region to stop further transcription (Ye *et al.*, 2014). Degradation of these negative components is then required to end repression of the positive loop thereby starting a new cycle of transcription (Sahar & Sassone-Corsi, 2012; Buhr & Takahashi, 2013; St John *et al.*, 2014).

The 24-hour phase observed in mammals in largely due to the delay observed in the accumulation of the PER: CRY repressor complex in the cytoplasm, and its degradation in the nucleus (St John *et al.*, 2014). However, other regulatory factors also contribute to the relative plasticity of the circadian system, such as posttranslational modifications (PTMs), including phosphorylation, acetylation, sumoylation, and ubiquitylation (Cardone *et al.*, 2005; Lee *et al.*, 2008); these participate in controlling the timing between the activation and repression of circadian transcription (Lowrey & Takahashi, 2011). Rhythmicity of these modifications is vital for maintenance of *in vivo* circadian rhythmicity, playing an important role in maintaining the stability of clock proteins, thereby ensuring the tightness and amplitude of observed rhythms (McClung, 2011; van Ooijen *et al.*, 2011; Mazzoccoli *et al.*, 2012).

The nuclear receptor (NR) feedback loop is an additionally important regulatory loop interconnected within the primary TTFL. In addition to activating transcription of *Per* and *Cry* genes, Clock:Bmal1 also activates transcription of NRs: *REV-ERB* (reverse transcript of erythroblastosis gene) α/β, and retinoic acid-related (RAR) orphan receptor (ROR)α, β/δ, and γ (Guillaumond *et al.*, 2005; Teboul *et al.*, 2009). REV-ERBα/β acts as a negative transcriptional regulator through binding with ROR-specific

response elements (ROREs) in the promotor region of target genes, thereby inhibiting binding of the positive transcriptional regulator RORα (Fontaine & Staels, 2007; Burris, 2008; Teboul *et al.*, 2009). Furthermore, these NRs have a direct role in regulating the expression of core clock genes (*Bmal1, Clock*, and *Cry1*). Interaction of RORα with peroxisome proliferator-activated receptor- γ (PPAR-γ) coactivator-1α (PGC-1α), promotes BMAL1 expression (Akashi & Takumi, 2005). Conversely REV-ERBα competes with RORα for the common regulatory elements (e.g., NCoR1-HDAC3; 1/histone deacetylase 3 nuclear receptor corepressor complex). The dichotomous actions of the RORα/PGC-1α activator complex and the REV-ERBα/nuclear corepressor repressor complex NCoR1-HDAC3 drive the rhythmic expression of Bmal1 (Preitner *et al.*, 2002).

Whilst the molecular regulation of the circadian is necessarily complex, the pervasive expression of this core clock machinery demonstrates the importance of proper circadian alignment in integrating physiological function throughout various organs, tissues, and cell-types (Mohawk *et al.*, 2012; Albrecht, 2017; Dierickx *et al.*, 2018).

Table 2.1 – Name, definition, and basic function of the 'core' circadian clock machinery involved in the transcription-translation feedback loop.

Name	Definition	Function	Reference
E-box	Enhancer box	Promoter region that regulates cellular transcriptional activity.	Hao *et al* (1997)
RORE	Retinoic acid-related orphan receptor response element.	Promoter region that regulates cellular transcriptional activity.	Cook, Kang & Jetten (2015)
CLOCK	Circadian Locomotor Output Cycles Kaput	Forms heterodimer with BMAL1 which binds to and activates the E-box thereby stimulating transcription and translation of Per and Cry.	Buhr & Takahashi (2013)
NPAS2	Neuronal PAS domain protein 2	Paralog of CLOCK. Forms heterodimer with BMAL1 which binds to and activates the Ebox thereby activating transcription and translation of Per and Cry.	Buhr & Takahashi (2013)
BMAL1 (Arntl)	Brain and Muscle ARNT-Like 1	Forms heterodimer with CLOCK which binds to and activates the Ebox thereby activating transcription and translation of Per and Cry.	Buhr & Takahashi (2013)
Cry1,2,3	Cryptochrome 1, 2, 3	Form a complex with Period proteins. Inactivates Ebox thereby inhibiting transcription and translation of CLOCK and BMAL1.	Ko & Takahashi (2006)
Per1,2,3	Period 1, 2, 3	Form a complex with Cryptochrome proteins. Inactivates Ebox thereby inhibiting transcription and translation of CLOCK and BMAL1.	Ko & Takahashi (2006)
NR1D1/2(REV-ERBα/β)	Nuclear receptor subfamily 1 group D member 1/2	Repression of BMAL1 gene expression through binding with RORE sites.	Guillaumond *et al* (2005)
ROR-α/β/γ	Retinoic-acid related orphan receptors	Transcriptional activator for BMAL1 through binding with RORE sites.	Guillaumond *et al* (2005)

2.1.2 Circadian regulation of metabolism

Aside from understanding the fundamental concepts of the circadian clock as discussed in section 2.1.1, it is also important to consider the downstream mechanisms of regulation beyond core clock gene expression, as well appreciating the range of processes modulated by circadian metabolism. Broadly, this is separated into two categories based on location: the central clock, and the peripheral clocks.

2.1.2.1 Central clock

The central circadian clock is located in the brain within a distinct region of the hypothalamus, termed the suprachiasmatic nucleus (SCN). Despite its relatively small size (~10,000 neurons), the SCN is the principle circadian clock, which anticipatorily coordinates daily cycles of physiology and behaviour with light-dark cycles (Mohawk *et al.*, 2012). As such, the SCN is reliant upon the ability to translate photic input into coordinated biological rhythms, which can then be translated to the peripheral tissues in the human body (Berson, 2003).

The principal photic organ in mammals is the eye, in which the retina is responsible for transmitting photons along neuronal pathways to the brain to generate visual images (Nguyen *et al.*, 2022). Within the retina exists intrinsically photosensitive retinal ganglion cells, which express melanopsin in response to light, thereby generating impulses that project to various regions of the brain associated with circadian rhythms and sleep - including the sub-paraventricular zone, the intergeniculate leaflet and importantly, the SCN (Provencio *et al.*, 1998; Pickard & Sollars, 2012; Do, 2019). The light-stimulated secretion of melanopsin generates an action potential within a cell, which, when carried to the SCN, prompts the release of glutamate and pituitary adenylate cyclase-activating polypeptide (Mure *et al.*, 2016). These compounds then bind to SCN neuron bound receptors causing depolarisation and calcium influx thereby triggering an intracellular signalling cascade, which results in the phosphorylation of CREB (cyclic adenosine monophosphate response element binding protein). The phosphorylated CREB is then able to bind to cAMP responsive elements in the promotor region of target genes (including PER), thereby phase adjusting core clock machinery through light input (Lundkvist *et al.*, 2005; Welsh *et al.*, 2010).

2.1.2.2 Peripheral clocks

Peripheral circadian clocks are located within non-SCN regions of the organism, including other regions of the central nervous system (Figure 2.1) (Takahashi et al. 2008). The importance of proper circadian alignment in integrating physiological function is apparent by the pervasive expression of molecular clock machinery throughout these peripheral organs, tissues and cell-types, with 3-16% of all mRNA in each tissue exhibiting rhythmic daily expression (Mohawk *et al.*, 2012; Albrecht, 2017; Dierickx *et al.*, 2018).

In order to coordinate rhythmic responses in both the SCN and peripheral tissues with the light-dark cycles across a 24-hour period, a variety of signalling pathways are required. These include, but are not limited to autonomic stimulation, endocrine action, and body temperature modification (Mohawk *et al.*, 2012; Albrecht, 2017). Individual neurons within the SCN are connected with more than one autonomic circuit involving both sympathetic and parasympathetic innervation of a single tissue, or sympathetic innervation of two different peripheral tissues, including the adrenal medulla, adrenal cortex, thyroid gland, liver, and pancreas (Bartness *et al.*, 2001; Cailotto *et al.*, 2005). Primarily, control of circadian rhythms through the autonomic nervous system occurs through the daily rhythm in melatonin release from the pineal gland (Reiter, 1991). Following secretion from the pineal gland, melatonin is secreted into the systemic circulation, where it acts to entrain peripheral tissues (e.g., pancreatic islets and gastrointestinal tract) (Slominski *et al.*, 2012). Melatonin also acts as a sleep-inducing compound, therefore directly influencing behavioural rhythms and demonstrating the usefulness of melatonin as a marker of circadian rhythmicity (Benloucif *et al.*, 2005). Finally, shifts in internal body temperature act to transfer information received at the SCN to peripheral oscillators. Specifically, core temperature is lower during sleep (i.e., dark) and increases when awake (i.e., light) (Buhr *et al.*, 2010). Importantly, the SCN itself is not sensitive to temperature changes, thus preventing feedback that could alter entrainment (Brown *et al.*, 2002).

Liver: Circadian control of hepatic transcripts regulates metabolism. Through a combination of techniques, it is estimated that between 6-10% of genes analysed display robust circadian rhythms in mouse hepatocytes, with specific gene clusters targeting both carbohydrate and lipid metabolism (Akhtar *et al.*, 2002; Robles *et al.*,

2014). Interestingly, rhythmic expression of novel genes in the liver do not appear to display rhythmicity in the SCN, suggesting a degree of tissue specificity (Akhtar *et al.*, 2002). Again, knock-down rodent models have been employed to demonstrate the importance of these rhythms in nutrient metabolism. In particular ablation of the SCN in mice results with severe insulin resistance and liver-specific knock-down of core-clock machinery impairs hepatic lipid homeostasis (Coomans *et al.*, 2013b; Zhang *et al.*, 2014a; Lee *et al.*, 2015).

Muscle: Rhythmic physiology in skeletal muscle regulates metabolism. Skeletal muscle plays a vital role in maintaining glucose homeostasis following ingestion of a meal, accounting for ~40-85% of exogenous glucose disposal (DeFronzo *et al.*, 1981b; Gachon *et al.*, 2017). Exercise also plays a role in glucose homeostasis, potentiating the uptake of glucose into the skeletal muscle through insulin independent mechanisms (Richter & Hargreaves, 2013). The maintenance of circulating glucose within a tight physiological range is important as glycaemic variability across 24 h is an established risk factor for the development of diabetes and cardiovascular disease (Cavalot *et al.*, 2011; Van Dijk *et al.*, 2013). Therefore, considering that periods of feeding and activity are typically aligned with cycles of light-dark the importance of aligning skeletal muscle physiology for the maintenance of metabolic health is evident (Dibner & Schibler, 2015; Wehrens *et al.*, 2017b). This is further supported by rodent models, in which deletion of core clock machinery (BMAL1) within skeletal muscle results in reduced GLUT4 mRNA and protein expression, therefore reducing insulin sensitivity (Andrews *et al.*, 2010; Dyar *et al.*, 2013; Harfmann *et al.*, 2016).

Whilst much is what is known about the importance of circadian rhythmicity in skeletal muscle comes from rodent models, recent evidence from *in vitro* and *in vivo* human models has broadened our understanding of these rhythms in the context of humans. Perrin *et al* (2015) provided the first evidence of a high amplitude cell-autonomous clock within primary human skeletal muscle myotubes, with rhythmic profiles observed in both *BMAL1* and *PER2*. Follow up work aimed to disentangle the effects of the endogenous circadian clock on skeletal muscle gene transcription from external factors. Using genome-wide transcriptome analysis in skeletal muscle biopsies collected from human subjects placed under a semi-constant routine, high amplitude oscillations for the core clock genes *BMAL1, NPAS2, CLOCK, PER2, PER3, CRY2,*

NR1D1(REV-ERBa) and *RORA* were observed (Perrin *et al.*, 2018a). Interestingly, under these conditions in which participants received hourly isoenergetic feedings across waking hours (0700-2200 h), peaks in transcript accumulation clustered at 1600 h (for genes implicated in muscle force production and mitochondrial activity) and at 0400 h (for genes implicated in immune function and inflammation), with rhythmicity also present for genes linked to glucose, lipid and protein homeostasis (Perrin *et al.*, 2018a).

Adipose: Rhythmic genes in body fat coordinate physiology with behaviour. Despite the prominent role of white adipose tissue (WAT) for energy storage in the form of triacylglycerols (TAG), WAT is also an important organ that acts in an endocrine and paracrine fashion to regulate metabolism (Mohamed-Ali *et al.*, 1998; Coelho *et al.*, 2013). It is therefore unsurprising that murine models have observed 24-h variations in ~10-20% of the WAT transcriptome (Ptitsyn *et al.*, 2006; Zvonic *et al.*, 2006). The importance of these 24-h variations, particularly in core clock genes, is evident from knockdown models in mice. *Per-2* deficiency results in altered lipid metabolism, with a reduction in total TAG and non-esterified fatty acids (Grimaldi *et al.*, 2010). Furthermore, increased adipogenesis, adipocyte hypertrophy and obesity have been observed following *Bmal1* knockdown in mice (Shimba *et al.*, 2011; Guo *et al.*, 2012; Paschos *et al.*, 2012). Recent evidence sheds light on the prevalence of circadian oscillations in human WAT. Firstly, Otway *et al* (2011) observed meaningful temporal oscillations in both core clock (*PER1, PER2, PER3, CRY2, BMAL1, REVERB-A*, and *DBP*) and metabolic (*RIP140*, and *PGC1A*) genes under diurnal conditions. In order to unmask the effects of these diurnal influences, follow-up research placed participants under a laboratory-controlled constant routine, observing similar rhythmic profiles to Otway *et al* (2011) suggesting a good degree of conservation between diurnal and constant conditions (Christou *et al.*, 2019).
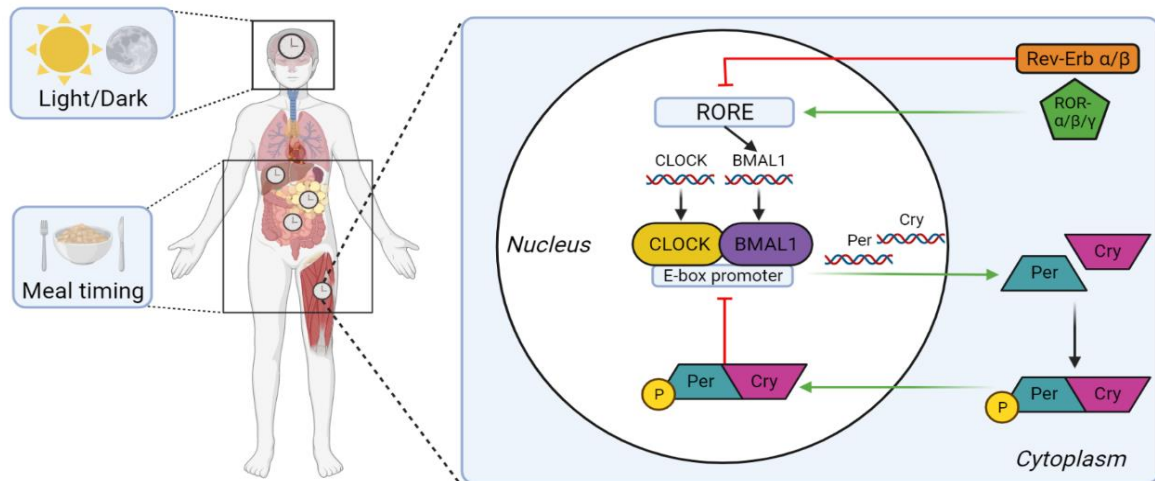
Figure 2.1. *Left hand side;* The central clock is located in the brain in the suprachiasmatic nucleus (SCN) and is robustly driven by regular cycles of light and dark. Core clock machinery is also present in numerous metabolically important peripheral tissues such as the liver, skeletal muscle, adipose tissue, and gut. These tissues are the primary target of the metabolic effects of meal timing. *Right hand side;* Graphic depiction of the cycle of core clock machinery within the nucleus and cytoplasm.

2.1.3 Methods used to investigate circadian rhythms

Behavioural cycles in humans (e.g., sleep-wake, rest-activity, fast-feed) occur alongside endogenous circadian cycles to temporally regulate metabolism. It is therefore difficult to assess the independent effects of each cycle on metabolism. However, there are several methods that can be employed to disentangle the influence of endogenous cycle from that of the behavioural cycle. Due to the location of the SCN in the brain, direct assessment of function can only be carried out in samples collected post-mortem (Hofman *et al.*, 1996; Dai *et al.*, 1998). Whilst this is advantageous in being able to directly assess function, this method is flawed in that lack of detail about behaviour and light exposure before death prevents full understanding of function.

Rodent models have highlighted the links between meal timing, circadian rhythms and health. However, there are important limitations to the applicability of this knowledge, emphasising the need to focus on human research. Firstly, mice display nocturnality relative to human diurnality (Eckel-Mahan & Sassone-Corsi, 2015). Moreover, mice continuously graze at irregular but frequent intervals through the night, in contrast to the typical intermittent scheduling of regular meals at infrequent intervals throughout the day typical of humans (Ellacott *et al.*, 2010). These distinct feeding behaviours are

reflective of the specific anatomy of each species; rodents have a high surface area and metabolic rate relative to mass, which drive an unrelenting requirement for more consistent energy delivery via their disproportionately large splanchnic organs (Ellacott *et al.*, 2010; Gonzalez & Betts, 2018). Additionally, differing sleep requirements based on mammal size provide further key differences in metabolic regulation between humans and rodents that emphasise the need for human circadian evidence (Zepelin & Rechtschaffen, 1974; Capellini *et al.*, 2008).

Inverted sleep-wake cycles can take advantage of how the circadian system is relatively slow in re-training to shifted behavioural/environmental cycle. Protocols employing inverted sleep-wake cycles are typically characterised by extended periods of wakefulness, whereby sleep is typically displaced by ~12 h to the daytime (Figure 2.2) (Qian & Scheer, 2016; Qian *et al.*, 2018). Participants may also be placed under conditions controlling physical activity, body posture, and nutritional state. As such, the endogenous cycle is still evident despite inversion of the typical behavioural cycle. For example, employment of inverted sleep-wake cycle can delineate the effects of time of day from specific sleep effects on glucose metabolism (Van Cauter *et al.*, 1991). Whilst this method clearly shows the anticipatory nature of the endogenous clock, the potential for the gradual rise in homeostatic sleep pressure due to forced wakefulness to influence the outcome variable of interest is an inherent limitation (Franken & Dijk, 2009).
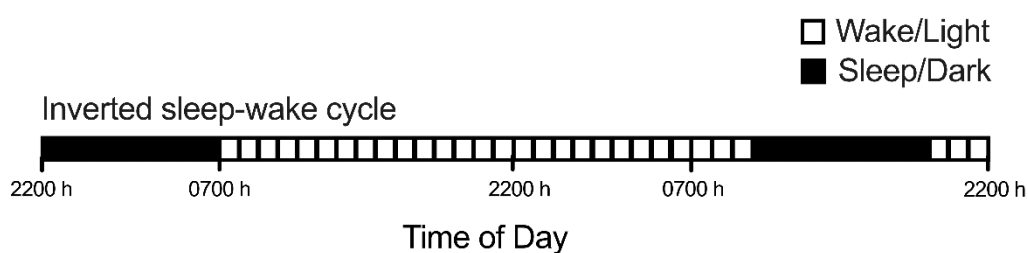
Figure 2.2 – Schematic representation of an inverted sleep-wake cycle protocol.

Forced desynchrony involves putting participants through non-24-h behavioural cycles under conditions of dim-light. The objective of such protocols is to minimise the influence of increased sleep disruption on homeostatic sleep pressure (Qian & Scheer, 2016; Stack *et al.*, 2017). Typically, the ratio of sleep:wake is maintained at 1:2 (Figure 2.3). Under conditions of forced desynchrony the internal circadian period is still expressed, and the behavioural cycle is distributed evenly across all circadian phases allowing for investigation of the effects of the circadian system separate from those of the behavioural cycle. This method is therefore inherently beneficial for determining endogenously driven rhythms, such as the observed rhythm in resting metabolic rate (Zitting *et al.*, 2018).
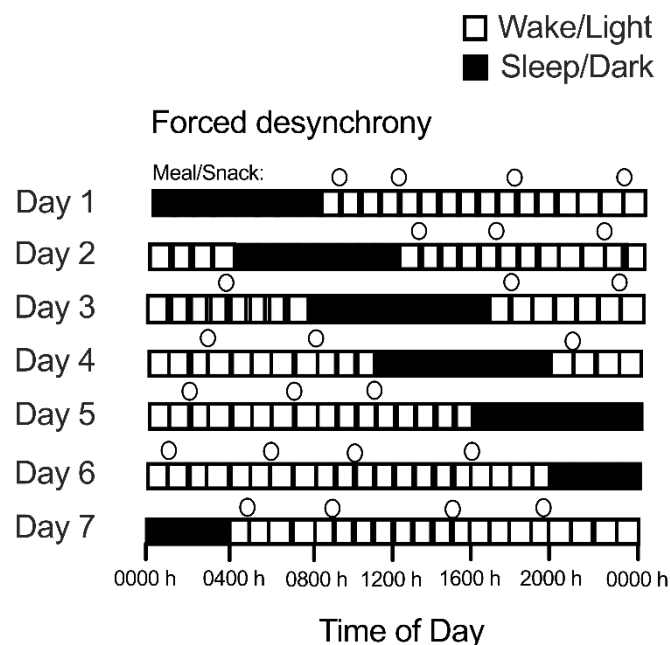
Figure 2.3 – Schematic representation of a forced desynchrony protocol. Daily shifting of behavioural cycles (e.g., sleep/dark) prevents entrainment of rhythms allowing for determination of whether circadian rhythms persist (i.e., are endogenously driven) or are disrupted (behaviourally driven).

Circadian misalignment protocols typically involve shifting normal daytime behaviours to the night. Protocols employing circadian misalignment to dissect the effects of the endogenous circadian system from external influences typically contrast a misalignment protocol with an alignment protocol using within-participants randomised crossover designs (Figure 2.4) (Qian & Scheer, 2016). The independent effects of alignment relative to misalignment can then be assessed through averaging the

responses to various meals between conditions to understand: the behavioural effect (e.g. breakfast aligned & breakfast misaligned *vs* dinner aligned & dinner misaligned); the circadian phase effect (e.g. breakfast aligned & dinner misaligned *vs* breakfast misaligned & dinner aligned); and the circadian misalignment effect (e.g. breakfast aligned and dinner aligned *vs* breakfast misaligned and dinner misaligned).
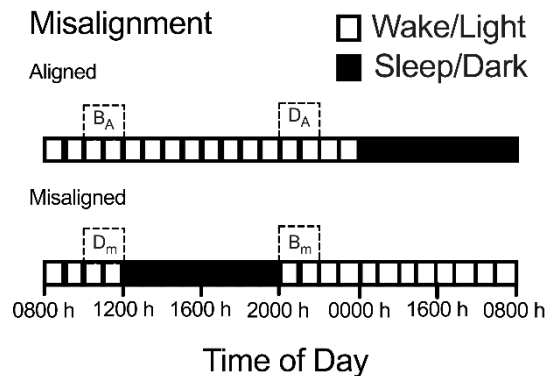
Figure 2.4 – Schematic representation of a circadian misalignment protocol. B_A = Breakfast aligned, D_A = Dinner aligned. B_M = Breakfast misaligned, D_M = Dinner misaligned.

Constant routine (CR) protocols were developed with consideration of the influence of periodic changes in both environmental and behavioural cues upon rhythmicity in physiological variables of interest (Figure 2.5). This is especially relevant when assessing rhythmic characteristics of the core circadian clock (e.g. gene expression) (Duffy & Dijk, 2002). Therefore, CRs are specifically designed to reduce or eliminate the behavioural drivers of circadian rhythms. Particularly, this refers to asking participants to remain awake in order to control the influence of the sleep-wake cycle, with even distribution of energy intake across the circadian cycle to minimise the influence of cycles of fasting-feeding. Once under these conditions of CR, rhythms of interest can be me measured at a frequency that is dictated by the necessary resolution required, but also that minimises any participant discomfort (Otway *et al.*, 2011; Perrin *et al.*, 2018a; Christou *et al.*, 2019). However, much as with inversion of the sleep-wake cycle, the gradual increase in homeostatic sleep pressure across prolonged wakefulness may negatively influence the rhythm in the outcome measure of interest. Furthermore, whilst CRs are inherently designed to remove the influence

behavioural rhythms on the endogenous clock, it is also important to acknowledge the ecological invalidity in which rhythms during CR are investigated (e.g. prolonged wake, or constant nutrition for 24 h), which could alter or even subvert the rhythm under investigation (Scheer *et al.*, 2013; McHill *et al.*, 2018). As a solution to this, semi-constant routines can be used to gain information about diurnal effects. Semi-constant routines are very similar in nature to constant routine protocols, however normal sleep is allowed, and constant nutritional state is only maintained during waking hours (Loizides-Mangold *et al.*, 2017; Perrin *et al.*, 2018b; Templeman *et al.*, 2021b).
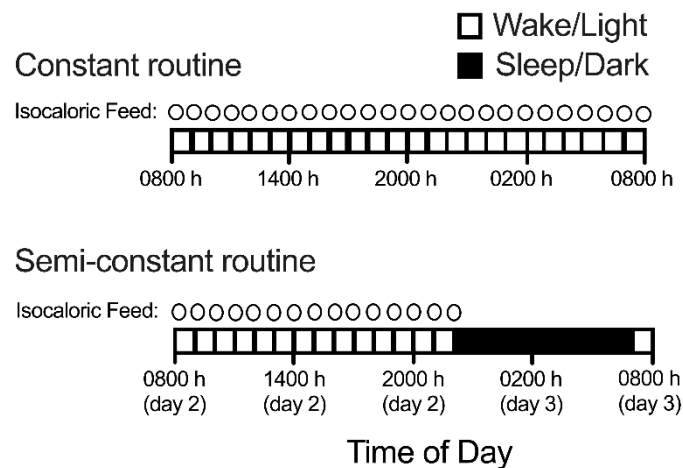
Figure 2.5 – Schematic representation of constant and semi-constant routine protocols.

2.1.4 Circadian rhythms in nutrient metabolism

2.1.4.1 Glucose metabolism

In the post-absorptive state (i.e., food has been digested, absorbed, and stored) blood glucose ($C_6H_{12}O_6$) concentration is maintained within a tight physiological range, however, this is not reflective of the degree of flux ($\sim$2 mg·kg^{-1}·min^{-1}) between the blood and active tissues (Fery et al., 1990; Wasserman, 2009). This high level of flux is important for maintaining delivery of fuel to vital organs, in particular the brain, and highlights the acute responsiveness of human metabolism to achieve such high fuel demand (Wasserman, 2009). The regulation of blood glucose through these physiological mechanisms is vital to prevent prolonged exposure to blood glucose concentrations outside of a tight physiological range which pose significant threats to life (Cavalot et al., 2011).

Blood glucose homeostasis is achieved through the action of two primary antagonistic hormones: insulin and glucagon. Both hormones are produced in and secreted predominantly from the pancreas, with insulin originating in the β-cells and glucagon in the α-cells of the pancreatic islets (Barg, 2003). In times of low systemic glucose availability (e.g., the post-absorptive/fasted state), glucagon secretion is initiated through the action of voltage-dependent sodium (Na^+) and calcium (Ca^{2+}) channels, which act to maintain action potentials when glucose is low. Influx of Ca2+ following depolarisation of the membrane results in glucagon secretion, supported by the activity of ATP-sensitive potassium (K_{ATP}) channels (Gromada et al., 1997; MacDonald et al., 2007). The subsequent increase in the glucagon:insulin ratio upregulates hepatic glycogenolysis and gluconeogenesis (GNG), serving to prevent hypoglycaemia through the conversion of substrates (e.g. lactate, pyruvate, and alanine) into glucose, maintaining substrate delivery to the brain (Wasserman, 2009). Following ingestion of a mixed-macronutrient meal, insulin secretion is stimulated from pancreatic β-cells. As blood glucose concentration rises, pancreatic β-cell glucose concentration also rises, resulting in the phosphorylation of the transcription factor pancreatic and duodenal homeobox 1 (PDX1), ultimately resulting in increased transcription and expression of insulin-coding genes (Andrali et al., 2008). Increased β–cell glucose concentration upregulates adenosine triphosphate (ATP) production via glycolysis, closing energy sensitive potassium channels, disrupting membrane potential (Ashcroft, 2005). The resulting depolarisation causes opening of voltage sensitive calcium channels (Catterall, 2011). The resulting net inward flow of calcium ions results in exocytosis of secretory vesicles, thereby resulting in insulin secretion (Eliasson et al., 2008).

Insulin secretion from the pancreas lowers blood glucose concentration through two means: inhibition of hepatic glucose output and increasing the uptake of glucose into peripheral tissues (Ferrannini et al., 1985; Edgerton et al., 2017). The former effect of insulin on hepatic glucose production occurs primarily through an alteration in the glucagon:insulin ratio (Edgerton et al., 2017). An increase the concentration of insulin at the α-cells of the pancreatic islets inhibits glucagon secretion, thereby inhibiting hepatic glucose production (Maruyama et al., 1984; Ishihara et al., 2003; Diao et al., 2005). Additionally, insulin-stimulated inhibition of adipose tissue lipolysis decreases the release of non-esterified fatty acids (NEFAs) and glycerol from adipose tissue as

well as gluconeogenic precursors from skeletal muscles (Edgerton et al., 2017). The resultant reduction in hepatic fatty acid oxidation thereby inhibits gluconeogenesis (Lewis et al., 1997; Mittelman & Bergman, 2000). The increase in glucose uptake occurs through an increased number and intrinsic activity of GLUT4 transporters at the cell membrane of myocytes and adipocytes. This occurs when insulin binds to the extracellular portion of the transmembrane insulin receptor, resulting in phosphorylation of tyrosine kinase at the intracellular portion of the transmembrane receptor (Lane et al., 1990). Insulin-receptor substrates downstream of the phosphorylated tyrosine kinase are then also phosphorylated, and coordinate with phosphatidylinositol-3-kinase (PI3K) to synthesise phosphatidylinositol (3',4',5')-triphosphate (PIP3) (Hemmings & Restuccia, 2012). Interaction between PIP3 and 3'-phophainasitide dependent kinase-1 (PDK1) then phosphorylates protein kinase B (PKB; also known as Akt), resulting in translocation of GLUT4 to the cell membrane (Shepherd & Kahn, 1999).

Glucose metabolism across the day displays a robust diurnal rhythm. Whilst basal blood glucose can be relatively elevated upon waking (i.e., the dawn phenomenon), post-prandial glucose tolerance is generally lower in the evening than in the morning (Van Cauter et al., 1989; Van Cauter et al., 1992; Simon et al., 1994; Van Cauter et al., 1997; Qian & Scheer, 2016). This has been shown consistently across the literature following oral glucose ingestion (Van Cauter et al., 1997), intravenous glucose administration (Van Cauter et al., 1989) and identical meals across the day (Van Cauter et al., 1992), as well as during continuous enteral provision of nutrients (Simon et al., 1994).

Core clock machinery is implicated in the circadian regulation of glucose homeostasis. The CLOCK:BMAL1 heterodimer is an important mediator for the transcription of coactivators involved in circadian synthesis of glucose regulatory hormones (Feng & Lazar, 2012). The negative component of the TTFL is also implicated in regulating glucose homeostasis, with CRY and REV-ERB-α proteins acting to inhibit gluconeogenic gene expression through regulation of CREBP activity and regulation of Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphotase (G6Pase) expression respectively. Conversely, ROR-α activates G6Pase (Sato et al.,

2004; Jordan & Lamia, 2013). Cellular energy sensing through AMPK (Adenosine Monophosphate activated protein kinase) also plays a role in modulating 24-hour profiles of glucose homeostasis through enhancement in the degradation of Per and Cry (Lamia et al., 2009). Importantly, the associated increase in insulin sensitivity following the postprandial upregulation of AMPK activity directly interacts with Clock, deacylating Bmal1 and Per2, thus exerting an effect within the TTFL (Asher et al., 2008; Hardie et al., 2012).

Diurnal rhythms in β-cell responsiveness, insulin secretion/clearance, and insulin sensitivity can partially explain 24-h variations in glucose tolerance. Following oral glucose ingestion, mixed meal consumption, and/or intravenous insulin stimulation, *β-cell* responsiveness has consistently been shown to be higher during the morning relative the rest of the day (Baker & Jarrett, 1972; Aparicio *et al.*, 1974; Saad *et al.*, 2012; Morris *et al.*, 2015b). Despite this, peak insulin secretion rate and therefore total insulin secreted following meal ingestion doesn't tend to peak until later in the day (Boden *et al.*, 1996; Saad *et al.*, 2012). However, these data, combined with the robust diurnal rhythmicity of systemic glucose, are suggestive of a reduction in peripheral insulin sensitivity across the day. The reduction in peripheral insulin sensitivity is a common observation in the literature, demonstrated using a variety of techniques, reporting up to a 34% reduction in whole body insulin sensitivity in the evening relative to the morning (Lee *et al.*, 1992). This diurnal rhythmicity in insulin sensitivity is in part explained by rhythmic activation in skeletal muscle insulin signalling. In particular, *AKT*, 5′-adenosine monophosphate-activated protein kinase (*AMPK*) and TBC1 domain family member 4 (*TBC1D4*) phosphorylation display robust diurnal rhythmicity in mice (Basse *et al.*, 2018). Oscillatory profiles in transcription factors in skeletal muscle relating to glucose homeostasis and oxidative phosphorylation may further contribute towards rhythms in peripheral insulin sensitivity (Perrin *et al.*, 2018a). Equally, the degradation of hepatic glycogen overnight to maintain glucose delivery to the brain may also contribute to greater insulin sensitivity in the morning relative to the evening (Davidson, 1981; Iwayama *et al.*, 2020). However, it is remarkable that no studies have collected simultaneous serial measures of skeletal muscle metabolism and systemic glucose/insulin under controlled diurnal conditions over 24-h and should be an immediate focus of human circadian research.

2.1.4.2 Lipid metabolism

The principal role of adipose tissue is to both store and release large quantities of energy in the form lipids. Specifically, the lipids are stored in the form of 3 non-esterified fatty acids (NEFA) bound to a glycerol backbone, forming triacylglycerol (TAG), consequently, the most commonly studied lipids in the systemic circulation are NEFA and TAG.

In the postprandial state, lipid storage is stimulated through an increase in circulating insulin. Binding of insulin to the insulin receptor on the adipocyte membrane causes tyrosine phosphorylation of insulin receptor substrates and binding of PI3K (Duncan *et al.*, 2007). The resultant phosphorylation of PKB in turn phosphorylates phosphodiesterase 3B (PDE) (Langin, 2006). Consequently, cyclic adenosine monophosphate(cAMP) is degraded, inactivating protein kinase A (PKA), thereby reducing the phosphorylation and activity of lipases and perilipin proteins (Londos *et al.*, 1985; Zhang *et al.*, 2005). Triacylglycerol and cholesterol from the ingested meal are absorbed and re-esterified in the cells of the intestinal wall and secreted into the circulation as chylomicron particles. However, the circulating TAG present in chylomicrons are too large to transfer into the interstitial fluid and therefore cannot be directly stored within the adipocytes (Frayn *et al.*, 1995). Importantly, as they travel through the circulation, interaction with many other particles, especially apolipoprotein CII, identifies chylomicrons as a target for lipoprotein lipase (Wolska *et al.*, 2017). The postprandial increase in LPL hydrolyses the TAG in the lipoprotein releasing fatty acids, which are then able to diffuse into the interstitial space to be taken up by the adipocytes (Frayn *et al.*, 1994; Eriksson *et al.*, 2003; Olivecrona, 2016). Importantly, the combined action of insulin in inhibiting lipolysis and activating lipoprotein lipase activity establishes a diffusion gradient across the capillaries in order to facilitate uptake of fatty acids in adipocytes/myocytes (Sadur & Eckel, 1982; Hamilton, 1999). This transport is facilitated through membrane-bound transporters including fatty acid transport proteins (FATPs) and CD36 (Pohl *et al.*, 2004; Koonen *et al.*, 2005; Xu *et al.*, 2013). Upon entering the cell, fatty acids are esterified to form triacylglycerol, before joining the lipid droplet for storage (Czech *et al.*, 2013). De-novo lipogenesis is another form of lipid storage, albeit from non-lipid sources (e.g., carbohydrates) that occurs within the liver (Ameer *et al.*, 2014). De novo genesis is not the default method of lipid

storage, however under conditions of consistent overfeeding, DNL helps to maintain metabolic homeostasis (Solinas *et al.*, 2015).

In the post-absorptive state, release of lipids from the adipose occurs through the action of lipolysis. During periods of low energy availability (e.g., during an extended overnight fast), white adipose tissue shifts towards greater net release of fatty acids and glycerol through lipolysis for use as energy substrates by other organs. Briefly, lipolysis is the process by which TAG are hydrolysed to form diacylglycerol (DAG), then monoacylglycerol (MAG), with the liberation of a FA at each step (Duncan *et al.*, 2007). Hormone sensitive lipase (HSL) plays a vital role in regulating this process and its action is particularly responsive to fluctuations in catecholamines (e.g., norepinephrine) and insulin (Steinberg & Khoo, 1977). Catecholamines exert a strong lipolytic effect in adipose tissue and are the principal activators of fasting-induced lipolysis (Galster *et al.*, 1981; Jensen *et al.*, 1987). Binding of norepinephrine to β-adrenergic receptors on the plasma membrane of adipocytes results in transmission of a stimulatory signal to adenylyl cyclase to generate cyclic AMP (Collins, 2011). Protein kinase A (PKA) then binds with this cAMP increasing its activity to catalyse the phosphorylation of HSL at multiple sites (Holm, 2003). Conversely, the potent inhibitive effect of insulin on lipolysis suggests lower insulin concentrations in the post-absorptive state are important for the upregulation of lipolysis (Frayn *et al.*, 1994; Frayn *et al.*, 1996; Samra *et al.*, 1996b; Manolopoulos *et al.*, 2012).

Lipid metabolism displays diurnal oscillation. Tight regulation of both lipogenesis and lipolysis is important as excess systemic lipids as well as impaired storage of lipids are risk factors for the development of metabolic disorders such as cardiovascular diseases and obesity (Miller, 2009; Gustafson *et al.*, 2015). It is therefore unsurprising that lipids are extensively regulated by the circadian system (Dallmann *et al.*, 2012). In contrast to rhythms in carbohydrate metabolism, lipid metabolism favours progressively elevated circulating non-esterified fatty acids (NEFA), triglyceride, and cholesterol later in the day and overnight (Zimmet *et al.*, 1974; Morgan *et al.*, 1999; Pan & Hussain, 2007; Ang *et al.*, 2012; Dallmann *et al.*, 2012; Yoshino *et al.*, 2014). Other systemic lipid species also exhibit rhythmicity, observing later afternoon/early evening peaks in phospholipids, lysophosphatidylcholines, and lysophosphatidylethanolamines (Ang *et al.*, 2012). Interestingly, diurnal rhythms of lipid species also exist within skeletal muscle; phosphatidylinositol,

phosphatidylcholine, sphingomyelins, ceramides, and glycosylceramide lipids exhibited peak levels around 0400 h while phosphatidylethanolamine, phosphatidylserine, and TAG peak at earlier time points (2000 h and 0000 h, respectively) (Loizides-Mangold *et al.*, 2017). Importantly, lipid species displaying rhythmic profiles in the latter study may impact on insulin signalling and on the development of insulin resistance, therefore further emphasising the metabolic importance of rhythmic alignment of lipid metabolism.

Diurnal variation in lipid metabolism may be explained by rhythms in regulatory factors such as insulin. Importantly, rhythms in circulating lipids are not due to food intake across the day and therefore can be largely explained by rhythmic physiology in both lipid storage and mobilisation (Yoshino *et al.*, 2014; Held *et al.*, 2020). Specifically, a combination of animal and human studies suggests a net shift in fatty acid metabolism from oxidation towards lipogenesis occurs throughout the day, with circadian regulation of intestinal triglyceride absorption, acylcarnitine's, mitochondrial oxidative capacity, VLDL secretion, and insulin secretion all contributing to this daily variance (Marrino *et al.*, 1987; Lee *et al.*, 1992; Pan & Hussain, 2007; Ang *et al.*, 2012; Pan *et al.*, 2013; Yoshino *et al.*, 2014; van Moorsel *et al.*, 2016; Sprenger *et al.*, 2021).

2.1.4.3 Protein Metabolism

Proteins in the human body play an important role in many aspects of metabolism, including architectural support for movement, enzymatic catalysis of metabolic processes, cell signalling, as well as providing fuel in certain circumstances. Aside from water, protein is the largest single component with regards to the mass of most tissues (Frayn, 2019). The majority of this bodily mass is proteins (e.g., actin and myosin) stored in skeletal muscle (~40% total body mass/~60% total body protein) (Poortmans *et al.*, 2012). Due to the lack of dedicated storage pool of proteins in excess of their functional necessity (e.g., glycogen for carbohydrate), resting post-absorptive protein turnover is higher than carbohydrate and triglyceride (Poortmans *et al.*, 2012). Therefore, it is essential that a fine balance is struck between ingestion/digestion/absorption, synthesis, and utilisation of protein, in order to maintain amino acid homeostasis (Liu & Barrett, 2002).

Digestion of ingested proteins begins in the stomach, whereby the major gastric protease pepsin hydrolyses ingested proteins into di- and tri-peptides, as well as N-terminal amino acids (e.g. leucine and phenylalanine) (Freeman & Kim, 1978; Erickson & Kim, 1990). (Meyer & Kelly, 1976). The di- and tri-peptide stimulated increase in cholecystokinin and secretin stimulates the production of hydrolytic enzyme precursors, from the acinar cells of the pancreas (Götze *et al.*, 1972; Meyer & Kelly, 1976). Activation of these enzyme precursors by enteropeptidase (also secreted from the pancreas) results in the further breakdown of the peptide bonds that comprise these proteins and polypeptides (Neurath & Dixon, 1957; Freeman & Kim, 1978; Erickson & Kim, 1990). The products of protein digestion are then absorbed for transport to the liver via the portal vein through a variety of independent processes. In particular, whereas amino acid transport across the epithelial cells occurs via sodium transport associated carrier-mediated active transport systems, larger peptides are actively transported against a hydrogen ion concentration gradient by transporters known as PEPT1 and PEPT2 (Schultz & Curran, 1970; Gray & Cooper, 1971; Liang *et al.*, 1995; Wang *et al.*, 2017).

The human body does not continuously accumulate or lose protein under "normal" life conditions. Necessarily, the rate of amino acid oxidation must balance the rate of ingested/absorbed protein. The liver is the most important organ in achieving this balance, not only due to the fact it is the first organ to receive amino acids following intestinal absorption, but also due to the abundance of enzymes present to remove nitrogen from amino acids through the synthesis of urea (Felig *et al.*, 1969; Felig & Wahren, 1971; Frayn, 2019). Catabolism of amino acids is broadly characterised by the loss of an amino group by the process of transamination. The resulting 2-oxoacid (e.g., pyruvate, 2-oxoglutarate, oxaloacetate) can then directly enter catabolic (e.g., acetyl CoA) or anabolic (e.g., gluconeogenesis) pathways (Frayn, 2019). The oxidative deamination of these 2-oxoacids yields the ammonium ion (NH_4^+), which alongside a nitrogen atom from the amino group of aspartate, enters the urea cycle in order to remove nitrogen from the body.

Catabolism of amino acids is acutely regulated by substrate supply; in the fed state this is determined by dietary amino acids, and in the fasted state by net rate of body protein breakdown (de Blaauw *et al.*, 1996; Witard *et al.*, 2014). Substrate supply also plays a role as a chronic determinant of hepatic amino acid metabolism, hormones

such as glucagon and cortisol (Brillon *et al.*, 1995). Specifically, glucagon appears to regulate the activity of amino acid transporters in order to increase amino acid uptake (Boden *et al.*, 1984).

Digestion of ingested protein appears to display 24-h variations, however human evidence is sparse. Peptides implicated in the digestion of dietary protein have shown time of day variation. In rats, pepsinogen activity, and chymotrypsin secretion peak during the active period (i.e. the equivalent of the daytime for humans), an effect that persists under constant fasting, suggesting anticipation of food intake and therefore endogenous control of this rhythm (Barattini *et al.*, 1993). Pepsin secretion also displays circadian variation in humans (Fiorucci *et al.*, 1995). Transport of the products of protein digestion also have 24-h variations, with an anticipatory peak in intestinal PEPT1 transcription and protein expression prior to the active phase in rats, which persists in the fasting state (Qandeel *et al.*, 2009a; Qandeel *et al.*, 2009b).

Circulating amino acids in humans show diurnal rhythmicity. The majority of amino acids (including all essential, some non-essential and some conditionally essential) display circadian rhythmicity, with peak systemic concentrations occurring between 1200-2000 h and with lowest values at 0400-0800 h (Feigin *et al.*, 1967; Wurtman *et al.*, 1967; Feigin *et al.*, 1968; Grant *et al.*, 2019). This rhythmicity occurs independent of diet, suggesting control by endogenous circadian system, rather than behavioural (Wurtman *et al.*, 1967; Feigin *et al.*, 1968). Considering dietary protein is not the only source of circulating amino acids, variation in the generation and release of amino acids from assorted tissues may underpin this rhythm, including rhythmicity in protein digestion and absorption (Barattini *et al.*, 1993; Fiorucci *et al.*, 1995; Qandeel *et al.*, 2009a; Qandeel *et al.*, 2009b). Necessarily, due to the cyclic nature of the TTFLs discussed in Chapter 2.1.1, it is unsurprising that rhythmicity is present in genes involved in protein turnover in mammalian cells (Duffield *et al.*, 2002). With regards to the largest deposit of protein in the body (i.e. skeletal muscle) knock-out models of *Bmal1*, *Myod1*, and *Rev-erbα* impair the development and function of skeletal muscle, further supporting the strong relationship between the body clock and protein metabolism (Kondratov *et al.*, 2006; Andrews *et al.*, 2010). Interestingly, recent transcriptomic analysis of regular human skeletal muscle samples across 24-h supports this, showing rhythmic transcription of *MYOD1,* which is a key regulator of myogenesis and a direct target of the *BMAL1:CLOCK* complex (Andrews *et al.*, 2010;

Perrin *et al.*, 2018a). Specifically, *MYOD1* was consistently higher during the day before sharply declining overnight, suggesting rates of myogenesis are likely higher during the day relative to the night. Stable isotope tracer methodology supports this, reporting 33% lower protein synthesis and 38% lower protein oxidation rates during night than the day, with no apparent time-of-day difference in the rate of protein breakdown – although tissue incorporation of tracers was not concurrently assessed, suggesting further research of protein synthesis rates across 24-h is needed using gold standard techniques (Garlick *et al.*, 1980; Adam & Oswald, 1981).

As the largest component of total body protein, rhythmic clocks in the skeletal muscle play an important role in anticipating the transition between fasting and feeding. In particular, this includes the repression of genes involved protein catabolism in the hours prior to systemic metabolic and hormonal cues that arise upon waking (Dyar *et al.*, 2018). However, evidence in humans for rhythmicity of protein metabolism is limited (especially within skeletal muscle), with much work focussing on rodent models. Considering the drawbacks of such models to investigate circadian metabolism in humans (Chapter 2.1.3) it is clear that further work is needed.

2.1.4.4 Autophagy

Autophagy is a cellular process with important roles in energy balance. Primarily, autophagy is characterised by the rearrangement of subcellular membranes in order to sequester cytoplasmic organelles for proteolysis (Klionsky & Emr, 2000). To date, 3 types of autophagy have been characterised: macro-, micro-, and chaperone mediated autophagy. For the purpose of this thesis, autophagy hereafter refers to macro-autophagy.

Autophagy is characterised by several distinct steps (Figure 2.6). Initiation of autophagy through nutrient deprivation, for example, prepares cells to construct a double membrane vesicle known as the autophagosome. At the molecular level this is achieved through dephosphorylation of the serine-threonine protein kinase ULK1, which then disassociates from the mTORC1 complex, rendering ULK1 enzymatically active in order to commence autophagy (Chang & Neufeld, 2009; Hosokawa *et al.*, 2009; Jung *et al.*, 2009). Increased activity of ULK1, in addition to mTORC1-

independent modulation of autophagy through AKT and EGFR, results in phosphorylation of the Beclin-1-Vps34 complex (Di Bartolomeo *et al.*, 2010; Wang *et al.*, 2012; Wei *et al.*, 2013). Vps34 produces phosphatidylinositol-3-phosphate (PI3P), which recruits an effector protein (DFCP1) to promote the initiation of double membrane vesicle nucleation (Axe *et al.*, 2008). This process results in the scaffolding of ATG proteins (such as LC3) to the pre-autophagosome membrane, which mediate the completion of the autophagosome. Specifically, these ATG proteins are grouped into 2 ubiquitin-like conjugation systems: ATG12-ATG5-ATG16L and ATG8-phosphatidylethanolamine (LC3-PE a.k.a LC3-B). In the first system, ATG12 is conjugated to ATG5 through the action of ATG7 and ATG10, this conjugate then binds to ATG16L, which then participates in LC3-PE conjugation (Mizushima *et al.*, 1998a; Mizushima *et al.*, 1998b; Fujita *et al.*, 2008). In the second system, LC3 is generated from Pro-LC3 by the ATG4 protease, which is then conjugated to phosphatidylethanolamine by ATG7, ATG3 and the ATG12–ATG5– ATG16L complex (Ichimura *et al.*, 2000; Kabeya *et al.*, 2000; Tanida *et al.*, 2004). LC3-B is then localised to the autophagosomal membrane, in order to participate in the formation and elongation of autophagosomes. The completed autophagosome engulfs proteins and organelles targeted for degradation by autophagy receptor or adaptor proteins (Ichimura *et al.*, 2008; Kirkin *et al.*, 2009; Filimonenko *et al.*, 2010; von Muhlinen *et al.*, 2012). Once matured, the autophagosome fuses with the lysosome to form the autophagolysosome, in which the tethered proteins/organelles are digested by lysosomal enzymes. Termination of autophagy is achieved through reactivation of the mTOR pathway by nutrients (e.g., amino acids) generated from autolysosome.
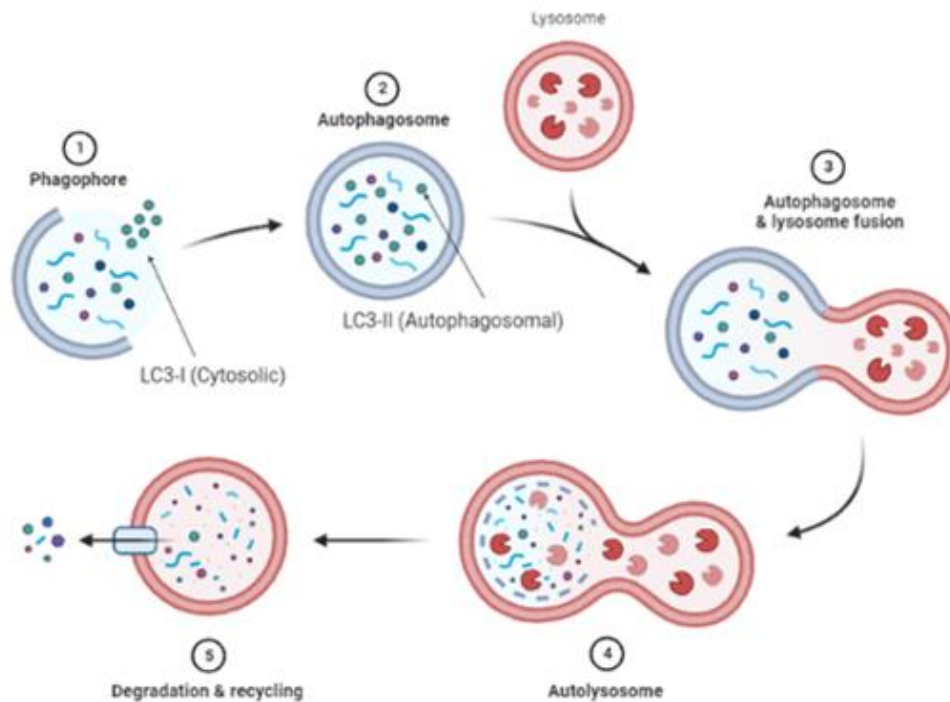
Figure 2.6 – Cellular autophagy is characterised by 5 distinct phases 1) Induction and LC3 Translocation: initiated by external/internal stimuli (e.g., nutrient depletion); 2) Autophagosome formation: Unwanted cytosolic proteins and aging organelles are sequestered by a double membrane vesicle, coordinated by complexes of autophagy proteins 3) Autophagosome and lysosomal fusion 4) Autolysosome: LC3 protein regulates traffic between autophagosome to lysosome. 5) Degradation: breakdown of the autophagic vesicle and its contents.

Autophagy is a conserved biological process that contributes to nutrient and cellular homeostasis. Under conditions of nutrient deprivation, upregulation of autophagy recycles proteins so that the products can be re-entered into energy yielding pathways to preserve energy homeostasis (Kim & Lee, 2014). Due to the central role of mTOR in the regulation of autophagy, amino acids play a particularly key part in this regulation. An increase in the presence of circulating essential amino acids (e.g., leucine) activates mTORC1, thereby inhibiting autophagy through distinct transcriptional, and post-translational mechanisms (Zoncu *et al.*, 2011; Bar-Peled *et al.*, 2012; B'Chir *et al.*, 2013). Conversely, during amino acid deprivation (e.g., during periods of prolonged fasting), autophagy is upregulated through translocation of TFEB,

a key regulator of lyosomal biogenesis, from the lysosome to the cell nucelus to induce transcription of genes related to autophagy (Settembre *et al.*, 2011).

Glucose metabolism is also closely related to autophagy at the cellular level. Under conditions of glucose deprivation, an increase of the cellular AMP:ATP ratio activates the energy-sensing AMPK, thereby inhibiting mTORC1, inducing autophagy (Gwinn *et al.*, 2008; Kim *et al.*, 2011; Hardie *et al.*, 2012). Primarily, it is thought that autophagy regulates glucose metabolism through gluconeogenesis derived from amino acids produced in autophagy (Ezaki *et al.*, 2011). Some evidence also suggests autophagy may participate in the breakdown of glycogen through the action of starch-binding domain-containing protein 1 (STBD1), which tethers glycogen to autophagic membrane structures for lysosomal degradation (Jiang *et al.*, 2010). Relatively less is known about the role of autophagy in lipid metabolism. However, some evidence suggests triglycerides may be liberated from the lipid droplet through a process called "lipophagy" (Singh *et al.*, 2009). A regulatory role of autophagy has been reported in reverse cholesterol transport (i.e., release of cholesterol from the lipid droplet for transport to the liver), as well as in the formation of cytoplasmic lipid droplets (Shibata *et al.*, 2009; Shibata *et al.*, 2010; Ouimet *et al.*, 2011). Importantly, feedback of nutrients generated as a result of autophagy act to inhibit autophagy, thereby preventing excessive breakdown of cellular components during periods of starvation.

Autophagy can be assessed by quantification of LC3 (ATG8). Specifically, this can be achieved through various techniques, including but not limited to: western blotting, fluorescence microscopy, immunohistochemistry, as well as flow and multispectral cytometry (Klionsky *et al.*, 2016). Autophagy is a highly dynamic, multi-step process, which can be modulated at several stages (Kim & Lee, 2014). Accumulation of autophagosomes (as seen by changes in GFP-LC3 puncta and LC3-B by transmission electron microscopy or western blot respectively) could either reflect a reduction in autophagosome turnover, or an imbalance between the rate of autophagosome turnover with increased autophagosome formation (Kovács *et al.*, 1986; Kovács *et al.*, 1987; Lucocq & Hacker, 2013). However, changes in GFP-LC3 or LC3-B may also result from the induction of a different pathway, such as LC3-associated phagocytosis (Bestebroer *et al.*, 2013). As such, it is generally recommended that the use of markers such as LC3-B to assess autophagy is complemented by measures of overall

autophagic flux, allowing for accurate interpretation of results (Klionsky *et al.*, 2016). Specifically, inhibition of autophagic flux can assess the accumulation of a given organelle, an organelle marker, a cargo marker, or the entire cargo at the point of blockage, which can then be compared to a non-inhibited control in order to assess the rate of autophagic flux (Seglen *et al.*, 1981; Eskelinen *et al.*, 2011). However, caution must be taken with this approach, as it does not assess complete autophagy due to the experimental block inhibiting lysosomal proteolysis, thereby preventing the evaluation of lysosomal functions.

Cyclic induction of autophagy provides a link between clock and metabolism. Due to the explicit role of autophagy in protein turnover, it is unsurprising that this process is characterised by diurnal rhythmicity. Timing cues that drive circadian autophagy are strongly related to clock and nutrition. Specifically, liver-specific knockdown of BMAL1 dampens rhythmic autophagy gene expression, and reduces autophagic flux, suggesting cell autonomous control of the endogenous circadian system upon autophagy (Ma *et al.*, 2011). Likewise, nutritional status (e.g., fast-fed cycles) is a potent entrainment signal for peripheral tissues, with autophagy tending to peak during the day in mice, a time during which their food intake is low (Ma *et al.*, 2011; Ryzhikov *et al.*, 2019). Furthermore, restricted feeding appears to reset the phase of the peripheral clocks alongside recovering the rhythmic expression of autophagy genes (Pfeifer, 1972; Ma *et al.*, 2011). Rhythmic transcriptional control of autophagy is one proposed mechanism of observed rhythms. In particular, rhythmicity of C/EBPβ stimulates autophagy gene expression, inducing protein breakdown in hepatocytes (Ma *et al.*, 2011; Ma & Lin, 2012). Furthermore, rhythmic transcription of TFEB, a key gene in lysosomal biogenesis has also recently been reported in skeletal muscle taken from humans under diurnal conditions (Perrin *et al.*, 2018a). Additionally, both mTOR and AMPK pathways undergo circadian regulation, although the role of this circadian regulation in the rhythmic control of autophagy remains relatively unexplored (Lamia *et al.*, 2009; Greenhill, 2019).

Rhythmic autophagy is coordinated with circadian regulation of energy homeostasis and proteome/organelle remodelling. Rhythmic autophagy contributes towards the maintenance of energy homeostasis. Under conditions of fasting-induced autophagy,

Atg7-deficient mice display diminished diurnal rhythmicity in branched chain amino acids and glucose in blood plasma, the liver, and skeletal muscle (Ezaki *et al.*, 2011). Disruption of the rhythm in autophagy through BMAL1 deletion is associated with impaired hepatic gluconeogenesis, leading to hypoglycaemia during the late light phase, when autophagic flux typically peaks (Pfeifer, 1972; Lamia *et al.*, 2008; Ryzhikov *et al.*, 2019). Autophagy is also linked to lipid metabolism, and whilst comparatively less is known about this relationship, it is likely that coordination of rhythmic autophagy with lipid metabolism further optimizes the supply of nutrients during cycles of fasting and feeding (Ma *et al.*, 2012; Rennert *et al.*, 2018). Aside from energy homeostasis, a role for cyclic autophagy in selective proteomic remodelling of the liver has also been proposed (Reddy *et al.*, 2006). Furthermore, the importance of cyclic autophagy has been evidenced in the rhythmic abundance of organelles such as mitochondria, peroxisomes, and endoplasmic reticulum (Uchiyama, 1990).

In summary, autophagy is a cellular process that is inherently important for maintaining energy balance through regulation of nutrient and cellular homeostasis. Importantly, this process is induced cyclically across the day, providing a novel link between clock and metabolism whereby rhythmic regulation of autophagy is coordinated with circadian regulation of energy homeostasis and proteome/organelle remodelling. However, no past research has investigated responses of autophagic flux to different feeding patterns in humans. Given the prominence of this process in multiple physiological pathways, it is important to now study the role of autophagy in mediating the transition between fasting and feeding in humans.

2.1.5 Circadian Rhythms in Energy Balance

The first law of thermodynamics states that energy cannot be created or destroyed, only transformed from one form to another. Necessarily, any shift in body weight is reflective of imbalance between energy entering and energy expended by the body. Therefore, there are 2 primary components that comprise energy balance: energy intake (EI) and energy expenditure (EE; radiant, conductive, and convective heat losses, as well as physical activity) (Hall et al., 2012).

2.1.5.1 Energy intake and appetite

Energy intake is comprised of the chemical energy ingested and absorbed from food and fluids. Energy intake is driven by appetite, which is a broad concept that can be characterised by the qualitative and sensory aspects of eating, as well as the degree of responsiveness to environmental factors alongside the homeostatic responses to physiological stimulation (Blundell et al., 2010). Within the concept of appetite there are several key terms: hunger (the drive/urge to eat); satiation (process that leads to meal termination); and satiety (process leading to inhibition of further eating) (Blundell et al., 2010).

Appetite can be assessed subjectively and objectively. Appetite sensations are subjective and are therefore typically assessed using validated visual analogue scales (VAS) referring to the individual components of appetite (Stubbs *et al.*, 1993). Scales employed to assess appetite in this way usually have a question e.g. "How hungry do you feel" on a 100 mm horizontal line, anchored with opposing extremes e.g. "I am not hungry at all" vs. "I have never been hungrier". This is an indirect measure of appetite, and it is therefore useful to combine this assessment technique with objective assessments such as the consumption of an *ad libitum* test meal. Notably, this combined method of assessing appetite is most suited for assessing appetite acutely (i.e., following a single or consecutive meals). Longer-term assessment of appetite can be performed with the use of weighed food records as these allow for free living quantification of energy intake, which can be employed over longer periods of time (e.g. during a dietary intervention) (Ortega *et al.*, 2015). However, under-reporting of energy intake is prevalent using this method, which can also influence the food behaviour (i.e., the types of food consumed) and therefore the use of weighed food records is not without limitation (Livingstone & Black, 2003; Ortega *et al.*, 2015).

The hypothalamus is the key region of the brain involved in appetite regulation. Specifically, the hypothalamic arcuate nucleus (ARC) acts to receive and integrate long- and short-term signals regarding appetite (Kaiyala *et al.*, 1995). The location of appetite inhibiting (pro-opiomelanocortin – POMC & cocaine-and amphetamine-regulated transcript - CART) and stimulating (agouti-related peptide – AgRP & neuropeptide Y -NPY) neurons within the ARC specialises this region for appetite regulation (Cone *et al.*, 2001). Importantly, this region of the brain has incomplete

isolation from the blood brain barrier, meaning the milieu of circulating gut hormones can stimulate these neurons (Haddad-Tovolli *et al.*, 2017).

In the postprandial state, satiety is regulated by a sensory system linking the gut to the appetite sensory centres within the hypothalamus. The gastro-intestinal tract acts in an endocrine fashion; the gut in particular is responsible for secreting a number of peptides in response to nutrient intake. These include: ghrelin, glucagon-like peptide-1 (GLP-1), pancreatic peptide (PP), peptide YY (PYY), cholecystokinin (CKK), and oxyntomodulin (Perry & Wang, 2012). Ghrelin is a 28-amino acid peptide released primarily from the stomach that acts to stimulate appetite (Kojima *et al.*, 1999). Ghrelin binds to the growth hormone receptor in the hypothalamus and is suggested to increase appetite through the binding with NPY/AgRP co-expressing neurons (Willesen *et al.*, 1999; Bailey *et al.*, 2000; Wren *et al.*, 2001b). The strong appetite-stimulating effects of ghrelin and the consequential increase in food intake (~30-70% increase) have been observed in both lean and obese humans (Wren *et al.*, 2001a; Druce *et al.*, 2005; Druce *et al.*, 2006).

Glucagon-like pepetide-1 is a product of the glucagon gene and is expressed in the most abundant source of endocrine cells in the gut, L-cells (Herrmann *et al.*, 1995). Secretion of GLP-1 is stimulated by the presence of nutrients in the lumen of the gut; however, some evidence suggests that additional neural or endocrine mechanisms may also stimulate secretion (Holst, 2007; Watkins *et al.*, 2021a). Generally, GLP-1 acts through the ARC as an appetite suppressant with both central- and peripheral-administration of GLP-1 resulting in enhanced satiety, as well as a reduction in food intake in humans (Larsen *et al.*, 1997; Vilsbøll *et al.*, 2007; van Bloemendaal *et al.*, 2014).

Pancreatic peptide is a 36-amino acid peptide primarily synthesized and secreted from the endocrine pancreas (Lonovics *et al.*, 1981). In the post-absorptive state, circulating concentrations are low and rise proportionally with feeding to suppress appetite (Track *et al.*, 1980; Batterham *et al.*, 2003). Within the hypothalamus, PP targets neuropeptide Y receptors with a specific affinity for the Y4 receptor, of which knockdown in mice completely abolishes food intake reduction in response to PP (Blomqvist & Herzog, 1997; Balasubramaniam *et al.*, 2006).

Peptide YY is a 36-amino acid peptide also generated and secreted from the L-cells of the intestine. The predominant circulating form is the 34-amino acid form (PYY3-36), which rises within ~1 h of feeding, influenced by both composition and energy content of the meal (Adrian *et al.*, 1985; Grandt *et al.*, 1994). Peripheral administration of PYY inhibits food intake via the Y family of G protein-coupled receptors expressed within the hypothalamic ARC (Keire *et al.*, 2000; Asakawa *et al.*, 2003; Lumb *et al.*, 2007).

CCK is synthesized and secreted from the duodenum and jejunum, where it acts locally to inhibit gastric emptying and stimulate gallbladder contraction in order to initiate satiety (Buffa *et al.*, 1976; Dufresne *et al.*, 2006). CCK is secreted mainly in response to the presence of the digestion products of proteins and lipids but also in response to gastric distension (Kissileff *et al.*, 2003; Kissileff *et al.*, 2019). With regards to the neurophysiological effects of CCK, it is abundant within the hypothalamus exerting regulatory effects on appetite through innervation of the paraventricular nucleus of the hypothalamus (PVH) (Moran & Schwartz, 1994; D'Agostino *et al.*, 2016).

Finally, oxyntomodulin (OXM) is co-secreted with GLP-1 in proportion to energy content of the ingested meal (Druce & Bloom, 2006). Central and peripheral administration reveals the appetite and food intake suppressive effects of OXM, alongside increasing energy expenditure chronic administration results in reduced body mass (Wynne *et al.*, 2005; Scott *et al.*, 2018).

Long-term endocrine signalling through leptin and insulin also acts to regulate appetite. Leptin is exclusively expressed by white tissue adipocytes and therefore acts as a chronic signal of adiposity in healthy humans (Baskin *et al.*, 1999). Primarily, leptin acts to inhibit appetite through both inhibition of the NPY/AgRP co-expressing neurons, and stimulation of the POMC-expressing neurons in the ARC (Jacob *et al.*, 1997; Thornton *et al.*, 1997; Sahu, 1998, 2003). Importantly, the action of leptin on these neurons is reflected in eating behaviour, with higher serum leptin levels correlating with greater feelings of fullness (Heini *et al.*, 1998). In accordance with the thermodynamic principles of energy balance, leptin is therefore said to be one of the

core physiological components that controls body (fat) mass in humans (Hall *et al.*, 2012). Insulin appears to play a similar role in longer-term feeding behaviour, with circulating levels also reflective of adiposity (Rocha *et al.*, 2011). Furthermore, insulin also acts to inhibit appetite through inhibition of appetite suppressing neurons (NPY/AgRP) (Kalra *et al.*, 1999; Loh *et al.*, 2017). Aside from these similarities, insulin also has a potentiating effect upon leptin, stimulating leptin release from WAT and therefore exerting a synergistic effect upon appetite (Heini *et al.*, 1998; Kieffer & Habener, 2000).

Despite the acute hormonal responses to regulate food intake, appetite does not appear to be simply regulated by a homeostatic energy balance mechanism. Rhythms in appetite have been observed in both forced desynchrony and constant routine protocols, whereby hunger typically peaks in the evening (~2000 h), when satiety is generally lowest; and hunger is lowest during the early hours of the morning (~0800 h) (Scheer *et al.*, 2013; Sargent *et al.*, 2016; Rynders *et al.*, 2020; Templeman *et al.*, 2021b). This robust rhythmicity occurs independent of time since waking, time between meals, and of energy content of the meal (Scheer *et al.*, 2013). Furthermore, this oscillatory pattern in hunger appears to match the typical pattern of energy intake in western society whereby energy intake is lowest in the morning and highest in the evening (NHANES, 2016). Furthermore, the diurnal pattern seen in these hormones acts to suppress hunger/promote fullness during sleep, when the delivery of nutrients is not expected (Templeman *et al.*, 2021b).

Notwithstanding robust rhythmicity in subjective appetite, regulatory hormones appear to track behavioural cycles, rather than circadian phase. Diurnal rhythmicity driven by meal intake has been observed in GLP-1. In a study of healthy normal weight individuals consuming 5 identical meals across the day, GLP-1 was at its lowest ~1000 h and rose to peak ~1700 h (Galindo Munoz *et al.*, 2015). Diurnal variation in PYY has been observed in women consuming 3 meals across the day, where PYY peaked at ~1400 h, falling to its lowest value ~0400 h (Hill *et al.*, 2011; Rynders *et al.*, 2020). Interestingly, the postprandial PYY response to each meal was correlated with energy content, therefore suggestive of a meal driven pattern. Rhythmicity in pancreatic peptide and cholecystokinin has been observed in humans with their respective peaks at 1500 and 1700 h (Johns *et al.*, 2006). Furthermore, day-night rhythmicity has been

observed for oxyntomodulin for mice, which again appears to be driven by the behavioural effects of meal timing (Landgraf *et al.*, 2015). However, no human evidence of this rhythm currently exists. Under conditions of forced desynchrony and constant routine, leptin and ghrelin also appear to track behavioural phase (i.e., meal timing)), rising during waking hours to peak at the onset of sleep (Shea *et al.*, 2005; Scheer *et al.*, 2009). Delayed meal timing results in proportional phase shifts in leptin supporting this notion (Schoeller *et al.*, 1997). A recent study employed a semi-constant routine (i.e., hourly feeding during waking hours), observing diurnal rhythmicity in leptin (peak: 0035 h trough ~ 1200 h) and ghrelin (peak 0826 h) that seemingly misaligned with diurnal rhythms in hunger and fullness (**Figure 2.7**) (Templeman *et al.*, 2021b). Phase separation between peaks in satiety hormones and ratings of hunger was also observed by Rynders *et al.* (2020). Collectively, the evidence therefore suggests that oscillations in subjective appetite follow a circadian phase and are regulated by the SCN, whereas the respective regulatory peptides are responsive to meal timing and therefore track a behavioural phase (Poggiogalle *et al.*, 2018).
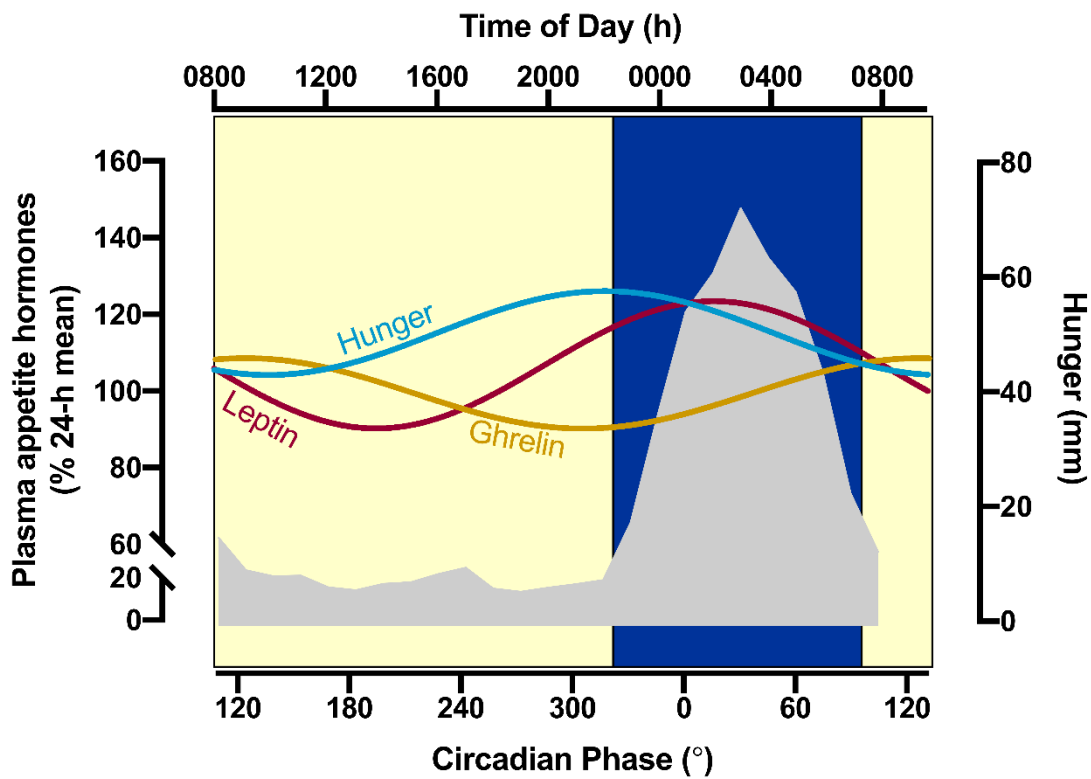
Figure 2.7 – Diurnal rhythms in unacylated ghrelin, leptin, and subjective hunger under conditions of semi-constant routine (i.e., hourly feeding during waking hours only) relative to melatonin profile (grey) and light/dark (yellow/blue respectively). Data re-illustrated from (Templeman *et al.*, 2021b).

2.1.5.2 Energy expenditure

Total energy expenditure (TEE) is comprised of 3 components: resting metabolic rate (RMR), dietary induced thermogenesis (DIT), and activity energy expenditure (AEE). RMR accounts for ~70% of Total EE (TEE) and is defined as the energy required to maintain normal cellular function, largely dictated by body size, composition, and recent energy imbalance (Johnstone *et al.*, 2005). Processing of ingested macronutrients necessarily requires energy expenditure, and this in what is referred to as DIT. This occurs in a hierarchal nature whereby isocaloric ingestion of protein results in a greater thermic effect than that of carbohydrate, which in turn is greater than that of fat (Westerterp-Plantenga *et al.*, 1999a; Westerterp, 2004a; Bray *et al.*, 2015). Normally, DIT is assumed to be a fixed percentage of EI (~10%), but variation

between and within individuals is common (Westerterp-Plantenga *et al.*, 1999a; Westerterp, 2004b). The remainder of TEE is made up of AEE, traditionally this is defined as any bodily movement produced by skeletal muscles that results in energy expenditure, however isometric thermogenesis (i.e., 'heat production as a result of increased muscle tension without performing any physical work') may also be included in AEE (Caspersen *et al.*, 1985; Dulloo *et al.*, 2017).

Energy expenditure is central to the regulation of body mass and health. This is apparent from observational evidence, showing those who are more physically active (i.e., expend more energy on a daily basis) are typically healthier than age- and BMI-matched individuals (Richardson *et al.*, 2004; Ischander *et al.*, 2007). This is reflected in the multitude of intervention strategies that focus on increasing energy expenditure to improve health (Jakicic & Davis, 2011; Elagizi *et al.*, 2020). Any scenario in which energy expended exceeds that of energy consumed would result in negative energy balance (i.e., weight loss) (Hall *et al.*, 2012). Typically, energy metabolism in humans is highly adaptive, such that the homeostatic response to weight loss or gain is in favour of energy balance (i.e., change in body mass is necessarily accompanied by a shift in RMR and therefore TEE), this is not only regulated by leptin but also by weight loading (i.e., gravitostat) (Hall *et al.*, 2012; Ohlsson *et al.*, 2020). However, weight gain can result in metabolic dysregulation such that positive energy balance leads into a positive feedback cycle, encouraging greater intake rather than less, although the mechanisms of this process are yet to be fully elucidated (Hall *et al.*, 2022).

Resting metabolic rate varies across 24-h. Logically, energy expenditure profiles across 24 h are reflective of sleep-wake cycles, whereby all components of energy expenditure are higher during waking hours relative to sleep. Due to its role in coordinating the central and peripheral clock machinery, higher core temperature is observed during waking hours relative to sleep (Buhr *et al.*, 2010). Constant routine protocols suggest rhythmicity is present in RMR, unmasking highest heat production, as well as oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$), during the biological morning (Krauchi & Wirz-Justice, 1994; Spengler *et al.*, 2000). However, a recent 26-h CR protocol observed no rhythm in EE across the experimental period (Rynders *et al.*, 2020). This is likely explained by the provision of hourly/regular snacks

during CR, which contradicts the recommendations for best practice in assessing resting metabolic rate (Jequier *et al.*, 1987; Compher *et al.*, 2006). Stronger evidence for rhythmic RMR therefore, comes from a recent 21 day forced desynchrony protocol, in which participants underwent eighteen, 28-hour days, with the equivalent of 10 hours sleep opportunity per 24 hours (Zitting *et al.*, 2018). Employment of this protocol revealed REE was lowest during the late biological night and was at its highest ~12-h later in the biological afternoon-evening.

The thermic effect of food displays morning evening-difference. Several studies report a clear morning evening difference whereby DIT is ~20-44% higher in the morning relative to an identical meal in the evening (Romon *et al.*, 1993; Bo *et al.*, 2015; Morris *et al.*, 2015a). Using circadian misalignment, Morris *et al* (2015a) reported no significant influence of behavioural cycle upon the DIT independent of endogenous circadian phase, suggesting the morning-evening difference is primarily driven by the endogenous circadian system. However, in this study the morning meal was preceded by a 12-h fast relative to only an 8.5-hour fast for the evening meal, suggesting that behavioural factors were not completely controlled for (Morris *et al.*, 2015a). Recent evidence indicates that this is apparent rhythmicity in diet-induced thermogenesis can be accounted for by the underlying circadian variation in resting metabolic rate (Ruddick-Collins *et al.*, 2021).

Physical activity thermogenesis is higher throughout waking hours in individuals with a normal sleep cycle. Logically, most physical activities cannot be performed when sleeping, contributing to the day-night variation. Therefore, studies investigating activity patterns across the day have focused on the daytime. In particular, one week of physical activity monitoring in males aged 71-91 years revealed higher moderate-vigorous and light- intensity physical activity during the morning (peak 1000-1100 h), with sedentary behaviours increasing throughout the day (Sartini *et al.*, 2015). However, an investigation of physical activity patterns in 45 - 65-year-olds revealed several contrasting patterns of daily PA (Jansen *et al.*, 2018). This is unsurprising given that timing of physical activity is highly dependent on external factors (e.g., work) (Sherwood & Jeffery, 2000). Understandably, it is difficult to study diurnal patterns in physical activity under controlled conditions due to the free-living nature of most

activities; daily activity patterns are therefore likely driven by behaviour, rather than an internal circadian clock. Interestingly, evidence is emerging of a relationship between time of day and physical activity in the opposite direction, whereby the response to exercise is dependent upon time of day - this will be covered in more detail in Chapter 2.1.5.3.

2.1.6 Zeitgebers in circadian physiology

Zeitgeber directly translates to 'time giver' and refers to external time cues that can phase shift and thereby entrain circadian rhythms. Aschoff's (1954) criteria can be used to characterise given behaviours as zeitgebers or not. Firstly, if a zeitgeber is switched off, the biological periodicity, if sustained, should begin to deviate from the ~24-h period duration. Necessarily, due to entrainment, some rhythms may persist for a short while after switching off a zeitgeber. Conversely, switching on a zeitgeber results in entrainment to periodicity of that zeitgeber. Secondly, reversal of a continuously periodic zeitgeber or environmental conditions (e.g., light-dark) must lead to an inversion in the biological rhythm, temporal shifts of the zeitgeber also result in phase shift of rhythms (both delay and advance must be observed). Finally, depending on the strength of the zeitgeber, changes in biological rhythm should be expected with an increase or decrease of the zeitgeber frequency. Additionally, removal or adjustment of the zeitgeber and other masking factors must occur in order to determine the true effects of the zeitgeber (Aschoff, 1954). In the context of the deleterious health consequences of circadian misalignment, the practice of synergistic use of zeitgebers to potentially reduce the chronobiological strain is an important consideration. Notably, the most commonly reported zeitgebers in the literature include light, sleep, exercise, and meal timing.

2.1.6.1 Light

Fundamentally, light is a form of radiation within a specific range of the electromagnetic spectrum that acts to align circadian clocks in the SCN and peripheral tissues through photosensitive retinal ganglions located in the eye (Pickard & Sollars, 2012; Do, 2019; Nguyen et al., 2022).

Light exposure regulates both the phase of circadian rhythms as well as suppressing melatonin. The SCN appears to be particularly sensitive to the phase shifting effects of light in the biological morning (phase advance of ~2 h) and biological evening (phase delay of ~3 h) (Duffy & Czeisler, 2009). Light exposure also acts to regulate circadian rhythms independently from phase shifting of circadian clocks through altering melatonin production. Specifically, whereas exposure to light suppresses melatonin, dim-light stimulates melatonin production. Notably, the relationship between melatonin and light conditions indicates the role of melatonin in regulating sleep in humans with pinealectomy resulting in negligible melatonin levels and associated decrements in sleep (Macchi *et al.*, 2002). Furthermore, whereas presence of melatonin during the day is strongly associated with daytime naps (Lockley *et al.*, 1997), pharmacologically induced suppression of melatonin production during the day diminishes sleepiness, with exogenous melatonin restoring night-time sleep (De Leersnyder *et al.*, 2001a; De Leersnyder *et al.*, 2001b). It is thought that the tight association between circadian melatonin profiles and sleep propensity are, in part, mediated by alterations in thermoregulation, such that an increase in melatonin production results in a decrease in core body temperature via heat loss induction (Kräuchi *et al.*, 2000). Finally, not only do acute lighting conditions alter phase shifting and melatonin suppression, but evidence also suggests that previous photic history alters sensitivity of melatonin suppression and the ability of the circadian clock to phase reset (Chang *et al.*, 2011).

Rhythmic melatonin production persists under inverse light-dark cycles, highlighting its use as a reliable means of estimating the timing of the internal circadian clock within the SCN (Benloucif *et al.*, 2008; Rahman *et al.*, 2018). Furthermore, circulating levels of melatonin are remarkably robust to various external factors, such as diet, further evidencing their utility in assessing circadian rhythmicity in humans (Kräuchi *et al.*, 2002). As such, it is common within circadian research to express physiological rhythms relative to melatonin profiles. More specifically, observed rhythms are adjusted based on the dim-light melatonin onset (DLMO), which is predictably defined as the start of melatonin production in response to evening dim-light conditions (Danielsson *et al.*, 2012). In doing so, rhythms can be reported based on circadian phase, accounting for inter-individual variance in rhythmicity, thereby increasing the

generalisability of such measures (Otway *et al.*, 2011; Wehrens *et al.*, 2017a; Templeman *et al.*, 2021b).

Glucose metabolism is intrinsically linked with cycles of light dark. Dampened, or abolished glucose rhythms in rats following pinealectomy highlights the role of the light-melatonin axis in regulating glucose metabolism across the day (la Fleur *et al.*, 2001). In humans, low melatonin (i.e., during the circadian day) is associated with higher postprandial *β*-cell function and insulin sensitivity (Garaulet *et al.*, 2020). Forced desynchrony through daytime sleep resulted in a 2-fold decrease in insulin sensitivity (SI) relative to a group maintaining nocturnal sleep patterns (Leproult *et al.*, 2014). Equally, a 12-h inverted behavioural and environmental cycle (i.e., night shift work), resulted in a reduction in glucose tolerance in both healthy adults and chronic shift workers, explained in part by a decrease in pancreatic β-cell function and insulin sensitivity (Morris *et al.*, 2015b; Morris *et al.*, 2016). Furthermore, acute manipulation of light in the morning and evening also holds the ability to disrupt glucose metabolism, resulting in 30% and 19% increase in insulin resistance respectively (Cheung *et al.*, 2016; Harmsen *et al.*, 2022).

Lipid metabolism is also altered in response to light exposure. Melatonin is responsible for orchestrating the relationship between lipid homeostasis and light exposure, influencing transcription in the liver and intestine (Hong *et al.*, 2020). Constant bright light exposure in rats increases circulating lipid levels, abolishes circadian rhythms in plasma fatty acids, and increases fat mass (Dauchy *et al.*, 2010; Fonken *et al.*, 2010). In humans, bright light exposure before and during breakfast increases postprandial triacylglycerol levels in type 2 diabetes mellitus patients and healthy young men (Versteeg *et al.*, 2017). Manipulation of light exposure in humans also modulates lipid oxidation, which may be modulated by altered secretion of melatonin in response to different light stimuli (Ishihara *et al.*, 2021).

Energy balance is related to light exposure. Exposure to bright light typically coincides with the waking period in humans, such that energy expenditure and energy intake are typically highest during this period; it is therefore unsurprising that a strong relationship between light exposure and body weight exists (Reid *et al.*, 2014). Animal models have demonstrated exposure to constant light stimuli resultants in increased food intake

during the typical sleep period, alongside a decrease in energy expenditure, and therefore weight gain, despite similar energy intake to rodents maintaining a normal light-dark cycle (Fonken *et al.*, 2010; Coomans *et al.*, 2013a). Likewise, supplementation of melatonin in animal models improves long-term body weight outcomes (Tan *et al.*, 2011). Interestingly, it appears that the effect of light on body mass is mediated by changes in energy expenditure rather than reduced energy intake (Terrón *et al.*, 2013). Reversal of the wake-sleep cycle by stimulated night shift work results in a reduction in total daily energy expenditure (McHill *et al.*, 2018). Along with similar energy intake, and decreased levels of the satiety hormones leptin and peptide YY, reversal of light/dark cycles appears to create positive energy balance (McHill *et al.*, 2018). Furthermore, manipulation of morning/evening light stimuli may attenuate the increase in hunger associated with sleep restriction by decreasing ghrelin concentrations (Figueiro *et al.*, 2012).

The longer-term implications of light exposure on circadian metabolism remain an important area of investigation (Webler *et al.*, 2019). Whilst light has arguably the strongest entrainable effect on circadian metabolism, it is apparent from studies investigating circadian misalignment that it is the interaction of light with other established zeitgebers (e.g. sleep, meal timing) that broadly regulates our metabolism across the day (McHill *et al.*, 2018). In particular, a recent review by Lewis *et al* (2020) suggested that studies investigating the effects of meal timing on circadian rhythms should report light exposure throughout experimental protocols in order to confirm or reject a behaviour as a zeitgeber (Aschoff, 1954).

2.1.6.2 Sleep

Sleep is an actively regulated, periodically occurring state of reduced consciousness, muscle relaxation, and altered responsiveness to stimuli, occurring in humans (Zielinski *et al.*, 2016). Sleep is inherently linked with light exposure, evidenced by broad alignment between cycles of sleep-wake and dark-light respectively. The sleep-wake cycle, therefore, is perhaps one of the clearest examples of the circadian clock in humans. Sleep duration in mammals generally depends on the size of the animal (Herculano-Houzel, 2015).

The architecture of sleep can be divided into 2 types, non-rapid eye movement (nREM – comprised of 3/4 stages), and rapid eye movement (REM). These 2 distinct types of sleep, and their respective stages are evident from electroencephalogram (EEG) trace (i.e., electrical activity) of the brain. During wakefulness, brainwaves emanating from the cortical region of the brain are typically desynchronised, high-frequency, low-amplitude waves (~14-30-Hz) (Fuller *et al.*, 2006). It is thought that this is reflective of cognitive, motor, and perceptual functions that occur during waking. Stage 1 nREM sleep is defined as the switch from wakefulness to sleep. This stage is characterised by a reduction in conscious awareness of the external environment, reduction in heart rate, breathing and eye movements as well as a general slowing of the EEG trace (~4- to 7-Hz theta) (Zielinski *et al.*, 2016). More time is spent in stage 2 nREM than other sleep stages and is typically a lighter period of sleep that precedes deeper sleep. Heart rate and breathing continue to slow, body temperature drops, eye movements cease, and muscles relax further concomitant with the appearance of "sleep spindles" and "K-complexes" in the EEG (i.e., brief bursts of brain activity) (Schönauer & Pöhlchen, 2018; Lechat *et al.*, 2021). Stages 3 and 4 nREM, therefore, is a period of deep sleep typically occurring during the first half of the night. Heart and breathing rate are at their lowest levels and delta brain waves are apparent from the EEG trace (~1-3 Hz; slow-wave sleep - SWS) (Zielinski *et al.*, 2016). Rapid eye movement sleep cycles periodically with nREM and is predominantly characterised by a switch to high-frequency, low amplitude EEG, more consistent with those seen during waking (Zielinski *et al.*, 2016). In contrast to wake, electrooculogram (EOG) reveals rapid eye movements and electromyogram (EMG) reveals atonia of skeletal muscle tissue with the exception of extraocular, inner-ear and respiratory muscles, which are still required to function (Aserinsky & Kleitman, 1953). Furthermore, breathing rate increases and becomes more irregular, and heart rate and blood pressure increase to near waking levels (Zielinski *et al.*, 2016).

There are two internal biological mechanisms that interact to regulate sleep. The first of these mechanisms is the homeostatic control of sleep (a.k.a. sleep propensity), which functionally describes the homeostatic drive to sleep (Figure 2.8). Secondly, circadian rhythms act to control cycles of sleep-wake through the temporal organisation of neurobehavioral, physiological, and biochemical factors (Fuller *et al.*, 2006). It is the interaction throughout the day between these two processes that

regulates cycles of sleep-wake, whereby one reflects increased feelings of sleepiness (i.e. homeostatic drive to sleep) and the other the output of the circadian clock (i.e. the drive to stay awake) (Dijk & Czeisler, 1995). It is thought that the somnogen adenosine plays a critical role in sleep propensity across the day (Porkka-Heiskanen *et al.*, 1997). In particular, adenosine accumulates throughout waking hours, which upon reaching a sufficient concentration inhibits neural activity in the wake promoting circuitry within the forebrain, thereby activating sleep-promoting mechanisms (Saper *et al.*, 2001). Unsurprisingly due to the inherent relationship between cycles of light-dark and sleep-wake, circadian rhythmicity in melatonin is one of the primary regulators of sleep-wake cycle; low presence of circulating melatonin during the day due to bright light exposure maintains wakefulness and increased presence with dim-light increases the drive to sleep (De Leersnyder *et al.*, 2001a; De Leersnyder *et al.*, 2001b). Interestingly, circadian rhythmicity in sleep propensity and sleep structure has been observed in humans through forced desynchrony protocols. Cajochen *et al* (2002) reported that the circadian clock facilitates frontal EEG activation during the waking period through the generation of an arousal signal, thereby preventing intrusion of low-frequency EEG that become more prominent throughout the day, driving the sleep homeostat. Using similar methods, circadian variation has been observed in in EEG during REM, with the nadir in alpha activity (i.e. 8.25-10.5 Hz) coinciding with the end of the period of highest circulating melatonin (i.e. close to the crest of REM) (Dijk *et al.*, 1997). Conversely, during nREM sleep, variation in EEG activity (~0.25 - 11.5 Hz) is predominantly dependent on prior sleep rather than circadian phase, with lowest values coinciding with the phase of melatonin secretion. Furthermore, high-amplitude circadian rhythms in the frequency range of sleep spindles antiphase to melatonin rhythm were observed (Dijk *et al.*, 1997).
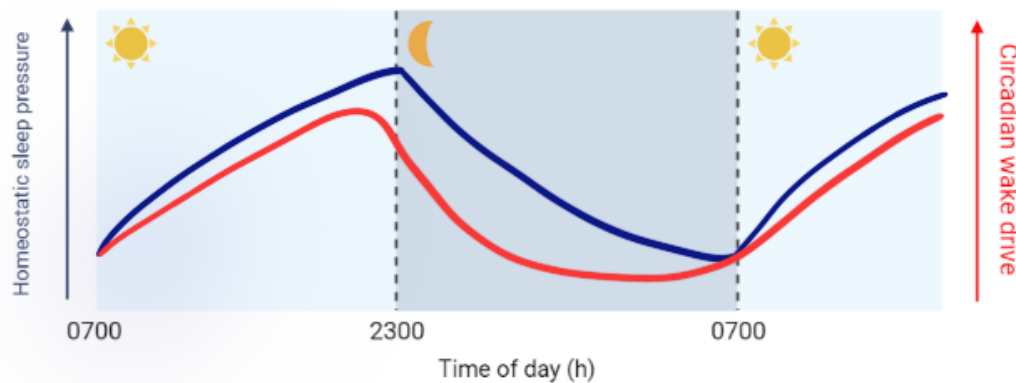
Figure 2.8 – Figure depicting the diurnal profiles of homeostatic sleep pressure and circadian wake drive (i.e., arousal).

The function of sleep is multifaceted. Sleep appears to have multiple purposes not only within a physiological context but also with regards to learning, memory consolidation, and motor skills (Sejnowski & Destexhe, 2000). From a metabolic standpoint, sleep can be seen as a recovery period whereby the body is able to deal with cell damage and fuel depletion that occurs during waking hours (Sharma & Kavuru, 2010). Necessarily, this evident from contrasting substrate metabolism and energy expenditure to wake. Logically, sleep is proposed to conserve energy and therefore energy expenditure during sleep is lower than that during wake, which may allow for recovery from wake-associated cell damage (Berger & Phillips, 1995; Jung *et al.*, 2011). Sleep is also characterised by a dramatic decrease (~30-40%) in glucose utilisation, alongside similar or increased systemic glucose and insulin levels, primarily driven by the reduced fuel demands of the brain, but also due to reduction in skeletal muscle tone (Boyle *et al.*, 1994; Simon *et al.*, 1994; Scheen *et al.*, 1996; Van Cauter *et al.*, 1997). Under normal conditions of nocturnal sleep, the effects of circadian rhythmicity and sleep are superimposed. Employment of an inverted sleep-wake cycle protocol results in a similar profile of decreased glucose tolerance during sleep-deprivation (i.e., the usual nocturnal sleep period), suggesting a robust time-of day effect. Furthermore, a sharp increase in systemic glucose during daytime sleep highlights the sleep dependent effects upon glucose metabolism (Van Cauter *et al.*, 1991).

Collectively, the effects of sleep on appetite regulating peptides such as ghrelin and leptin act in favour of energy conservation during overnight fasting. Feeding and sleep

are mutually exclusive events that occur periodically during a 24 h period. Despite this, sleep regulates energy balance regulation through modulation of the 24 h secretion of leptin and ghrelin. Under normal feeding circumstances, 24 h leptin displays diurnal rhythmicity whereby circulating levels increase throughout the day culminating in a nocturnal maximum (~0000 h), reflecting the impact of meal intake (Schoeller *et al.*, 1997; Templeman *et al.*, 2021b). This rise in leptin during sleep is driven by both the circadian clock, as well as sleep-related mechanisms. A study employing an 8 h shift in bedtime alongside continuous enteral nutrition delineated the effects of sleep and the circadian clock showing that elevation in leptin over 24 h results from both the circadian clock as well as sleep (Simon *et al.*, 1998). Whilst leptin is mostly tonic (as opposed to episodic) in its effects on appetite, it has been proposed that the acute elevation in circulating leptin concentrations during sleep facilitates the maintenance of prolonged fasting overnight (Morselli *et al.*, 2012). Interestingly, it also appears that sleep architecture is influenced by leptin secretion, with administration of exogenous leptin in lean rats increasing slow wave sleep, whilst decreasing REM; likewise, leptin deficiency disrupts typical sleep architecture (Sinton *et al.*, 1999; Laposky *et al.*, 2006). Sleep also appears to have a stimulating effect on total ghrelin secretion (Cummings *et al.*, 2004; Liu *et al.*, 2008a; Spiegel *et al.*, 2011). However, in contrast to the typically appetite-stimulating effects of ghrelin, it is likely that the sleeping increase in ghrelin acts to decrease energy expenditure (Lim *et al.*, 2010). In particular, the sleeping profile of ghrelin appears to be the result of a post-meal rebound with an inhibitory effect of sleep. A reduction in the ratio between acylated and total ghrelin during sleep suggests a decrease in the activity of ghrelin-O-acyl-transferase (GOAT), thereby reducing the appetite-stimulating effects of this hormone (Lim *et al.*, 2010; Spiegel *et al.*, 2011). This is further supported by a recent study of humans under controlled diurnal conditions, in which a gradual increase in unacylated ghrelin concentration was observed throughout the night (Templeman *et al.*, 2021b)

Models of sleep restriction highlight the importance of sleep quality and quantity on metabolism and health. Differing models of sleep disruption have been employed across the literature. Generally, sleep deprivation refers to a limitation of sleep duration throughout the night, with studies either employing partial (i.e., limiting the habitual duration) or total (i.e., no sleep) deprivation in order to investigate the importance of

sleep. Partial sleep deprivation can either be achieved by prescribing sleep opportunity in a single 'bolus' (e.g., 4 h sleep opportunity) or in broken sleep (e.g., 2 periods of sleep separated by an extended waking interval) (Donga *et al.*, 2010; Sweeney *et al.*, 2017). A further method of sleep disruption involves sleep fragmentation, whereby sleep is disrupted by more regular, but shorter waking periods (e.g., waking every hour for ~5 mins) (Stamatakis & Punjabi, 2010; Gonnissen *et al.*, 2013). Whilst the extent of metabolic perturbation largely depends on the model of sleep disruption employed, this generally allows for wider understanding of the dose response relationship between sleep, metabolism, and health.

Restriction of sleep appears to have a particularly potent effect on next-day glucose control. Total sleep deprivation, for example, impairs fasting glucose values in a duration dependent manner, with higher fasting glucose reported after 120 h but not after 24-72 h of sleeplessness (Kuhn *et al.*, 1969; Vondra *et al.*, 1981; Wehrens *et al.*, 2010; Benedict *et al.*, 2011). However, it appears that the effects of total sleep deprivation are more evident when examining postprandial glucose control, with studies reporting either elevated postprandial glucose responses and/or reduced insulin sensitivity following 24-126 h of sleeplessness (Kuhn *et al.*, 1969; VanHelder *et al.*, 1993; Wehrens *et al.*, 2010; Benedict *et al.*, 2011). Partial sleep deprivation also perturbs glucose metabolism the following day, with studies reporting impaired glucose clearance and whole-body insulin sensitivity with as little as a single night of disrupted sleep or sleep fragmentation (Donga *et al.*, 2010; Gonnissen *et al.*, 2013; Wang *et al.*, 2016; Sweeney *et al.*, 2017).

Equally, disrupted sleep alters both fasting and postprandial metabolism of lipids. Total sleep deprivation increases circulating lipid species (e.g., phosphatidylcholine) and triacylglycerides (Davies *et al.*, 2014; Chua *et al.*, 2015). Partial sleep restriction also perturbs lipid metabolism, impairing postprandial lipaemia and adipocyte function, and increasing circulating fatty acid intermediates (Broussard & Brady, 2010; Bell *et al.*, 2013; Ness *et al.*, 2019).

Appetite regulatory peptides are also altered by sleep restriction, with total sleep and partial deprivation resulting in a reduction in the amplitude of diurnal variation in leptin (Mullington *et al.*, 2003; Spiegel *et al.*, 2004; Pejovic *et al.*, 2010). Likewise, ghrelin is

also affected by sleep disruption, typically resulting in higher ghrelin levels relative to a habitual night of sleep (Spiegel *et al.*, 2004; Schmid *et al.*, 2008). Interestingly, Benedict *et al* (2011) reported that the elevation in ghrelin with total sleep deprivation occurs in rebound to an initial inhibition of total ghrelin levels during the first half of the night. With the observed changes in appetite regulatory peptides, it is unsurprising that ratings of hunger increase with increased levels of acute sleep deprivation (Schmid *et al.*, 2008). Specifically, Spiegel *et* al (2004) reported an 18% decrease in leptin accompanied by a 23% increase in ghrelin with partial sleep deprivation resulted in an ~23% increase in hunger, which was primarily driven by increased ghrelin:leptin.

It appears that sleep deprivation (both partial and total) favour increased energy intake. Studies of acute partial sleep deprivation in men and women support this, reporting a ~20% increase in energy intake during the subsequent day (Bosy-Westphal *et al.*, 2008; Brondel *et al.*, 2010). A recent meta-analysis of all partial sleep deprivation studies investigating energy intake found that partial sleep deprivation resulted in an ~385 kcal·d^{-1} (95%CI 252-517 kcal·d^{-1}) increase in energy intake (Al Khatib *et al.*, 2017). Studies reporting greater energy intake following a night of sleep deprivation restricted sleep through earlier waking time, such that participants were awake for a longer duration before feeding relative to the habitual sleep group (Nedeltcheva *et al.*, 2009; St-Onge *et al.*, 2011). In contrast, the one study to report no change in food intake following 2 nights of 4-h sleep opportunity relative to 2 nights of 8-h sleep opportunity with participants woken at the same time in both conditions (Schmid *et al.*, 2009). As rhythms in appetite regulatory hormones tend to favour energy intake during waking hours it is possible that methodological differences in sleep deprivation may explain the apparent increase in energy intake (Templeman *et al.*, 2021b).

Despite a relatively clear effect of sleep disruption on energy intake, the evidence surrounding the effects of sleep curtailment on energy expenditure are conflicting. In particular, it appears that the individual components that comprise energy expenditure are differentially affected. Of the studies employing total sleep deprivation, Benedict *et al* (2011) reported a reduction in RMR and postprandial EE. Conversely, Jung *et al* (2011) reported *higher* postprandial EE and 24-h energy expenditure, which when analysed further was driven primarily by increased night-time energy expenditure. With

regards to partial sleep deprivation, Nedeltcheva *et al* (2009) and St-Onge *et al* (2011) both reported no change in free-living EE with partial sleep deprivation as assessed with doubly-labelled water, nor RMR assessed via indirect calorimetry. However, in the latter study, a trend for reduced peak activity alongside a reduction in the time spent in high intensity physical activity during the 6-day period of restricted sleep was apparent (St-Onge *et al.*, 2011). Notably, further studies of partial sleep deprivation suggest that sleep disruption *per se* is not sufficient to disrupt RMR, and that circadian disruption alongside partial sleep deprivation may be necessary to alter RMR and potentially TEE (Buxton *et al.*, 2010; Buxton *et al.*, 2012; Zitting *et al.*, 2018). With regard to the substrates that contribute towards energy expenditure, it appears that the effects of sleep disruption are not quite clear, with one study reporting that fragmentation of sleep increases respiratory quotient and carbohydrate oxidation and reduced fat oxidation (Hursel *et al.*, 2011).

Given the collective acute effects of sleep disruption on metabolic control, it is unsurprising that longer term disruption of sleep (i.e., typically seen in rotational/shift workers) is associated with poorer health outcomes. Epidemiological evidence highlights a relationship between sleep and health whereby those sleeping less, or at unusual times, are at higher risk of mortality, obesity, type 2 diabetes, hypertension, cardiovascular disease, and coronary heart disease (Itani *et al.*, 2017). Sleep is therefore an important factor when considering the effectiveness of interventions to improve health. For example, Nedeltcheva *et al* (2010) studied the effect of sleep duration (5.5 *vs* 8.5 h sleep opportunity) on weight-loss achieved via moderate calorie restriction. Whilst total weight loss was similar between groups, restriction of sleep to 5.5 h per night for the 2 week period undermined the loss of adiposity seen in the control group, thereby resulting in greater fat-free tissue loss (Nedeltcheva *et al.*, 2010). This suggests that sleep contributes towards the maintenance of fat-free body mass during times of decreased energy intake, therefore supporting the role of sleep as a means of energy conservation/restoration in humans.

In summary, sleep is a critical facet of daily life for many reasons. In particular, it appears that the collective physiological effects of sleep upon multiple facets of metabolism act to conserve energy. Importantly, studies of sleep restriction, particularly partial sleep deprivation, highlight the metabolic importance of sleep.

However, the more common occurrence of sleep fragmentation is relatively under-researched, especially in relation to its influence on next day metabolic control and therefore requires further investigation.

2.1.6.3 Exercise/Physical activity

In a similar fashion to sleep, patterns of physical activity and/or exercise broadly align with cycles of light/dark. Therefore, it is unsurprising that exercise displays a strong relationship with circadian metabolism.

Time of day alters the molecular physiological response to exercise as well as exercise outcomes. As exercise is typically characterised by major physiological changes, circadian rhythmicity directly influences the response to exercise (Wolff & Esser, 2019). Studies in humans have revealed robust rhythms in both skeletal muscle oxidative metabolism and strength (Giacomoni *et al.*, 2005; van Moorsel *et al.*, 2016). At the molecular level, established exercise response pathways (i.e., *mTORC1* and *PGC1*) have been identified as being downstream of the molecular clock, thereby facilitating time-of-day effects upon the response to exercise (Um *et al.*, 2011; Ramanathan *et al.*, 2018). Additionally, timing of exercise can also modulate the outcomes of regular exercise, with a recent study in mice reporting that exercise training late in the active phase attenuated the accumulation of adipose tissue typically seen on a high-fat diet relative to exercise early in the active phase, which may be explained by the established rhythm in skeletal muscle oxidative capacity, peaking in the evening (i.e. late in the active phase) (van Moorsel *et al.*, 2016; Dalbram *et al.*, 2019).

Exercise also acts as a circadian time cue, with several core clock genes showing responsiveness to acute bouts of exercise. In particular, *BMAL1*, *PER2*, and *CRY1* seem to be responsive to acute exercise. Zambon *et al.* (2003) reported increased expression of *BMAL1*, *PER2*, and *CRY1* in leg muscle following 10 sets of 8 repetitions of isotonic knee extension at 80% of 1-repetition maximum. Similarly, assessment of clock gene expression in human leukocytes in response to acute exercise found that *BMAL1* was upregulated following morning *and* afternoon exercise, whereas *CRY1* expression was only upregulated by morning exercise (Tanaka *et al.*, 2020). Recent

meta-analysis of the effects of exercise on human skeletal muscle clock gene expression, alongside data from electronic muscle stimulation in myotubes of mice, provide further evidence that exercise acutely increases the expression of and phase shifts *PER2* expression (Small *et al.*, 2020). Conversely, not all clock genes appear to be responsive to exercise, with little evidence of a response in CLOCK, *PER1*, and *CRY2* following acute exercise (Zambon *et al.*, 2003; Tanaka *et al.*, 2020).

Several mechanisms for the time cue effects of exercise have been proposed. Exercise is implicitly linked with circadian metabolism through the energy sensing properties of AMPK, which is activated by exercise to restore energy balance (Kahn *et al.*, 2005; Long & Zierath, 2006). AMPK targets the *CRY* proteins for degradation, thereby providing a molecular mechanism through which exercise can regulate the molecular clock (Lamia *et al.*, 2009). Recent evidence also suggests that the effects of exercise (i.e. muscle contraction) on skeletal muscle clocks are mediated through calcium-dependent channels (Small *et al.*, 2020). Pharmacological treatment of myotubes to increase cytosolic calcium concentrations mimicked the exercise-induced upregulation of *PER2* expression, whereas treatment to block calcium channels negated the effect of stimulation-induced contraction on *Per2* expression (Small *et al.*, 2020).

2.1.6.4 Meal timing

Meal timing is a key consideration that can be referred to in both absolute and relative terms. Typical considerations of diet include both the quantity (i.e., how much we eat) and quality (what we eat). However, a growing body of evidence now implicates a third consideration of diet, timing (when we eat). Meal timing can be referred to in both absolute terms (i.e., given time of day of meals - clock time), as well as relative terms (i.e., relative to other related events across the day such as sleep, exercise, and critically, other meals).

2.1.6.4a Absolute meal timing

Time of day alters the physiological response to a meal. Given the influence of molecular clockwork across multiple tissues and metabolic processes it is unsurprising that the absolute (i.e., clock time) time of eating elicits differential physiological

responses across the day. This is evident from studies comparing the physiological responses to identical meals consumed at different times of day (Leung *et al.*, 2019). For example, Van Cauter *et al.* (1992) reported twofold greater total area under curve and 2-h area under curve for glucose and insulin in the evening than in the morning. Data from mice suggests that transcripts driven by food intake fail to sustain their rhythms in the absence of food intake and therefore display circadian and ultradian rhythmicity that tracks feeding behaviour (Vollmers *et al.*, 2009). However, the effect of rhythmic feeding behaviour is not independent of the endogenous clock, which modulates the expression of these food driven transcripts. Specifically, feeding increases the amplitude of core clock oscillations and their immediate target genes, thereby causing robust rhythmic transcription. The circadian clock then modifies and enhances the transcriptional response to feeding cues (Lamia *et al.*, 2009; Vollmers *et al.*, 2009). Therefore, it is the interaction between the clock and feeding patterns that ultimately determines the wider circadian transcriptome.

Evidence from a human study of shifting meal timing supports mouse data in showing that transcripts in peripheral tissues track feeding behaviour. Wehrens *et al.* (2017a) investigated clock gene expression as well as rhythmicity in glucose, insulin and triglycerides during 2 constant routine protocols. One following a lead-in diet with standardised meal timings at 0.5, 5.5, and 10.5 h after waking, and the other following a lead-in diet where all meals were delayed by 5 h. Cosinor analysis revealed a significant effect of meal timing on *PER2* phase in adipose tissue, but not on the phase of *PER3* or *BMAL1*. Shifting mealtimes by 5 hours also resulted in a corresponding 5-hour shift in glucose acrophase, whilst also reducing blood glucose concentration during the follow up constant routine (Wehrens *et al.*, 2017a). Similarly, Gu *et al.* (2020) found a 4-h shift in dinner timing resulted in a corresponding 4-h delay in postprandial glucose and insulin peak, further supporting the notion that rhythms largely track feeding behaviour.

Meal timing targets the peripheral clock rather than the central clock. Whilst rhythmicity of the central clock persists under conditions of prolonged fasting, meal timing ultimately dictates the timing of peripheral clocks. Initial work in rodent models of circadian physiology demonstrated a potent effect of feeding on circadian transcripts in the liver (Stokkan *et al.*, 2001; Kawamoto *et al.*, 2006; Vollmers *et al.*, 2009). Likewise, shifting meal timing in humans results in an alteration in clock gene phase

in adipose tissue and circulating metabolites, with no corresponding changes in the temporal patterns of melatonin or cortisol (i.e., robust markers of the central clock) (Wehrens *et al.*, 2017a). Furthermore, lack of entrainment of rhythms in these markers to non-photic cues in totally blind humans, further suggests that the role of meal timing is predominantly focused on the peripheral clock (Mistlberger & Skene, 2005). The differential resynchronisation rate of murine clocks to food (Damiola *et al.*, 2000; Stokkan *et al.*, 2001), as well as the lack of shift in circulating *PER3* rhythms shown by Wehrens *et al.* (2017a) hints at tissue-specific responsiveness of peripheral tissue clocks. Mechanistically, these tissue-specific responses are likely resultant of the established effect of feeding-associated factors (e.g., nutrients and hormones) on the entrainment of peripheral gene rhythms. In particular, feeding responsive hormones such as insulin, glucagon and IGF-1 appear to be especially capable of modulating clock gene and or protein expression in multiple tissues (Tahara *et al.*, 2010; Mukherji *et al.*, 2015; Sun *et al.*, 2015; Ikeda *et al.*, 2018; Crosby *et al.*, 2019). However, no studies have investigated 24-h muscle transcripts in response to contrasting feeding patterns. In light of the importance of skeletal muscle as a metabolic tissue as well as the established rhythmicity in skeletal muscle transcripts under controlled diurnal conditions (DeFronzo *et al.*, 1985; Perrin *et al.*, 2018a), it is now important to assess the effects of feeding patterns and meal timing on these outcomes.

Eating at a time when the body is not expecting nutrients results in unfavourable metabolic responses. This especially applies to late eating, which has been associated with greater fat mass (McHill *et al.*, 2017). A recent randomised crossover trial reported exaggerated postprandial TAG with late (2200 h) *versus* regular (1800 h) dinner, alongside lower exogenous fatty acid oxidation (assessed using $[^2H_{31}]$ palmitate) (Gu *et al.*, 2020). Interestingly, fat oxidation is lower with late eating when assessed using whole-body calorimetry (Sato *et al.*, 2011). Taken together, late eating may inhibit both oxidation of both endogenous and exogenous lipid therefore providing some explanation to the association between late eating and fat mass (McHill *et al.*, 2017). Furthermore, late eating also negatively impacts glucose metabolism, with a greater increase in postprandial glucose following late dinner than regular dinner, as well as a significantly higher mean daily glucose with late *versus* regular dinner (Sato *et al.*, 2011; Gu *et al.*, 2020). It unsurprising perhaps that chronic late eating modulates

changes in body mass. In particular, a weight-loss intervention in overweight-obese women revealed that eating a late evening meal resulted in less weight loss than those consuming an early evening meal (Madjd *et al.*, 2020). Equally, early evening meal consumers in this study also had greater reductions in total cholesterol, triglyceride, HOMA IR, and fasting insulin after the 12-week intervention. These detrimental metabolic effects are most likely driven by desynchrony between endogenous rhythms and behavioural patterns. For example, it is well established that under controlled conditions glucose tolerance peaks in the biological morning and is at its lowest in the biological evening (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Van Cauter *et al.*, 1997). It is somewhat unsurprising therefore that shifting meals well into the biological evening results in poorer metabolic and health outcomes. Interestingly, feeding of a large dose of protein at 0400 h has knock-on effects during a subsequent meal upon waking, resulting in greater postprandial glycaemia (Smith *et al.*, 2020). Notably, 0400 h is the reported time during which circulating amino acids are suggested to peak (Feigin *et al.*, 1967, 1968), such that the body is not anticipating an influx of nutrients (in this case protein), and may be ill-equipped to deal with a bolus at this time.

Time restricted eating (TRE) may be a promising intervention for weight-loss and improved glycaemic control. Briefly, TRE is an intervention characterised by a restriction in the time between the first and last energy intake of the day. This definition is somewhat ambiguous as restriction of the eating window can take multiple forms, as covered by a recent review (Templeman *et al.*, 2020). Research in animal models provided early evidence of the promise of time restricted feeding (TRF), showing attenuated weight-gain in response to a high-fat diet, lowered fat mass and serum cholesterol as well as improved glucose tolerance and decelerated cardiac aging (Hatori *et al.*, 2012; Sherman *et al.*, 2012; Chaix *et al.*, 2014; Gill *et al.*, 2015). More recent studies have begun to explore the effects of TRE in humans, showing that shortening the eating window induces weight-loss, reduces fat mass, and improves insulin sensitivity, blood pressure, appetite control, and glycaemic excursions across the day relative to later/longer eating windows (LeCheminant *et al.*, 2013; Antoni *et al.*, 2018; Sutton *et al.*, 2018; Jamshed *et al.*, 2019; Parr *et al.*, 2020; Xie *et al.*, 2022). Whilst the mechanisms that underlie these beneficial adaptations are yet to be fully elucidated, it is likely that these benefits may be due to coincidence of the eating

"window" and peak physiological function of a variety of metabolic processes such as glucose metabolism, and skeletal muscle oxidative capacity (Van Cauter *et al.*, 1992; van Moorsel *et al.*, 2016). This is apparent when shifting dinner timing by 4-hours, which results in higher postprandial glucose, delayed triglyceride peak, lower NEFA and dietary fatty acid oxidation (Gu *et al.*, 2020).

The time and relative proportion of specific macronutrient ingestion may be an important determinant of circadian metabolism and health. Pivovarova *et al.* (2015) examined the effects of changing dietary macronutrient composition on the expression of core clock genes assessed in circulating monocytes. Following 6 weeks of low-carbohydrate high-fat diet (40% carbohydrates, 15% proteins, 45% fat), morning-afternoon amplitude in *PER1, PER2,* and *PER3* expression increased alongside mesor with no shift in acrophase. Notably, changes in *PER1* and *PER2* were associated with changes in LDL and NEFA, respectively. However, despite changes in these core clock genes, no change in the temporal patterns of robust markers of the central clock (cortisol and melatonin) were reported. Collectively this suggests that meal composition alters only the peripheral clock, rather than the central clock (i.e., SCN), with this effect being limited to altering amplitude only, rather than phase. Further work studied the specific timing of high- *versus* low-carbohydrate meals across the day (Kessler *et al.*, 2017; Kessler *et al.*, 2018). These studies contrasted 2 isoenergetic diets where either carbohydrate was predominantly consumed in the morning and fat in the evening (HC/HF), or fat in the morning and carbohydrate in the evening (HF/HC). Following 4 weeks of each diet, participants underwent metabolic assessment of 2 meals of which the order was dependent on previous dietary intervention (e.g., HC then HF if finishing the HC/HF diet). Greater glucose and insulin responses were seen when fed the high-carbohydrate meal, relative to the high fat meal, regardless of time of day, (Kessler *et al.*, 2017). Notably though, glucose tolerance was lower when a high-carbohydrate meal was consumed in the evening relative to the morning, which again was expected based on circadian literature (Qian & Scheer, 2016). Interestingly, while no effect of diet was reported in individuals with normal glucose tolerance, whole day glucose concentration was ~8% higher in participants with impaired glucose tolerance with HF/HC relative to the HC/HF diet. No effect of diet was found on fasting lipid profiles, nor was there any difference in daily lipid profile between diets (Kessler *et al.*, 2018). Collectively, given that meals of

differing macronutrient composition result in differing postprandial responses and that numerous feeding factors (including metabolites and hormones) have been implicated in the entrainment of peripheral gene rhythms, it appears that not only is timing of energy intake important but also that timing of specific nutrients needs consideration.

Given the metabolic importance of skeletal muscle in postprandial metabolism (DeFronzo *et al.*, 1981b), the apparent tissue-specific effects of meal timing, and the limitations of using murine models to study circadian rhythms (Section 2.1.3), further work investigating the effects of meal timing in this tissue is justified.

2.1.6.4b Relative meal timing

The metabolic/physiological response to a meal is influenced by the time of consumption relative to other interrelated events across the day. Western eating patterns are typically reflected by 3 meals across the day, and therefore the study of metabolic responses to sequential meals provides a greater degree of generalisability to free-living scenarios. The importance of meal timing relative to other meals is particularly evidenced by "the second meal effect", whereby the glycaemic response to the second exposure to exogenous glucose is lower than that of the first (Hamman & Hirschman, 1919). This was first observed using sequential oral glucose tolerance tests (OGTTs) but has since been replicated with intravenous infusion (Szabo *et al.*, 1969), as well as with mixed-macronutrient meals (Chowdhury *et al.*, 2015, 2016b).

Relative timing of food intake is intertwined with cycles of fasting and feeding. The human genome has evolved around periods of variable and often limited food intake and so is highly adapted with the relevant pathways to cope with periods of fasting. In the absence of energy intake, the body maintains life by oxidising endogenous fuels such as glycogen, lipids, and ketones (Nilsson & Hultman, 1973; Romijn *et al.*, 1990). Remarkably, the shifts in fuel source observed with prolonged fasting have been shown to be adequate to sustain life over a prolonged period (> 1 year) if energy stores are sufficient (Stewart & Fleming, 1973). The shift in metabolic substrate with fasting initially acts to maintain glucose homeostasis while ultimately aiming to spare muscle tissue. It is well established that prolonged fasting is characterised by reduced contribution of carbohydrate to metabolism alongside increased contribution of lipids evident in the respective reductions and increase in circulating glucose and elevated

blood lipids, as well as lower postprandial carbohydrate oxidation and higher lipid oxidation following a 72 hour fast (Horton & Hill, 2001; Tsintzas *et al.*, 2006). Study of prolonged fasting provides insight into the mechanisms involved in human obesogenic tendencies including hypothetical models of physiological body weight regulation such as the 'thrifty gene' hypothesis (i.e., genetically determined survival advantage during times of low energy availability (O'Rourke, 2014)) and the set point theory (i.e., the physiological drive to maintain body weight within a preferred range (Speakman *et al.*, 2011)). Equally study of prolonged periods of fasting also allows for study of the mechanisms that drive beneficial pathways in disease states including hypertension, atherosclerosis, diabetes, and cancer.

The physiological effects of fasting are determined by fasting duration. Initial requirements for glucose are supported by glycogenolysis and utilisation of muscle-derived amino acids as substrate for gluconeogenesis, with an increasing contribution of renal glucose production as the duration of the fast increases (Cahill, 1970; Rothman *et al.*, 1991; Ekberg *et al.*, 1999). During the early stages of a prolonged fast (~20 h), adipose tissue efflux of NEFA increases and adipocyte glucose uptake declines (Samra *et al.*, 1996a; Horowitz *et al.*, 2001; Gjedsted *et al.*, 2007). The increased efflux of NEFA from adipose continues after 48-72 h of fasting, facilitating the sparing of the protein by catabolism of lipid-derived fatty acids via beta oxidation, or in the case of red blood cells and the central nervous system, utilisation of lipid-derived ketone bodies (Cahill, 1970). Fasting-induced shifts in metabolic fuel are evident in changes in plasma metabolite profiles. Plasma glucose levels initially decline from day 1 to 2 before stabilising in days 3 and 4 (Zauner *et al.*, 2000). This is concurrent with a gradual rise in plasma NEFA across the day. While cholesterol remains stable across days 1 and 2 of a prolonged fast, a small increase is observed as the fast moves into days 3 and 4 (Zauner *et al.*, 2000). During a 10-d inpatient fasting protocol the shift to lipid metabolism is marked by the preferential release of unsaturated fatty acid species that surges 1–2 days after fasting onset Steinhauser *et al.* (2018). This coincides with both the onset of ketosis (and associated candidate metabolites) and a state of acute hypoleptinaemia and associated hypercortisolaemia, which speculatively may help orchestrate the shift from carbohydrate to lipid metabolism (Chan *et al.*, 2003).

Prolonged fasting markedly reduces in insulin sensitivity and glucose tolerance. This has been established using oral/intravenous glucose tolerance tests, and mixed meal ingestion, (Newman & Brodows, 1983; Mansell & Macdonald, 1990; Tsintzas *et al.*, 2006). The reported reduction in insulin sensitivity and glucose tolerance is due to changes in gene and protein expression in skeletal muscle, reflecting a shift towards preferential lipid oxidation over carbohydrate (Mansell & Macdonald, 1990; Tsintzas *et al.*, 2006). In light of the metabolic importance of both adipose and skeletal muscle (as well as any potential crosstalk between the 2 tissues), it is remarkable that no studies have made simultaneous measures of these with complementary measures of circulating metabolic markers in response to a prolonged fast. Specifically, simultaneous assessment of genes and proteins responsible for regulating substrate metabolism in the adipose tissue would provide novel insight into the metabolic flexibility of this tissue under severe energy deficit.

Prolonged fasting disrupts circadian clocks and may dysregulate metabolism. The complete absence of daily food intake for 24 h (i.e. fasting) can eradicate the circadian rhythm in hepatic gene expression that would otherwise occur with a regular meal pattern (Vollmers *et al.*, 2009). This is perhaps unsurprising given that feeding responsive hormones such as insulin, glucagon, and IGF-1 are potent modulators of clock gene and protein expression, and this effect is particularly heightened when animals are in a fasted-state (Tahara *et al.*, 2010; Mukherji *et al.*, 2015; Sun *et al.*, 2015; Ikeda *et al.*, 2018; Crosby *et al.*, 2019; Tuvia *et al.*, 2021). However, no studies have looked at expression of clock genes in human peripheral tissue in response to prolonged fasting.

Timing of exercise alters the physiological response to meals. Acutely, exercise in the fasted-state attenuates postprandial glycaemia/insulinaemia to a greater extent than post-meal exercise in healthy participants as well as those with type 2 diabetes, facilitated by contraction driven GLUT-4 translocation and negative carbohydrate balance (Haxhi *et al.*, 2013; Richter & Hargreaves, 2013; Taylor *et al.*, 2018; Johnson-Bonson *et al.*, 2021). There also appears to be an interaction between pre-exercise nutrition and the metabolic response to sequential meals, whereby exercise performed after breakfast results in an elevated glycaemic response to a sequential meal (Gonzalez *et al.*, 2013; Gonzalez, 2014; Edinburgh *et al.*, 2018). Fasted exercise has

a similarly beneficial effect on postprandial lipaemia, with 60 minutes of moderate intensity cycling resulting in an attenuation of the VLDL, TG, and insulin responses to a meal when compared to the same exercise performed 60 min postprandially (Enevoldsen *et al.*, 2004). This may be explained by higher activity of skeletal muscle LPL, as well as reduced hepatic secretion of VLDL (Herd *et al.*, 2001; Gill *et al.*, 2003). Notably, the aforementioned effects of exercise timing on postprandial metabolism appear to be modulated by fat content of the meal consumed (Haxhi *et al.*, 2013). Furthermore, timing of exercise around meals also acutely impacts 24-h energy balance, inducing negative daily energy balance (−400 kcal nCI: -230, -571 kcal) when morning exercise is performed in the fasted-state compared to the same exercise in the fed-state (492 kcal nCI: 332, 652 kcal) or when breakfast is followed by rest (7 kcal nCI: -153, 177 kcal) (Edinburgh *et al.*, 2019). When exercise is performed in the fasted-state over a 6-week period, it appears that the greater rates of lipid metabolism result in improved postprandial insulinaemia relative to when exercise is performed following carbohydrate ingestion (Edinburgh *et al.*, 2020).

Recent evidence has elucidated possible further relationships between timing of meals relative to sleep. Whilst shifting meal timing does not appear to alter objective or subjective sleep parameters (Wehrens *et al.*, 2017a), there appears to be a modulating role of habitual sleep timing on the response to late eating. Specifically, habitual early sleepers appear to be more susceptible to the effects of late eating on glucose control and fatty acid oxidation than those who are classed as habitual late sleepers (Gu *et al.*, 2020).

2.2 Breakfast and human health

2.2.1 Relationship between breakfast consumption and health

Breakfast has been investigated in regard to its role in human health. A relative wealth of observational evidence highlights a negative relationship between breakfast skipping and poorer cardiometabolic health (Fabry *et al.*, 1964a; Ruxton & Kirk, 1997; Siega-Riz *et al.*, 1998; Otaki *et al.*, 2017). However, until recently very little causal evidence existed to support this relationship. Within the last decade a series of randomised control trials has begun to shed light on the distinct relationship between breakfast and health (Betts *et al.*, 2011; Betts *et al.*, 2014; Chowdhury *et al.*, 2015; Betts *et al.*, 2016; Chowdhury *et al.*, 2016a; Chowdhury *et al.*, 2016b; Gonzalez *et al.*,

2017; Chowdhury *et al.*, 2018; Ruddick-Collins *et al.*, 2018; Chowdhury *et al.*, 2019; Ruddick-Collins *et al.*, 2022).

2.2.2 Acute physiological effects of breakfast consumption or skipping

Breakfast is an eating occasion with particular potential to serve as a zeitgeber and to modify subsequent responses, since this first meal of the day generally marks the end of the overnight period of darkness, sleeping, resting and fasting, whilst also preceding all other daily events. The capacity of breakfast to exert a marked influence on metabolic control later in the day is perhaps best illustrated by the 'second-meal effect', which describes how the glycaemic and insulinaemic responses to repeated carbohydrate ingestion are attenuated relative to an initial meal hours earlier (Hamman & Hirschman, 1919). This phenomenon was first observed using sequential oral glucose tolerance tests but has since been replicated with intravenous infusions (Szabo *et al.*, 1969) and mixed macronutrient breakfasts relative to extended morning fasting (Gonzalez, 2014; Chowdhury *et al.*, 2015, 2016b; Jakubowicz *et al.*, 2017). Studies of the second-meal effect following breakfast consumption/omission have typically focused on 2-meals (i.e., breakfast and lunch), although it does appear that worsened glycaemic control persists to the evening in individuals with type 2 diabetes (Jakubowicz *et al.*, 2015). Few studies have explored the precise mechanisms underpinning the second-meal effect (Lee *et al.*, 2011; Edinburgh *et al.*, 2017a; Edinburgh *et al.*, 2018). However, it is possible that maintenance of rhythmic clock gene expression plays a role, since the expected pattern of core clock gene expression in leukocytes is disrupted when habitual breakfast consumers omit their usual morning meal (Jakubowicz *et al.*, 2017).

Acutely, consumption and skipping of breakfast result in divergent, yet logical, subjective appetite responses in controlled conditions. More specifically, whereas consumption of breakfast suppresses subjective appetite, breakfast omission is characterised by increased subjective appetite across the morning (Chowdhury *et al.*, 2015; Allerton *et al.*, 2016; Chowdhury *et al.*, 2016b; Slater *et al.*, 2022). In light of the relatively clear effects on subjective appetite, the balance of evidence on objectively assessed appetite (i.e., energy intake assessed during *ad libitum* or buffet lunches) suggest breakfast omission is not fully compensated for during subsequent meals. When considering total morning intake (i.e., breakfast + lunch), the majority of studies

report lower total energy intake across the morning following breakfast omission (Gonzalez *et al.*, 2013; Levitsky & Pacanowski, 2013b; Chowdhury *et al.*, 2015, 2016b; Slater *et al.*, 2022), whereas only 1 study reports similar energy intake across the morning when breakfast is consumed relative to omitted (Astbury *et al.*, 2011). If total morning energy intake is split into breakfast and lunch, it appears that only partial compensation of energy intake (~33%) takes place at lunch when breakfast was omitted (Chowdhury *et al.*, 2015). Interestingly, however, this partial compensation is not apparent in individuals with obesity/overweight (Chowdhury *et al.*, 2016b) and this could reflect differences in appetite/energy balance regulation between populations (Galindo Munoz *et al.*, 2015; Hopkins & Blundell, 2016). It is also important to note that dinner is the largest single contributor towards daily energy intake in western societies. To this end, Levitsky and Pacanowski (2013b) investigated energy intake across an entire day (including afternoon and evening snacks). Total daily energy intake was greater when breakfast was consumed despite partial compensation for breakfast omission at lunch. Acute laboratory studies of breakfast omission *versus* consumption collectively hint at the *potential* for breakfast to induce negative energy balance; at least from an energy intake perspective and certainly when energy balance is considered over a single day.

Aside from subjective appetite and energy intake, breakfast ingestion acutely alters the temporal profiles of hormonal appetite regulators across the day. Omission of breakfast is characterised by elevations in both total and acylated ghrelin across the morning relative to consumption of a carbohydrate-rich breakfast in lean and obese populations (Chowdhury *et al.*, 2015, 2016b; Slater *et al.*, 2022). Notably, consumption of breakfast also results in a "second-meal effect" for total and acylated forms of ghrelin whereby plasma concentrations remain stable across the post lunch period relative to suppressed ghrelin when lunch is consumed after breakfast is omitted. Skipping breakfast also acutely shifts the diurnal phase of leptin such that the typical increase later in the day is delayed (Schoeller *et al.*, 1997; Chowdhury *et al.*, 2015).

Consistent with its effects on subjective fullness, PYY increases with breakfast consumption and remains elevated following lunch relative to breakfast omission, following which systemic PYY decreases (Leidy & Racki, 2010; Astbury *et al.*, 2011; Chowdhury *et al.*, 2015, 2016b; Slater *et al.*, 2022). Similarly, GLP-1 is elevated post-

breakfast ingestion, with no apparent differences following *ad libitum* lunch ingestion (Astbury *et al.*, 2011; Chowdhury *et al.*, 2015, 2016b). Collectively it appears that diminished post-lunch responses in appetite regulatory gut peptides are in line with subjective ratings of appetite (Astbury *et al.*, 2011; Chowdhury *et al.*, 2015, 2016b; Clayton & James, 2016).

In summary, acute laboratory investigations of breakfast consumption relative to omission result in divergent metabolic and behavioural responses. Consuming breakfast results in favourable glycaemic responses to a subsequent meal relative to breakfast omission yet doesn't necessarily influence energy intake at following meals. Consequently, omitting breakfast appears to be an effective method of eliciting an energy deficit across one day, when free-living factors (e.g., physical activity) are controlled for.

2.2.3 Longer-term physiological effects of breakfast consumption or skipping
Further to the acute metabolic effects of breakfast on the responses to subsequent meals later within the same day, recent research has also explored the longer-term effects (i.e., 6-week) of regular daily breakfast consumption *versus* extended morning fasting on free-living behavioural responses and any accumulated adaptation in metabolic control. Complete omission of breakfast (i.e., zero energy intake until midday) every day for 6 weeks resulted in significantly lower physical activity thermogenesis than when a regular morning feeding was prescribed – a finding that has been replicated both amongst lean adults and those with obesity (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). However, the higher morning physical activity thermogenesis was approximate to the extra-calories consumed with the breakfast and so energy balance was maintained. Therefore, whereas acute studies suggest that breakfast omission is effective at inducing negative energy balance, it appears then when translated to a free-living conditions compensation in physical activity erodes this deficit (Levitsky & Pacanowski, 2013b; Betts *et al.*, 2014; Chowdhury *et al.*, 2016a).

Chronic breakfast omission alters 24-hour glycaemia**.** In line with the acute effects of breakfast omission, lean individuals who skip breakfast display greater

afternoon/evening glycaemic variability across a 6-week period (Betts *et al.*, 2014). At the tissue level breakfast consumption alters adipose tissue gene expression (favouring greater lipid turnover) in lean individuals and improves insulin sensitivity in obese individuals (Gonzalez *et al.*, 2017). Aside from this, no other effects of regular breakfast on markers of cardiometabolic health nor any postprandial metabolic adaptation are apparent in lean populations or those with obesity (Chowdhury *et al.*, 2018; Chowdhury *et al.*, 2019).

2.2.4 Putative mechanisms that underpin the causal relationship between breakfast consumption and human health

Elevations in plasma glucose across the morning may drive higher PAEE when breakfast is consumed versus skipped. Speculatively, the postprandial increase in readily available metabolic substrate in the form of endogenous glucose both stimulates and fuels higher levels of movement (Smith *et al.*, 2017). Omission of breakfast has therefore resulted in less voluntary energy expenditure through low-intensity physical activities, associated with lower average blood glucose concentrations (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a; Smith *et al.*, 2017).

Breakfast consumption may maintain circadian rhythmicity. As little as 6 days of skipping breakfast is sufficient to delay the typical circadian rhythm seen in core temperature, but not melatonin, or core clock genes measured in leukocytes (Ogata *et al.*, 2020). This may be due to the relative alignment between breakfast, waking, and light exposure; 4 days of early time-restricted feeding appears to maintain temporal profiles of clock gene expression (Jamshed *et al.*, 2019). Meal frequency may also be responsible for the relationship between breakfast consumption and clock gene profiles. Eating 3 meals per day as opposed to 6 maintains periodicity of core clock gene expression in individuals with type 2 diabetes, and this may also be associated with the improvements in glycaemic control also resultant of less frequent meal consumption (Jakubowicz *et al.*, 2019). Despite this emerging evidence, very little research has explored the chronic (i.e., longer than 1 week) influence of breakfast consumption or omission upon clock gene expression. Given the tight relationship between physiological processes involved in energy sensing and core clock machinery, it is important that future research explores this.

2.2.5 Manipulation of breakfast to induce health benefits

2.2.5.1 Macronutrient composition of breakfast meals

Recent evidence shows that 64% of adults in the UK consume breakfast on a daily basis (Reeves *et al.*, 2013). Moreover, relative to the wide variety of foods that are typically consumed at all other meals throughout the day, the morning meal is limited to a narrow range of (typically) carbohydrate rich "breakfast foods". In particular, the first meal of the day typically tends to have the lowest protein content (**Figure 2.9**) (NHANES, 2016); with consideration of the aforementioned potential mechanisms that link breakfast with human health, modification of the protein content of the first meal of the day may help to further understanding of the mechanisms that link breakfast and health.
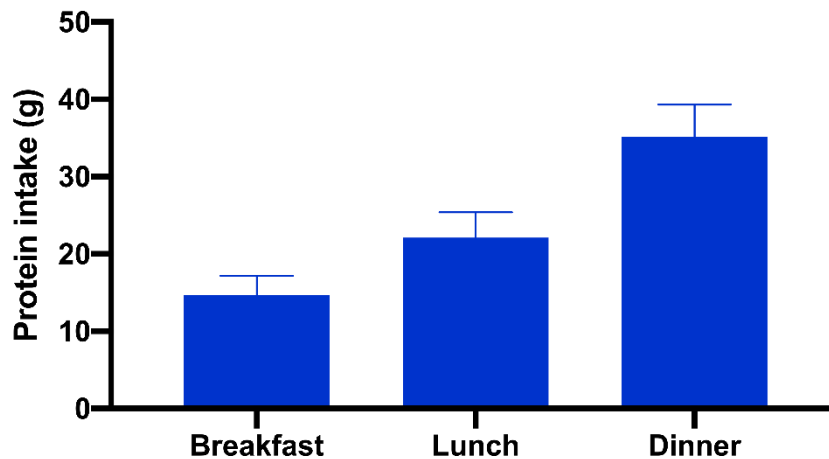
Figure 2.9: Mean ± SD data from NHANES >9000 adult men and women showing imbalanced distribution of protein intake across the day (NHANES, 2016).

2.2.5.2 Metabolic response to protein ingestion

Whey protein is known to be potent insulin secretagogue (Floyd *et al.*, 1970; van Loon *et al.*, 2000). In particular, whey protein is rich in essential amino acids, especially leucine, which has been demonstrated to exert acute and chronic effects at regulatory sites in multiple tissues/organs (e.g. pancreatic β-cells, liver, muscle and adipose tissue) to influence glucose homeostasis (Yang *et al.*, 2010b; Salehi *et al.*, 2012).

Insulin secretion is consequential of an increase in the intracellular ATP:ADP ratio within *β*–cells (Ashcroft, 2005; Eliasson *et al.*, 2008). There are two separate pathways

by which leucine directly stimulates insulin secretion through this mechanism. Firstly, catabolism of leucine increases cellular energy status through its deaminated metabolite α-ketoisocaproic acid (KIC), which is subsequently converted to acetyl-CoA prior to entry into the tricarboxylic acid (TCA) cycle (Gao *et al.*, 2003). Secondly, leucine enhances glutaminolysis through allosteric activation of glutamate dehydrogenase, a key enzyme in the conversion of glutamate to α-ketoglutarate, which itself is an intermediate in the TCA cycle (Liu *et al.*, 2008b; Smith & Stanley, 2008). The increase in ATP via leucine metabolism results in closure of energy-sensitive potassium channels, depolarising the cell membrane and generating an influx of calcium ions. This is followed by exocytosis of secretory granules, which discharge their contents (e.g., insulin) in the extracellular space (Seino *et al.*, 2011). Interestingly, some amino acids may also directly depolarise the cell membrane through co-transport with Na$^+$, thereby stimulating insulin secretion through activation of voltage-dependent calcium channels (Salvucci *et al.*, 2013).

Given the potent insulinotropic properties of both carbohydrate and protein, it is unsurprising that co-ingestion of protein with carbohydrate *per se* results in a strong elevation in postprandial systemic insulin (van Loon *et al.*, 2000; Manders *et al.*, 2014). Theoretically increased insulin secretion through this mechanism therefore improves postprandial glycaemia, demonstrated in several studies (Nuttall *et al.*, 1984; Gunnerud *et al.*, 2013; King *et al.*, 2018; Cunha *et al.*, 2020). However, not all studies report improved glycaemia with co-ingestion of protein with carbohydrate despite higher insulinaemia, potentially explained by non-isoenergetic meals (Allerton *et al.*, 2016).

Beyond the ability of protein to enhance insulin secretion, protein also delays gastric emptying (Stanstrup *et al.*, 2014), resulting in simultaneous suppression of systemic glucose appearance alongside accelerated glucose disposal (Manders *et al.*, 2006b; Betts & Williams, 2010b). Delayed gastric emptying prolongs the exposure of enteroendocrine cells to ingested nutrients and therefore suggests that improved glycaemic control following high-protein meal ingestion could be mediated by the secretion of incretin hormones**.** The incretin effect describes the influence of nutrient presence in the gastrointestinal tract (GI-tract) on insulin secretion, thereby suggesting greater regulation beyond that of increased exposure of the pancreatic *β*–cells to

glucose (Elrick *et al.*, 1964). Two intestinally-derived hormones are primarily responsible for the mediation of insulin secretion due to the presence of nutrients in the GI-tract: glucose-dependent insulinotropic peptide (GIP) (Dupre *et al.*, 1973), and glucagon like peptide-1 (GLP-1) (Bell *et al.*, 1983).

GIP is secreted from specific endocrine cells, known as the K cells which are most densely located in the duodenum, but can be found throughout the entire small intestinal mucosa (Mortensen *et al.*, 2003). GLP-1 is primarily secreted from L-cells of the intestinal mucosa. Secretion of both GIP and GLP-1 is stimulated by absorbable carbohydrates, lipids, and proteins, resulting in greatly increased circulating concentrations following ingestion of a meal (Orskov *et al.*, 1996; Bodnaruc *et al.*, 2016). Despite lower circulating concentrations, GLP-1 is more insulinotropic than GIP (Holst, 2007). Notably the postprandial time course of GIP and GLP-1 is determined by the distribution of intestinal endocrine cells, such that the postprandial rise in GIP precedes that of GLP-1 (Mortensen *et al.*, 2003; Rozengurt *et al.*, 2006). Both incretin hormones exert their insulinotropic effects through interaction with their specific β–cells membrane receptors (GLP-1R & GIPR). Briefly, binding of GIP/GLP-1 to their respective β–cell receptor causes activation of adenylate cyclase, via a stimulatory G protein, resulting an elevation of cyclic AMP levels, leading to increases in the intracellular calcium concentration, thereby augmenting the exocytosis of insulin-containing granules (Doyle & Egan, 2007; Mayendraraj *et al.*, 2022).

Whilst there is little evidence to suggest that protein *per se* stimulates a greater GLP-1 response relative to isoenergetic amounts of carbohydrate or fat (Elliott *et al.*, 1993; Carr *et al.*, 2008; Chungchunlam *et al.*, 2015), ingesting mixed macronutrient meals with high relative amounts of dietary protein may be an effective strategy for enhancing GLP-1 secretion (Belza *et al.*, 2013; van der Klaauw *et al.*, 2013; Watkins *et al.*, 2021a; Watkins *et al.*, 2021c). Less evidence exists directly comparing postprandial GIP responses to individual macronutrients in humans, although individual studies report an average ~0.31-fold increase in postprandial GIP per gram of protein relative to ~0.10 fold per gram of glucose and ~0.06 fold per gram of fats (Ahrén, 2022; Yamamoto *et al.*, 2022).

2.2.5.3 Protein and Energy Balance

Satiety is a multifaceted outcome not only dictated by the endocrine system, but also the cognitive, neural, and gastrointestinal systems (Pesta & Samuel, 2014). Relative to carbohydrate and fats, dietary protein is the most satiating macronutrient; dose-dependently enhancing satiety in acute settings (Barkeling *et al.*, 1990; Stubbs *et al.*, 1996; Crovetti *et al.*, 1998; Westerterp-Plantenga *et al.*, 2006; Smeets *et al.*, 2008; Soenen & Westerterp-Plantenga, 2008; Veldhorst *et al.*, 2008). Notably, this protein-induced satiety may also result in a reduction in subsequent energy intake (Johnson & Vickers, 1993).

There are several proposed mechanisms that may explain the potent satiating effects of dietary protein. Firstly, protein slows gastric emptying to a greater extent than carbohydrates, which is associated with greater satiety (Cooke & Moulang, 1972; Barker *et al.*, 1974; Burn-Murdoch *et al.*, 1978; Bergmann *et al.*, 1992). Delayed gastric emptying results in greater exposure of intestinal L and K cells to nutrients, which maximises the stimulation and release of gut hormones that modulate appetite (e.g., GLP-1 GIP, PYY, CKK etc) (Batterham *et al.*, 2003; Batterham *et al.*, 2006; Mace *et al.*, 2012; Watkins *et al.*, 2021b). Protein has a particularly potent effect upon GLP-1, acting through the calcium-sensing receptor expressed on L-cells in the intestine, inducing a large GLP-1 response which may influence appetite, especially when co-ingested with sufficient carbohydrate (Lejeune *et al.*, 2006; Smeets *et al.*, 2008; Chen *et al.*, 2020; Watkins *et al.*, 2021c). Equally, the thermogenic properties of dietary protein relative to both carbohydrate and fat have also been proposed as a possible mechanism for protein-induced satiety (Crovetti *et al.*, 1998; Westerterp-Plantenga *et al.*, 1999a; Livesey, 2001; Lejeune *et al.*, 2006). The range of values for the thermic effect of dietary protein is 20-30% kcal making it higher than either carbohydrate (5-10% kcal) or fat (0-3% kcal) (Acheson, 1993; Livesey, 2001; Sánchez-Peña *et al.*, 2017). Fundamentally, increased energy expenditure following a meal implies increased oxygen consumption and body temperature, thus promoting satiety (Westerterp-Plantenga *et al.*, 1999a; Westerterp-Plantenga *et al.*, 1999b). Whey protein in particular is particularly thermogenic relative to soy and casein, and is rapidly digested, making it an ideal candidate to target the thermic effect of feeding through the breakfast meal (Acheson *et al.*, 2011; Bray *et al.*, 2015).

Given the marked effects of protein upon gastric emptying, glycaemia, appetite, energy intake and the thermic effect of feeding, manipulation of the protein content of breakfast provides an insightful model through which the mechanisms that underpin the relationship between breakfast, metabolism, and health can be isolated and explored. Acutely, the effect of a higher protein breakfast on the glycaemic response to a subsequent meal is unclear. Whereas Meng *et al.* (2017) reported an attenuated glycaemic response to a subsequent meal following a high protein breakfast, the same effect was not apparent in (Allerton *et al.*, 2016). However, in the latter study, breakfast meals not only differed in macronutrient composition but also energy content. Furthermore, without characterisation of the metabolic response to the high protein breakfast in the study by Meng *et al.* (2017) it is difficult to understand the mechanisms underlying the attenuation in second meal glycaemia.

There is a relative paucity of studies investigating the longer term metabolic and behavioural effects of higher protein breakfasts. Of the studies to have been carried out, findings are inconsistent. Two weeks of altering protein content of the breakfast meal had no apparent effect on fasting glucose or insulin, nor did it effect postprandial responses of these biomarkers (Amankwaah *et al.*, 2017). Equally, no difference in non-breakfast energy intake was observed between groups. However, as little as 8-d of consuming a high protein breakfast resulted in a higher thermic effect of feeding, greater fat oxidation, and lower postprandial hunger relative to 8-d of carbohydrate-rich breakfast (Neumann *et al.,* 2016). A 24-week study in older adults supplemented both breakfast *and* lunch with a milk-based protein matrix or non-nitrogenous maltodextrin control (Norton *et al.*, 2016). Supplementation of protein at breakfast and lunch meals resulted in a more balanced distribution of protein intake across the day, accompanied by an increase in lean tissue mass (Wirth *et al.*, 2020). However, it is difficult to isolate the effects of supplementation at breakfast when the lunch meal was also supplemented.

No studies to date have investigated free-living energy balance and consequential changes in body mass, metabolic control, or appetite in response longer-term consumption of a protein-enriched breakfast meal. Future work should address this in order to improve our understanding of the mechanisms that underpin the relationship between breakfast and human health.

2.3 Thesis Objectives

The collective body of literature reviewed in this chapter highlights the need for studies investigating the metabolic and behavioural consequences of nutrient timing, and its various manifestations (i.e., absolute or relative) Given that meal timing itself can be considered in both absolute (i.e., clock time), and relative terms (i.e., to other inter-related events across the day), this thesis will take a multi-faceted approach to studying the metabolic and behavioural effects of meal timing, with focus on both absolute and relative timing.

In particular, whilst animal models have been invaluable in uncovering core-clock machinery, fundamental differences between rodent and human physiology (especially in feeding patterns) prevent generalisation to human models. On this basis it is pertinent to now study the effects of nutrient feeding patterns on circadian clocks in metabolically active tissues such as the skeletal muscle (Figure 2.10).

Equally, the metabolic effects of sleep fragmentation remain relatively under studied compared to total and partial sleep deprivation. Study of sleep fragmentation will help to improve our knowledge of the importance of sleep on next day metabolic regulation (Figure 2.10). Furthermore, no studies to date have investigated the metabolic effects of common stimulating remedies following a poor night of sleep (i.e., caffeinated coffee); this will add further ecological and novel context on typical daily behaviours.

Furthermore, work over the last 10 years has shed light on the links between breakfast, metabolic regulation, and health. The next important step is to now begin to isolate and exploit the potential mechanisms that underpin this relationship.
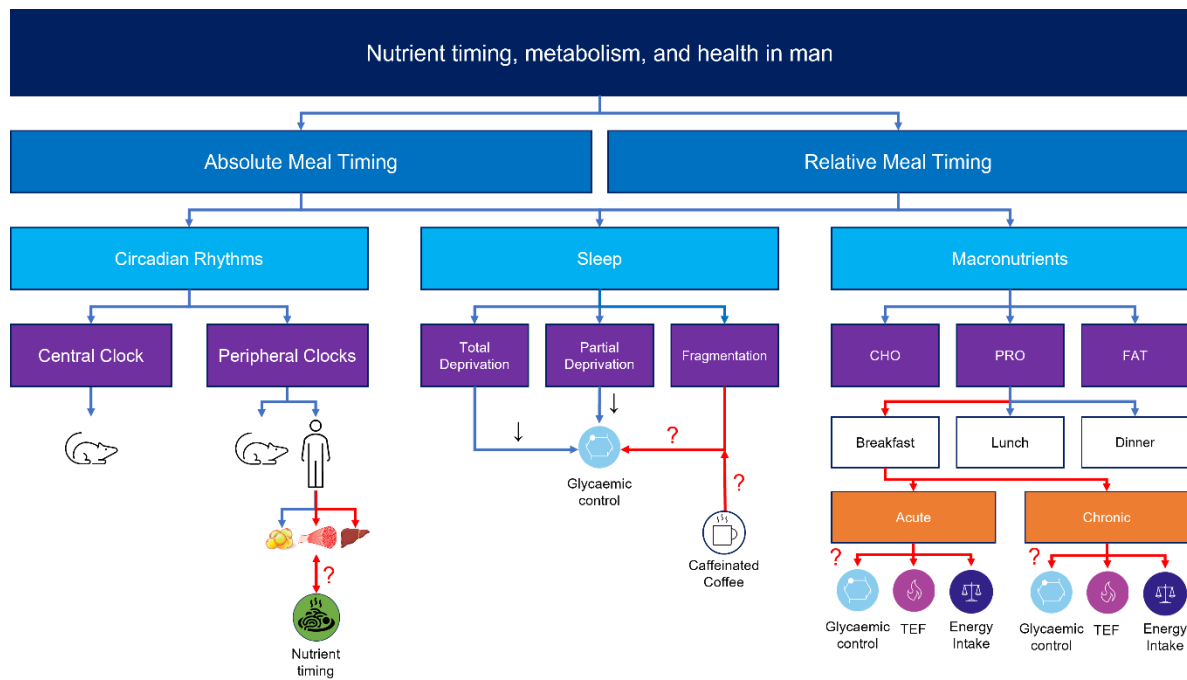
Figure 2.10 – Overview of the main themes and objectives of the thesis. Red arrows denote unknown relationships based on current literature. Question marks are related to the thesis objectives.

With these factors in mind, the objectives of this thesis are as follows:

1.   Characterise the diurnal hormonal and metabolite rhythms in the context of time series skeletal muscle gene expression.

2.   Contrast the metabolic response to 2 distinct temporal nutrient delivery patterns over 24-h.

3.   Determine whether hourly sleep disruption influences next morning metabolic control.

4.   To establish the acute (hours; day) metabolic response to sequential feedings when enriching a typical breakfast with whey protein.

5.   To establish the short/mid-term (days; weeks) free-living behavioural and molecular responses when introducing daily protein-enriched breakfasts.

Chapter 3 – General Methods

This chapter provides an account of all generic methodologies implemented during the studies in this thesis. Prior to data collection all studies received ethical approval. The data reported in Chapter 4 are the result of further analysis on historical samples for a project approved by the Southwest (Frenchay) NHS research ethics committee (14/SW/0123). The study described in Chapter 5 was also approved by this committee (18/SW/0176). The study outlined in Chapter 6 was approved by both the University of Bath Research Ethics Approval Committee for Health (REACH) (SES/HES: 18R1-019) and the Northwest NHS research ethics committee (18/NW/0573) due to it being run in conjunction with another study. Finally, the study outlined in Chapters 7 and 8 received ethical approval from the REACH at the University of Bath (EP 17/18 260) and the Yorkshire & The Humber NHS research ethics committee (20/YH/0310). All data collection was performed in the human physiology laboratories at the University of Bath. In all cases, the University of Bath acted as the research sponsor. All procedures were performed in accordance with the Declaration of Helsinki.

3.1 Sampling/Recruitment

Recruitment for all studies was performed using advertisements at the University of Bath, as well as the surrounding local area. Potential participants were asked to contact a member of the research team for more information if they were interested in participating, upon which they were provided with a full briefing on what participation would entail. Individuals who then expressed a willingness and ability to undertake the protocol were invited to provide both verbal and written informed consent to confirm their participation, provided that they were deemed eligible to participate in line with the defined criteria. Enrolled participants were reminded that consent was at all times voluntary, and that they held the right to withdraw their participation from the study at any point, without justification. Eligibility was assessed via the use of a health questionnaire, as well as a variety of validated scales to assess characteristics such as chronotype (Appendices A-I). To ensure protection of confidentiality, all participants were assigned a unique study code upon enrolment, which was used in place of identifiable information on all data collection tools.

3.2 Anthropometry

3.2.1 Height

The stature of all participants was assessed to the nearest 0.1 cm using a wall-mounted stadiometer (Seca; Hamburg, Germany). Participants were asked to remove their shoes and stand with their heels in contact with the baseplate whilst ensuring that the head, scapula, and buttocks were also in contact with the stadiometer. The moveable indicator was then lowered, until contact with the most superior point of the head was made during one deep inspiration and a recording was taken.

3.2.2 Body mass

Participants were asked to wear light clothing, remove all items from their pockets and remove their shoes for all assessments of body mass. In accordance with standards for best practice, all measurements were taken immediately post-void. Instruction was provided to participants to stand in a relaxed position with their weight spread evenly across the scale before body mass was recorded using an electronic scale to the nearest 0.1 kg (Tanita BC-543, Tanita, Amsterdam, NED). For repeated measures, such as in chapter 7 participants were asked to wear the same light weight clothing as they had at baseline, to ensure consistency of measurement. Body mass index ($kg·m^{-2}$) was then calculated as body mass (kg) divided by height (m) squared (Keys *et al.*, 1972).

3.3 Physiological measures

3.3.1 Expired gas collection and analysis

In order to concomitantly assess rates of thermogenesis and substrate oxidation through indirect calorimetry, expired gas samples were collected via the Douglas bag technique (Douglas, 1911). In accordance with guidelines for best practice, participants were required to rest in a semi-supine position for 20-minutes prior to providing a sample and were provided with a nose-clip and one-way respiratory valve mouthpiece at least 30-seconds prior to sample collection. This mouthpiece was attached to a fully evacuated 200 L Douglas bag via falconia tubing to create a closed system in which the sample could be collected. During sample collection, ambient recordings of barometric pressure and temperature were made alongside ambient

oxygen and carbon dioxide concentrations to account for changes over time (Betts & Thompson, 2012). Expired gas concentrations of O_2 and CO_2 were then analysed in a known volume of sample, using paramagnetic and infrared analysers, respectively (Mini HF 5200; Servomex Group Ltd, Crowborough, UK). All analysers used were calibrated using a two-point calibration with gases of known concentrations (O_2: 0-20.32%; CO_2: 0-8.10%; British Oxygen Company, UK). Total volumes of expired gas were determined using a dry gas meter (Harvard Apparatus, Holliston, USA) and temperature measured using a digital thermometer (Edale Instruments, Longstanton, UK). All values were converted to the standard temperature and pressure for a dry gas (i.e. 0°C and 760 mmHg). Application of the Haldane Transformation (Wilmore and Costill, 1973) allowed for calculation of oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$). Using these data, RER ($\dot{V}CO_2/\dot{V}O_2$) and the relative contributions of carbohydrate and lipids towards energy expenditure could then be calculated using indirect calorimetry.

3.3.2 Determination of in laboratory energy expenditure and substrate oxidation
The rate of substrate oxidation was estimated using oxygen consumption and carbon dioxide production values from analysed Douglas bags, in stoichiometric equations for resting and exercise metabolism where appropriate (Frayn, 1983; Jeukendrup & Wallis, 2005).

Resting (Frayn, 1983):
$$\text{Lipid Oxidation} = 1.67 \cdot \dot{V}O_2 - 1.67 \cdot \dot{V}CO_2 - 1.92 \cdot n$$

$$\text{Carbohydrate Oxidation} = 4.55 \cdot \dot{V}CO_2 - 3.21 \cdot \dot{V}O_2 - 2.87 \cdot n$$

Exercising (Jeukendrup & Wallis, 2005):
$$\text{Lipid Oxidation} = 1.695 \cdot \dot{V}O_2 - 1.701 \cdot \dot{V}CO_2 - 1.77 \cdot n$$

$$\text{Carbohydrate Oxidation} = 4.210 \cdot \dot{V}CO_2 - 2.962 \cdot \dot{V}O_2 - 0.40 \cdot n$$

Where $\dot{V}O_2$ and $\dot{V}CO_2$ are in L·min^{-1}, and n is grams of urinary nitrogen excreted per minute.

Energy expenditure was calculated based on glucose and lipids providing, 15.64 and 40.81 kJ·g^{-1}, respectively (Jeukendrup & Wallis, 2005). The equation employed to calculate lipid oxidation was based on oxidation of the representative TAG from human adipose tissue (palmoyl-stearol-olyeol glycerol; PSOG), and therefore stoichiometry references 55 carbon (Frayn, 1983). At rest, glucose was assumed to be the sole contributor to carbohydrate metabolism.

Calculations of energy expenditure in Chapters 4,5 and 7 assumed negligible contribution from protein oxidation. For Chapters 4, and 5 energy expenditure was measured on one occasion to determine nutrient delivery rates and therefore correction for protein oxidation was not required. Furthermore, in the context of the study described in Chapter 7, protein oxidation is not stimulated above basal levels when consuming a single bolus within a range of 0-20 g (Moore *et al.*, 2009) and therefore the addition of a bolus protein to the breakfast meal within this range (15 g) was not expected to significantly elevate the contribution if protein oxidation to energy expenditure. Finally, it is worth noting that similar studies have also assumed negligible protein oxidation contribution towards energy expenditure (Whyte *et al.*, 2010; West *et al.*, 2011). In Chapter 8, resting values of energy expenditure and substrate oxidation were corrected for rates of protein oxidation as fasting diets can lead to reductions in lean body mass

3.3.3 Urinary Nitrogen Extraction

Total urinary output was collected in a 500 mL container treated with 5 mL of 10% thymol isopropanol preserving solution for the duration of the breakfast postprandial period in Chapter 8. At the end of this 180 minute period total urine volume was measured and a homogenous 1 mL sample was frozen for subsequent analysis of urinary urea concentration, which is assumed to represent ~90% urinary nitrogen excretion (Skogerboe *et al.*, 1990). Furthermore, the first (0 min) and last (180 min) blood samples were analysed for plasma urea concentration. Assessment of these parameters allowed for estimation of protein oxidation using the method described by Jéquier and Felber (1987).

3.3.4 Blood sampling

During main trials, blood was sampled through an intravenous cannula (Venflon Pro, BD, Switzerland). This cannula was inserted into an antecubital vein or dorsal hand vein following sterilisation of the chosen site. Upon successful entry of the cannula into the vein, a sampling tube was attached (Single Lumen Bionector Octopus Extension, Vygon, UK) and the cannula was secured with a dressing. Samples were immediately distributed into specialised tubes coated in ethylenediaminetetraacetic acid (EDTA) as well as a serum tube containing polystyrene beads. Unless otherwise stated. All samples were centrifuged at 3466 x g for 10 minutes at 4°C for removal of the plasma or serum supernatant, which was subsequently aliquoted and frozen at -80°C pending analysis.

3.3.4.1 Arteriovenous sampling

Arterial blood is the best representation of exposure of the peripheral tissues to systemic metabolites and hormones (Copeland *et al.*, 1992). Despite this, the associated risks of arterial cannulation mean that venous cannulation is the default method of blood sampling used in metabolic research (Hall, 1971). However, this method assumes a directly proportional relationship between venous and arterial concentrations, which is violated by the fact that circulating metabolite concentration is dependent upon tissue sensitivity and hormone concentration. Given that these two variables have been shown to be altered by dietary intervention (Johnson *et al.*, 2016), venous samples alone are not sufficient to fully understand the effect of a given treatment/intervention on metabolite kinetics. This is particularly important to consider when thinking about the studies described in chapters 6 and 7.

As such, an alternative method of obtaining samples representative of arterial blood is required. To do so a heated-air hand-box can be used as a method of arterialising the blood through vasodilation of cutaneous capillary beds. This has been demonstrated to be an effective and practical method of obtaining samples that closely approximate arterial samples for key metabolites including glucose and lipids, as well as important regulatory hormones (McGuire *et al.*, 1976; Jensen & Heiling, 1991; Chen *et al.*, 2018). This method of heating was used specifically for the studies outlined in chapters 6 and 7 in line with the methods described by (Edinburgh *et al.*, 2017b).

Specifically, in these studies participants were instructed to place the hand of their cannulated arm in the heated-air hand-box (University of Vermont; VT, USA), which was temperature stable at 55ºC, at least 10 minutes prior to each sample to ensure meaningful oxygen saturation of the blood as shown previously (Kurpad *et al.*, 1994). Arterialised samples drawn from the cannula were handled in accordance with the description in section 3.3.3. However, arterialising blood is not always practical, especially in Chapters 4 and 5 where blood sampling was performed over a protracted period and when participants were provided with a sleep opportunity which may have disrupted by arterialisation of venous blood.

3.5 Muscle Biopsy Sampling and Storage

During the studies described in Chapters 4 and 5, six skeletal muscle biopsy samples were sampled from the *vastus lateralis* across a 24-h period at 4-hourly intervals (1200, 1600, 2000, 0000, 0400, 0800 h). The *vastus lateralis* was an ideal muscle to sample from both a scientific and practical perspective. Compositional analysis of the *vastus lateralis* in healthy young men and women reveals a relatively even distribution of fibre types (43% Type I/57% Type II), which allows for a more general understanding of skeletal muscle responses aside from fibre type (Staron *et al.*, 2000). Furthermore, estimates of postprandial glucose uptake by skeletal muscle were made in the femoral artery, which supplies the *vastus lateralis* demonstrating the metabolic importance of musculature in the upper leg (DeFronzo *et al.*, 1981b; Biondi & Varacallo, 2022). From a practical perspective the *vastus lateralis* is also a large muscle to sample from, therefore allowing for a serial sampling within 24 h, yet is equally safe, as it is not located too proximally to major blood vessels or bones.

Participants rested in a semi-supine position (i.e., torso ~60° above horizontal with legs lying flat) while a 3-5 mm incision was made in the anterior aspect of the thigh using a surgical blade under local anaesthetic (1% lidocaine: Hameln Pharmaceuticals Ltd., Brockworth, UK). Approximately 100 mg of wet tissue was then collected from the incision site using a Bergstrom needle adapted for suction (Bergstrom, 1962a; Tarnopolsky *et al.*, 2011). Samples were collected from each leg in alternating and ascending order with a 2-3 cm separation between skin incisions with randomisation (i.e., dominant, non-dominant) of the starting leg. For night-time samples, a further 2 incisions were made at 2100 h to minimise disruption to participants' sleep. Once

collected, muscle samples were immediately snap-frozen in liquid nitrogen prior to storage at -80°C.

3.6 Blood Analysis

3.6.1 Spectrophotometry

An automated spectrophotometric analyser (RX Daytona, Randox Laboratories, UK), calibrated against commercially available standards, and checked with quality controls, was used to analyse concentration of glucose, non-esterified fatty acids glycerol, triglycerides and urea in plasma samples. In principle, this technique measures the amount of light absorbed by a sample and determines concentration proportionally to the amount of light absorbed. Specifically, during analysis, samples are run through a series of enzyme catalysed reactions in order to eliminate cross-reactive materials and are then reduced yielding hydrogen peroxide. Subsequently, perioxidase-catalysed reactions create a dye from the hydrogen peroxide, which is directly proportional to the analyte of interest. As such, exposure to colorimetric methods at wavelengths ranging from 340-800 nm assesses absorbance which can be used to determine metabolite concentration.

3.6.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Commercially available ELISA techniques were employed to determine plasma concentrations of insulin (Crystal Chem, USA; Mercodia, Sweden), C-Terminal Telopeptide (IDS, UK), melatonin (Surrey Assays ltd, UK) and GLP-1 (Merck-Millipore, USA). Serum samples were analysed for Testosterone (R&D, USA) (Chapters 4 and 5), and Cortisol (Tecan, CH) (Chapter 4).

3.6.3 Assay performance

Table 3.1: Performance characteristics of biochemical analytical techniques. Values are mean

Analyte	Method	Coefficient of variation (%)	
		Intra-assay	Inter-assay
Glucose	Photometric	3.0	3.3
Triglycerides	Photometric	4.4	18.4
NEFA	Photometric	6.4	6.0
Glycerol	Photometric	12.4	17.7
Urea	Photometric	0.2	3.1
Insulin	ELISA	6.0	13.0
GLP-1	ELISA	1.2	1.4
Cortisol	ELISA	5.9	6.8
CTX	ELISA	18.7	26.5
Testosterone	ELISA	29.9	27.2

3.7 Subjective assessments of appetite and mood

In all studies described in this thesis, visual analogue scales (VAS) were employed to assess subjective ratings of appetite and mood. At the necessary intervals during each trial participants were provided with a booklet containing questions that related to aspects of appetite (e.g., "how strong is your desire to eat") and mood ("I feel anxious") (Appendix J). Each question was provided with a 0-100 mm scale, with defined extremes (e.g., "extremely weak" and "extremely strong"), on which they were asked to place an intersecting vertical line denoting their respective sensations relative to the two extremes and that specific time point. This vertical mark was measured horizontally to the nearest millimetre and this value was recorded as the rating.

In order to calculate appetite a composite equation was used, based on questions within the VAS booklet. These included: desire to eat, prospective consumption, satisfaction, and fullness. The equation used to calculate composite appetite is reported below.

$$\text{Composite Appetite} = ((\text{Hunger} + \text{Prospective consumption}) + (100 - \text{fullness}) + (100 - \text{satisfaction}))/4$$

3.8 Free living measures

3.8.1 Energy and Macronutrient Intake

In the context of the studies in chapters 5, 6 and 7, free-living energy intake was quantified using weighed food and fluid consumption records. Participants were set up on a digital food recording app (Libro, Nutritics, Ireland) and were asked to weigh and record all energy intake within a given monitoring period (as specified in Chapters 5, 7 and 8) (SmartWeigh foodscales). Participants were also asked to log time of consumption, branding information, as well as other information they deemed potentially relevant Where possible, participants were also asked to record the component ingredients for recipes and cooking methods employed. In scenarios whereby weighed food records were not possible (e.g., eating out) participants were asked to provide approximate estimations of portion size based on perception (e.g., small/medium/large) or UK household equivalents (e.g., 2 tablespoons). In the case of consumption of packaged foods, participants were encouraged to retain packaging to allow for greater precision in the analysis of energy and macronutrient intake.

3.8.2 Physical Activity Energy Expenditure

Free-living measures of physical activity energy expenditure were captured using Actiheart™ combined heart rate and accelerometry monitors. As described in Brage *et al.* (2007), monitors were placed at the level of the Xyphoid process and were held in place by either a modified heart rate strap, or adhesive pads, based on participant preference. Female participants could also wear the monitor higher on the chest (i.e., above the heart if this provided more comfortable. Monitors were worn continuously during periods of dietary monitoring and lifestyle standardisation, other than activities that would involve direct exposure to water (e.g., swimming). In this scenario, activities were recorded in an activity log and monitor counts of physical activity were adjusted accordingly based on the Compendium of Physical activities (Ainsworth *et al.*, 2011).

Default estimates of physical activity energy using Actiheart 4 software (2018) are made using a group calibration. However, an individual calibration can account for ~95% of the variance in the relationship between heart rate and physical activity intensity, therefore providing more precision at the individual level, which is vital when assessing changes in behavioural variables as the result of an intervention. As such, prior to undergoing lifestyle monitoring, participants in Chapter 8 were to undergo a

4x3 minute stage incremental treadmill protocol; level walking (3.2 km·h^{-1} + 0% gradient), brisk level walking (5.2 km·h^{-1} + 0% gradient), brisk uphill walking (5.8 km·h^{-1} + 10% gradient), level running (self-selected pace 9.0-12.0 km·h^{-1} + 0% gradient). The employment of 3-min stages ensured steady-state was achieved prior to assessment of heart rate (H7, Polar Electro; Kempele, Finland) and energy expenditure (via indirect calorimetry as described in section 3.3.2). Where this was not achieved, the stage would be extended until steady state was achieved. Testing would be ceased if heart rate exceeded 90% age-predicted maximum, or upon request of the participant.

Using data from the exercise test, the relationship between heart rate and energy expenditure from incremental stages was plotted as a segmental linear regression. In line with Actiheart 4 software input parameters, heart rates were rounded to the nearest 10 bpm and energy expenditure was accordingly interpolated from the relevant segment of the regression. When reading heart rate data, the Actiheart 4 software defines each 1-minute epoch based on the physiological plausibility of the value read with physiologically plausible values being defined as "OK". Implausible values are defined as either ≤30 beats·min^{-1}, ±100 beats·min^{-1} from the previous epoch, or a 75% change from the average of the last four epochs. In these scenarios the data is sorted in the following processes. Initially, the implausible raw value is replaced with a zero and defined as lost, with activity estimated using only accelerometry data where available. The inter-beat interval is then used to estimate heart rate where zeroes are present. If this estimate is within 30 beats·min^{-1} of the last valid epoch, then this estimate supplants the lost data. At this stage, gaps of less than five consecutive epochs are interpolated using the epochs from either side. The underlying principle of this approach is taken from the combined heart rate-accelerometry data assessed using the Actiheart™ monitor (Haskell *et al.*, 1993; Luke *et al.*, 1997; Rennie *et al.*, 2000; Brage *et al.*, 2004). Accordingly, on days with >10% of data missing, not including water-based activities, the data was removed from further analysis (Farooqi *et al.*, 2013).

The measurement of physical activity using Actiheart™ in Chapters 4 & 5 was employed in order to assess compliance with the 7-days of standardisation. By contrast, in chapter 8 energy expenditure was measured in order to quantify changes in physical activity energy expenditure as the result of the employed intervention.

Whilst doubly labelled water (DLW) could have also been utilised to assess TEE in this context, use of Actiheart™ avoids violation of the assumed respiratory exchange ratio of 0.85 associated with DLW through alteration of dietary composition (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Importantly, valid and reliable measurements of heart rate and activity counts have been demonstrated in the Actiheart™. Furthermore, good comparability in measures of physical activity energy expenditure derived from these data exists relative to DLW (Brage *et al.*, 2015). One important final consideration for the use of Actiheart™ is the 1-minute epochs in which data is sampled over long periods. This provided sufficient resolution to examine temporal shifts in physical activity energy expenditure of various intensities, which is especially important considering the temporal effect of breakfast consumption on physical activity energy expenditure (i.e., more physically active across the morning and specific to low-moderate intensity movements) (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a).

3.9 Statistical Analyses

Data with hourly resolution (i.e., blood metabolites, hormones and telopeptides) in Chapters 4 and 5 were adjusted to dim light melatonin onset (DLMO) for each participant as determined by the 25% method. The time at which plasma melatonin was at 25 % of each individual's 24 h peak was calculated and subsequently assigned at 0º of the circadian phase (Benloucif *et al.*, 2008). The time in minutes between the DLMO and midnight was then calculated for each participant and used to adjust 24-h profiles (Example in Appendix K). The resulting x-values were binned around half past the hour with average y-values plotted at half past the hour (Otway *et al.*, 2011; Spiegel *et al.*, 2011; Mantele *et al.*, 2012).

The primary aim of the study outlined in Chapter 4 was to objectively characterise diurnal rhythms in metabolic regulation both systemically and at the level of the tissue. Chapter 5 builds upon this, investigating how divergent feeding patterns alter these rhythms. As the duration of these studies was one circadian period, the cosine method was deemed an appropriate means of assessing rhythmicity for a given set of data (Bingham *et al.*, 1982; Refinetti *et al.*, 2007; Templeman *et al.*, 2021b). Fundamentally, the Cosinor approach compares goodness of fit (R^2) of a cosine wave fitted to the 24-

h profile of a given variable, relative to a horizontal line through the mean values (null). If the fitted cosine wave provides the best fit for the data relative to the horizontal line, then the dataset is deemed to be rhythmic and the mesor (rhythm-adjusted mean), amplitude (magnitude of the difference between mesor and peak/trough values) and acrophase (timing of rhythmic peak) are identified and reported (Refinetti *et al.*, 2007; Cornelissen, 2014).

Individual scores and/or paired differences (as appropriate) for data in this thesis requiring a single comparison of 2 level measurements were assessed for normality of distribution using the Shapiro-Wilk test and visual inspection of frequency distributions. A paired or independent *t*-test's was employed for data that was normally distributed where appropriate. While non-parametric data were assessed by the Wilcoxon test. Where multiple comparisons were required (i.e., Chapters 6.0, & 7.0) a repeated measures ANOVA (Condition x Time) was used to identify differences between experimental conditions. For Chapters 5.0 primary contrasts were examined using a two-way group x time mixed model of variance (ANOVA), with experimental group (Group) as a between subjects' factor and timepoint as a within subjects' factor (Condition x Time). Chapter 8.0 also used a two-way group x time mixed model of variance (ANOVA), with experimental group (Group) as a between subjects' factor and baseline-follow up or habitual-intervention (Time) as a within subjects factor. For time series data (i.e., postprandial responses), a three-way ANOVA was employed to include timepoint as an additional within subjects factor (Group x Time x Timepoint). This analysis was performed regardless of normality as the type 1 error rate of this model is typically close to the nominal value even when data are severely non-normally distributed (Maxwell & Delaney, 1990). The Greenhouse-Geisser correction was adopted for epsilon <0.75 and the Huynh-Feldt correction adopted for less severe asphericity (Greenhouse & Geisser, 1959). Interaction effects were then followed up using with a Ryan-Holm-Bonferroni stepwise correction to adjust the resulting *p*-values for multiple comparisons and thus avoid inflation of type I error rate (Ludbrook, 1998; Atkinson, 2002). Significant interaction effects were followed up with multiple paired/unpaired t-tests where appropriate to identify the location of variance. The Holm-Bonferroni stepwise corrections were then used to prevent inflation of the type 1 error rate by multiple comparisons (Ludbrook, 1998; Atkinson, 2002).

The potential for trial order effects in randomised crossover studies (i.e., Chapters 6 & 7) was examined by analysing the iAUC/tAUC, peak, and time to peak data of participants' first, second and third laboratory visits using a one-way ANOVA; significant effect of trial sequence were followed up with two-way condition x sequence ANOVA (Wellek & Blettner, 2012; Betts *et al.*, 2020).

For unpaired data (i.e., Chapters 4, 5 & 8), error bars on figures are 95 % confidence intervals to show the variability around each mean (O'Brien & Yi, 2016). For paired data (Chapters 6 & 7) error values on figures are normalised confidence intervals corrected for between-participant variation (SPSS, IBM v28 & Microsoft Excel v16.0.13801.21072). Normalised confidence intervals are calculated using an equation (Appendix L) that utilises the same error term generated from statistical testing. This correction is such that the magnitude of these confidence intervals infer the contrast between paired means at each time point rather than variance of individual values around each mean (Loftus & Masson, 1994). Using this approach, non-overlapping intervals between contrasted values would typically be deemed significantly different according to conventional null hypothesis testing (i.e., $p \leq 0.05$). Considering the 3-condition design of Chapters 6 & 7, normalised confidence intervals were required to be corrected for violations of sphericity. This correction was performed using the Greenhouse-Geisser epsilon (<0.75) with the Huynh-Feldt correction adopted for less severe asphericity (>0.75) epsilons to reduce the respective degrees of freedom for the omnibus error term, thus providing a valid reflection of the overall pattern across all 3 means. When reporting data such as iAUC, it was of theoretical interest to illustrate the independent contrasts between trials; the specific error terms for each contrast were therefore used to calculate separate pairs of normalised confidence intervals for each comparison (Loftus & Masson, 1994).

Chapter 4 Characterisation of 24-h skeletal muscle gene expression alongside metabolic and endocrine responses under diurnal conditions in healthy adults.

4.1 Introduction

The human circadian system, broadly characterised by both central (suprachiasmatic nuclei; SCN) and peripheral clocks (e.g., muscle, liver, adipose), allows for temporal coordination of physiological processes with cyclic environmental and behavioural events such as light-dark, waking-sleeping, activity-rest, and feeding-fasting.

Diurnal rhythmicity is evident in carbohydrate and lipid metabolism, whereby glucose tolerance is generally lower in the evening than in the morning, whereas lipid metabolism favours progressively elevated circulating lipids later in the day/overnight (Zimmet *et al.*, 1974; Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1992; Simon *et al.*, 1994; Van Cauter *et al.*, 1997; Morgan *et al.*, 1999; Pan & Hussain, 2007; Ang *et al.*, 2012; Yoshino *et al.*, 2014; Qian & Scheer, 2016). Diurnal regulation of insulin secretion/clearance and sensitivity drives rhythmicity in both carbohydrate and lipid metabolism, with lipid metabolism further dictated by rhythmic intestinal triglyceride absorption, mitochondrial oxidative capacity, and very low-density lipoprotein (VLDL) secretion (Marrino *et al.*, 1987; Lee *et al.*, 1992; Pan & Hussain, 2007; Ang *et al.*, 2012; Pan *et al.*, 2013; Yoshino *et al.*, 2014; van Moorsel *et al.*, 2016; Sprenger *et al.*, 2021). Equally, circulating catabolic and anabolic markers, such as cortisol and testosterone also exhibit rhythmicity across the day; testosterone tends to be lowest during the day but raises overnight to a morning peak, whereas cortisol tends to be at its highest during the morning/daytime and lowest across the night (Bremner *et al.*, 1983; Debono *et al.*, 2009). Aside from their broad roles in anabolic/catabolic pathways, both testosterone and cortisol also have distinct effects on carbohydrate and lipid metabolism and therefore diurnal variation in these hormones may further contribute to observed daily profiles in circulating glucose and lipids (Ottosson *et al.*, 2000; Christiansen *et al.*, 2007; Kelly & Jones, 2013).

Previous studies employing constant routine protocols to study daily variation in carbohydrate/lipid metabolism have provided valuable insight into *in vivo* circadian rhythmicity. However, glucose and lipid metabolism are strongly modulated by behavioural factors, including: fasting duration (Tsintzas *et al.*, 2006), physical activity/exercise (Sylow *et al.*, 2017; Edinburgh *et al.*, 2018), sleep (Donga *et al.*,

2010), and food/macronutrient intake (Chowdhury *et al.*, 2015, 2016b; Meng *et al.*, 2017; Yoshinaga *et al.*, 2021). During typical schedules, behavioural rhythms such as feeding-fasting are naturally aligned with cycles of light-dark and wake-sleep such that the majority of daylight hours are spent in the postprandial state, with the longest period of fasting across 24-hour period occurring at night (Ruge *et al.*, 2009b). Given the divergent responses of circulating insulin to feeding and fasting, alongside the potent entrainment effect of insulin upon circadian clocks, it is vital to study such metabolic rhythms in the context of these diurnal influences (Crosby *et al.*, 2019; Templeman *et al.*, 2021c; Tuvia *et al.*, 2021).

Skeletal muscle plays a central role in the storage, synthesis, and breakdown of nutrients, so the influence of this tissue in the diurnal variation of nutrient metabolism is particularly important to consider (DeFronzo *et al.*, 1985; Goodpaster *et al.*, 2001; Schenk & Horowitz, 2007). In the postprandial state skeletal muscle is responsible for ~40-85% of glucose uptake, some of which is incorporated into its storage form glycogen (DeFronzo *et al.*, 1981b; Shulman *et al.*, 1990). Skeletal muscle glycogen content and the ability to readily store and mobilise glycogen when required are all important determinants of insulin sensitivity and therefore metabolic health (Jensen *et al.*, 1997; Taylor *et al.*, 2018; Johnson-Bonson *et al.*, 2021). Skeletal muscle is also an important auxiliary store for lipids. The ability to turnover stores of intramyocellular lipids (IMCL) prevents accumulation of lipotoxic intermediates and therefore contributes to insulin sensitivity (Goodpaster *et al.*, 2001; van Loon & Goodpaster, 2006). Equally, skeletal muscle is the primary store of protein within the human body and is therefore the primary reservoir used to replace circulating amino acids taken up by other tissues. Through this process skeletal muscle continuously contributes towards the balance of protein synthesis with breakdown (Felig *et al.*, 1969; Biolo *et al.*, 1995).

Autophagy is a central process that regulates skeletal muscle protein turnover as well as glucose and lipid metabolism in constitutive conditions and in response to a variety of stimuli, including, nutrient deprivation, and amino acid starvation (Mizushima *et al.*, 2008; Luk *et al.*, 2021). Considering the importance of the skeletal muscle in providing flexibility in directing the response to nutrient availability it is remarkable that no studies to date have studied rhythmicity in the regulation of skeletal muscle metabolism alongside circulating metabolites and hormones involved in carbohydrate and lipid

metabolism. Moreover, skeletal muscle displays robust rhythmicity in core clock machinery, oxidative capacity, and lipid storage, further implicating the potential for this tissue to drive circadian rhythms in nutrient metabolism (van Moorsel *et al.*, 2016; Loizides-Mangold *et al.*, 2017; Perrin *et al.*, 2018b; Held *et al.*, 2020).

To enhance our knowledge of metabolic regulation across a 24-hour period of tightly controlled light-dark exposure and sleep-wake opportunity, it is now important to assess systemic hormonal and metabolite profiles alongside simultaneously collected skeletal muscle samples. To this end, the aim of the first study was to characterise temporal profiles in skeletal muscle gene expression in genes involved in carbohydrate metabolism, lipid metabolism, circadian clocks, and autophagy alongside systemic glucose, non-esterified fatty acids, glycerol, insulin, cortisol, testosterone, and C-terminal telopeptide (CTX), during a semi-constant routine whereby feeding-fasting was aligned with light-dark exposure and wake-sleep opportunity.

4.2 Methods and Materials

4.2.1 Approach to the research question

Given the descriptive and protracted nature of this study, a single-arm time series design was deemed appropriate. Whereas constant routine studies eliminate the influence of diurnal factors such as sleep-wake and fasting-feeding, the current study employed a semi-constant routine to study the diurnal influence of those factors. This protocol was characterised by designated wake and sleep opportunities, that are aligned with feeding and fasting, respectively. Given that most humans in middle-high income countries spend the majority of waking hours in the postprandial state, isocaloric snacks were ingested by participants every hour during waking hours to align feeding-fasting with wake-sleep and light-dark respectively (Ruge *et al.*, 2009b). Hourly feeds were prescribed to provide $6.66\%\cdot h^{-1}$ of measured 24 h resting metabolic rate across the 15 h waking period (i.e., 0800 – 2200 h). Feeding was therefore prescribed relative to measured resting energy requirements accounting for RMR as a primary driver of energy intake (Blundell *et al.*, 2012; Blundell *et al.*, 2015).

Hourly blood sampling was deemed both sufficient and feasible to detect diurnal rhythmicity in systemic hormones and metabolites (Cornelissen, 2014; Rynders *et al.*, 2020). Conversely, a different approach was required for muscle sampling due to the invasive nature of collecting these samples. Four hourly sampling was deemed appropriate to assess rhythmic expression of metabolic genes in this tissue while also minimising participant discomfort.

4.2.2 Research Design

A time-series design was employed to investigate temporal rhythms in skeletal muscle gene expression relating to carbohydrate metabolism, lipid metabolism, circadian clocks, and autophagy alongside plasma glucose, non-esterified fatty acids, insulin, glycerol, triglycerides, Cross-terminal Telopeptide (CTX) as well as serum cortisol and testosterone under conditions of semi-constant routine. Following a 7-day period of wake-sleep, meal-timing, and light exposure standardisation participants underwent a 37-hour in-patient visit to the resting laboratory at the University of Bath. During the final 24-hours of this visit participants had a designated sleeping opportunity and hourly isocaloric feedings during waking periods to preserve diurnal influences of

sleep-wake and fasting-feeding. Hourly blood samples were collected throughout day and night during sleep for assessment of rhythms in the systemic concentrations of glucose, non-esterified fatty acids, and insulin, along with melatonin and cortisol to provide a validated internal phase marker. Skeletal muscle samples were collected every 4-h from 1200 h for the remainder of the trial for assessment of mRNA expression. Ethical approval for the experimental protocol was obtained from the Cornwall and Plymouth NHS research ethics committee (reference: 14/SW/0123). All procedures were performed in accordance with the Declaration of Helsinki.

4.2.3 Participants

Ten healthy participants (9M;1F, **Table 4.1**) were recruited via local advertisement. Participant screening was undertaken through completion of a general health and validated chronotype questionnaires to assess habitual sleep patterns and diurnal preferences (Horne & Ostberg, 1976; Buysse *et al.*, 1989; Roenneberg *et al.*, 2003). All volunteers were fully briefed on the requirements of the study prior to provision of written informed consent.

Table 4.1: Participant characteristics of the study cohort. Data are presented as mean ± SD.

Characteristic	Mean ± SD
Age (y)	30 ± 10
Height (m)	1.81 ± 0.06
Body Mass (kg)	78.7 ± 7.0
Body Mass Index (kg·m^{-2})	24.1 ± 2.7
Resting Metabolic Rate (kcal·day^{-1})	1724 ± 314
Midsleep time (hh:mm)[*]	03:42 ± 01:13
Horne-Östberg Score	57 ± 11
Pittsburgh Sleep Quality Index	3 ± 2

[*]Determined from the Munich Chronotype Questionnaire (Roenneberg *et al.*, 2003)

4.2.4 Pre-experimental standardisation week

Participants adhered to a strict routine of feeding and sleeping in the 7-days prior to entering the laboratory, waking between 0600 and 0700 h and going to sleep between 2200 and 2300 h, confirmed using time-stamped voicemail. Upon waking, participants ensured at least 15 minutes of natural light exposure within 1.5 hours of waking, affirmed by wrist actigraphy using a light sensor, further confirming standardisation of sleep-wake patterns (Actiwatch™, Cambridge Neurotechnology; Cambridge, UK). Self-selected meals were scheduled at 0800, 1200 and 1800 h, with assigned snacking opportunities at 1000, 1500 and 2000 h. participants also completed a weighed record of all food and fluid intake on the final two days of this 7-day standardisation period.

4.2.5 Experimental Protocol

Following the standardisation week, participants reported to the laboratory at 1900 h on experimental day 1 in order to acclimatise to the laboratory environment. Laboratory conditions were standardised for the duration of their stay, with blackout-blinds to prevent the penetration of natural light and room temperature maintained at 20-25°C. During waking hours artificial lighting was set at 800 lux in the direction of gaze (0700-2200 h) and turned off (0 lux) during sleeping hours (2200-0700 h), during which time participants wore an eye mask. Participants remained in a semi-recumbent position throughout (i.e., head-end of bed elevated to 30°). Upon arrival, participants were shown to their bed and provided with a prescribed meal composed of a baked potato with butter and cheese, steamed vegetables (broccoli and mini corn), followed by a bowl of fresh strawberries, raspberries and blueberries (1245 kcal; 31% carbohydrate, 50% fat and 19% protein). An instant hot chocolate made with whole milk was then provided at 21:30 (242 kcal; 56% carbohydrate, 24% fat and 20% protein) before lights out at 2200 h.

Participants were woken at 0700 h and resting metabolic rate was immediately measured over 15 minutes using indirect calorimetry via the Douglas bag technique (Compher *et al.*, 2006). An intravenous cannula was fitted to an antecubital vein to allow for hourly 10 mL blood draws from 0800 h, alongside VAS during waking hours. Muscle biopsies were collected every 4 hours from 1200 h on day 2 through to 0800 h on day 3 as described in Chapter 3.5. After each set of measurements, an hourly feed was then ingested in the form of a meal-replacement solution (1.25 kcal·mL^{-1},

45% carbohydrate, 25% fat, 30% protein; Resource Protein, Nestlé; Vevey, Switzerland). Each hourly dose was prescribed to give $6.66\%\cdot h^{-1}$ of measured 24 h resting metabolic rate across the 15 h wake period. Plain water was consumed *ad libitum* and participants had access to mobile devices, on-demand entertainment, music and reading material throughout waking hours only. Toilet breaks were permitted in the first half of each hour as required.

The final set of waking measurements were collected at 2200 h, along with ingestion of the final prescribed feed. Following this, the lights were switched-off and participants were asked to wear an eye mask throughout the lights-out period. Blood samples continued throughout the night at hourly intervals without intentionally waking the participants. At 0700 h, participants were woken and immediately completed a set of VAS before a blood sample was drawn. The final set of measurements were made at 0800 h.
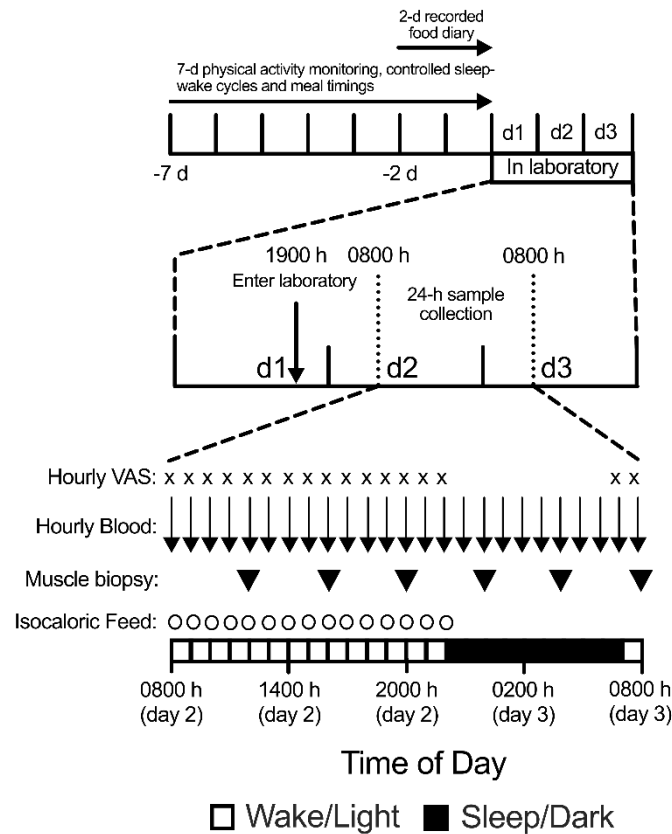
Figure 4.1 – Schematic representation of the study protocol. Participants began lifestyle standardisation 1 week (-7 d) prior to entering the laboratory for the experimental 24 h. d1 = experimental day 1, d2 = experimental day 2, d3 = experimental day 3.

4.2.6 Outcome Measures

Blood Sampling and Analysis – At each time-point, 10 mL of whole blood was drawn and immediately distributed into tubes treated with lithium heparin (melatonin) or ethylenediaminetetraacetic acid (EDTA; glucose, insulin, non-esterified fatty acids, glycerol-corrected triglycerides, glycerol, C-terminal telopeptide) or left to clot at room temperature for 15 minutes (Serum; cortisol and testosterone). Blood collection tubes were centrifuged for 10 minutes (3466 x g, 4°C), after which the supernatants were removed and stored at -80°C.

Hourly, plasma melatonin concentration was measured in the heparinised samples using a radioimmunoassay (Surrey Assays Ltd, UK). Plasma insulin (Mercodia, Sweden) C-Terminal Telopeptide (CTX) (ISD, UK), glucose, non-esterified fatty acids

(NEFA), glycerol and triglycerides (Randox, UK) were quantified in EDTA-treated plasma with cortisol (Tecan, CH) and testosterone (R&D Systems, Bio-Techne, US) quantified in serum. Assay performance is reported in Chapter 3.5.3.

4.2.7 Skeletal muscle sampling and analysis

Skeletal muscle samples were collected from the *vastus lateralis* under local anaesthesia (1% lidocaine: Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples were collected at 4-hourly intervals from 1200 until 0800 h (i.e., 6 in total) from a 3-5 mm incision in the anterior aspect of the thigh using a Bergstrom needle adapted for suction (Bergstrom, 1962b; Tarnopolsky *et al.*, 2011). Samples were taken from each leg in a randomly determined alternating order between dominant and non-dominant leg, ascending up the leg with skin incisions separated by 2–3 cm. Daytime biopsies were taken following the VAS and blood sample but before the prescribed feed. Prior to sleep, incisions for the night-time biopsies were made to minimise disruption to participants sleep. For night-time tissue biopsies (i.e. 0000 and 0400 h) participants were woken briefly but continued to wear the eye mask while samples were taken by torch-light. Samples were immediately snap-frozen in liquid nitrogen for subsequent storage at -80°C.

Samples were later homogenised in 2 mL Trizol (Invitrogen, UK) and centrifuged 2500 x *g* for 5 min at 4°C. The top layer and pellet were removed and 200 µl of chloroform was added per 1 mL of Trizol and mixed vigorously for 15 s. Samples were subsequently incubated at room temperature for 3 min prior to centrifugation at 2500 x *g* for 5min at 4°C. The aqueous phase was then removed and mixed with an equal volume of 70% ethanol prior to loading on an RNeasy mini column for extraction (Qiagen, Crawley, UK). All samples were quantified using spectrophotometry, with 2 µg of total RNA reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Assays from Applied Biosystems were used: AKT serine/threonine kinase 1 (*AKT1*) (Hs00178289_m1), 5'-aminolevulinate synthase 1 (*ALAS1*) (Hs00963537_m1), Basic helix-loop-helix ARNT like 1 (*ARNTL*) (Hs00154147_m1), beta-1,4-galactosyltransferase 5 (*B4GALT5*) (Hs00941041_m1), Circadian Locomotor Output Cycles Kaput (*CLOCK*) (Hs00231857_m1), Carnitine palmitoyltransferase 1B (*CPT1B;CHKB-CPT1B*) (Hs00189258_m1), cAMP responsive

element binding protein 5 (*CREB5*) (Hs00191719_m1), Cryptochrome circadian regulator 1 (*CRY1*) (Hs00172734_m1), Cryptochrome circadian regulator 2 (*CRY2*) (Hs00901393_m1), Citrate synthase (*CS*) (Hs02574374_s1), Casein kinase 1 epsilon (*CSNK1E*) (Hs01095999_g1), Cytochrome c, somatic (*CYCS*) (Hs01588974_g1), Eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*) (Hs00607050_m1), Fatty acid binding protein 3 (FABP3) (Hs00997362_m1), F-box protein 32 (*FBXO32*) (Hs01041408_m1), Forkhead box O3 (*FOXO3*) (Hs00818121_m1), Hexokinase 2 (*HK2*) (Hs00606086_m1), Heterogeneous nuclear ribonucleoprotein D like (*HNRNPDL*) (Hs00943609_m1), Myelin and Lymphocyte T-cell differentiation protein (*MAL*) (Hs00707014_s1), Mitogen-activated protein kinase 1 (*MAPK1*) (Hs01046830_m1), Mitogen-activated protein kinase 14 (*MAPK14*) (Hs01051152_m1), Mitogen-activated protein kinase 3 (*MAPK3*) (Hs00385075_m1), Mechanistic target of rapamycin kinase (*MTOR*) (Hs00234508_m1), Myosin heavy chain 1 (*MYH1*) (Hs00428600_m1), Myogenic differentiation 1 (*MYOD1*) (Hs00159528_m1), Neuronal PAS domain protein 2 (*NPAS2*) (Hs00231212_m1), Nuclear receptor subfamily 1 group D member 1 (*NR1D1*) (Hs00253876_m1), Nuclear receptor subfamily 1 group D member 2 (*NR1D2*) (Hs00233309_m1), Orthodenticle homeobox 1 (*OTX1*) (Hs00951099_m1), Pyruvate dehydrogenase kinase 4 (*PDK4*) (Hs01037712_m1), Period circadian protein 1 (*PER1*) (Hs00242988_m1), Period circadian protein 2 (*PER2*) (Hs01007553_m1), Period circadian protein 3 (*PER3*) (Hs00213466_m1), Peroxisome proliferator activated receptor delta (*PPARD*) (Hs04187066_g1), Peroxisome proliferator activated receptor gamma (*PPARG*) (Hs01115513_m1), PPARG coactivator 1 alpha (*PPARGC1A*) (Hs00173304_m1), Protein kinase AMP-activated catalytic subunit alpha 1 (*PRKAA1*) (Hs01562315_m1), Protein kinase AMP-activated catalytic subunit alpha 1 (*PRKAA2*) (Hs00178903_m1), Ribosomal protein S6 (*RPS6*) (Hs04195024_g1), Sirtuin 1 (*SIRT1*) (Hs01009006_m1), Sirtuin 3 (*SIRT3*) (Hs00953477_m1), Solute carrier family 2 member 4 (*GLUT4/SLC2A4*) (Hs00168966_m1), Transcription factor A, mitochondrial (*TFAM*) (Hs00273372_s1), Tumor protein p53 (*TP53*) (Hs01034249_m1), Uncoupling protein 3 (*UCP3*) (Hs01106052_m1). Real-time PCR was performed using a 7900HT PCR System (Applied Biosystems). The geometric mean of 18S ribosomal RNA (*18S*) (Hs03003631_g1), Actin alpha 1, skeletal muscle (*ACTA1*) (Hs05032285_s1), and Hydroxymethylbilane synthase (*HMBS*) (Hs00609296_g1) was used as an

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endogenous control. The comparative threshold cycle (Ct) method was used to process the data where ΔCt=Ct target gene – Ct endogenous control (Geometric mean of *18s, Actin, HMBS*). Data were then normalised to an internal calibrator and finally 24-h mean expression.

4.2.8 Statistical Analysis

Raw concentrations for circulating metabolic and endocrine markers were adjusted to dim light melatonin onset (DLMO) for each participant as determined by the 25% method (i.e., calculation of when 25 % of the peak melatonin concentration occurred) with the time of DLMO being assigned at 0° of the circadian phase (Benloucif *et al.*, 2008). The time in minutes between the DLMO and midnight was calculated for each participant and used to adjust 24-h profiles. The resulting x-values were binned around half past the hour with average y-values plotted at half past the hour (Otway *et al.*, 2011; Spiegel *et al.*, 2011; Mantele *et al.*, 2012).

Analysis of rhythmicity for all outcomes was conducted using the cosine method (Prism 9, Graphpad; CA, USA). In this approach a cosine wave is fit to the 24-h profile of a given variable and compared against a horizontal line through the mean values (null). If a cosine wave provides a better fit (R^2) for the data than the horizontal line then the dataset characterises diurnal (or 24-h) rhythmicity, with the mesor (rhythm-adjusted mean), amplitude (magnitude of the difference between mesor and peak/trough values) and acrophase (timing of rhythmic peak) all identified and reported (Refinetti *et al.*, 2007; Cornelissen, 2014). All data are presented as mean ± SD unless otherwise stated (e.g., figures are mean ± 95% Confidence Intervals).

This was an exploratory study and the first to serially sample skeletal muscle alongside systemic metabolites and hormones. Therefore, no formal sample size calculation was performed. However, based on similar research investigating circulating and adipose tissue rhythms under controlled conditions, an estimate of 10 participants was deemed sufficient to be able to detect rhythmicity in both blood and tissue markers (Shapiro *et al.*, 1988; Van Cauter *et al.*, 1992; Christou *et al.*, 2019).

4.3 Results

4.3.1 Melatonin

Peak plasma melatonin occurred at 0330 h ± 55 min with DLMO at 2318 h ± 55 min. Correction of to DLMO resulted in an average shift from clock time of 42 min (Range -35 – 117 min). *4.3.2 Metabolites*

All plasma metabolites displayed diurnal rhythmicity. Mean plasma glucose was rhythmic (p = 0.04, R^2 = 0.03, **Figure 4.2A**). The acrophase occurred at 0119 h and fell to the nadir in the afternoon, with a mean concentration of 4.83 ± 0.44 mmol·L^{-1} and amplitude of 0.17 mmol·L^{-1}. Plasma NEFA was also rhythmic peaking at 0456 h and falling to the nadir in the afternoon, with an amplitude of 0.15 mmol·L^{-1} and rhythm adjusted mean of 0.18 ± 0.05 mmol·L^{-1} (p < 0.01, R^2 = 0.38, **Figure 4.2B**). Likewise, diurnal rhythmicity was evident in plasma glycerol. Mean concentrations across the period were 0.02 ± 0.01 mmol·L^{-1} the diurnal rhythm was characterised by an amplitude of 0.08 mmol·L^{-1}, peaking at 0432 h with lowest values in the afternoon (p < 0.01, R^2 = 0.14, **Figure 4.2C**). Plasma triglycerides were also rhythmic with the acrophase occurring at 2314 h and falling to a nadir in the afternoon, with an amplitude of 0.13 mmol·L^{-1} and 24-h mean of 0.94 ± 0.32 mmol·L^{-1} (p < 0.01, R^2 = 0.06, **Figure 4.2D**). Finally, plasma Urea was rhythmic across the period, peaking at 0046 h with an amplitude of 0.66 mmol·L^{-1} and mean concentration of 7.45 mmol·L^{-1} (p < 0.01, R^2 = 0.08, **Figure 4.2E**).
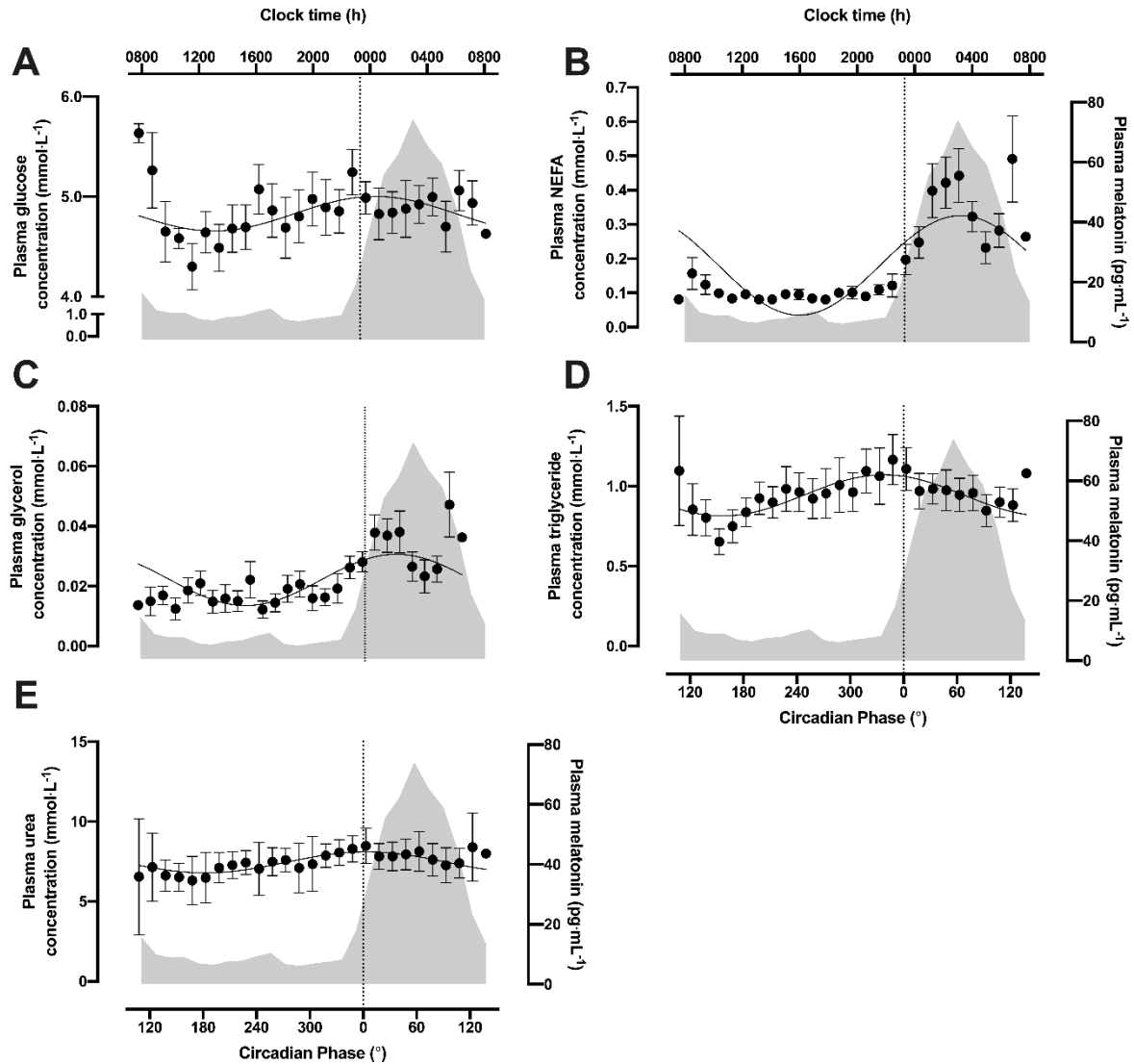
Figure 4.2 – 24-hour profile for dim light melatonin onset adjusted A) plasma glucose B) plasma non-esterified fatty acids C) plasma glycerol D) plasma triglycerides E) plasma urea. Solid lines denote the regression that best fits the data with the horizontal dotted line representing the 24-hour mean concentration used for the null comparison. The dotted vertical line denotes DLMO. The shaded areas represent 24-h melatonin profile.

4.3.3 Hormones and telopeptides

Plasma insulin was rhythmic, peaking at 1804 h before falling to an overnight nadir (p < 0.0001, R^2 = 0.08, **Figure 4.3A**). The diurnal rhythm occurred with an amplitude of 10.0 pmol·L^{-1}and a mean concentration of 43.4 ± 17.1 pmol·L^{-1}. 24-hour plasma CTX was also characterised by diurnal rhythmicity (p < 0.0001, R^2 = 0.19, **Figure 4.3B**). Peak concentration occurred at 0507 h and fell to the nadir during the afternoon, with an amplitude of 0.16 ng·mL^{-1} and mean of 0.29 ± 0.20 ng·mL^{-1}. Mean serum cortisol was also rhythmic, peaking at 1050 h with an amplitude of 22.3 nmol·L^{-1} (p < 0.0001, R^2 = 0.12, **Figure 4.3C**). Average cortisol concentration across the 24-h period was 232 ± 55 nmol·L^{-1}. Conversely, mean serum testosterone was not rhythmic with an average concentration of 70.2 ± 54.8 nmol·L^{-1} (p = 0.62, **Figure 4.3D**).
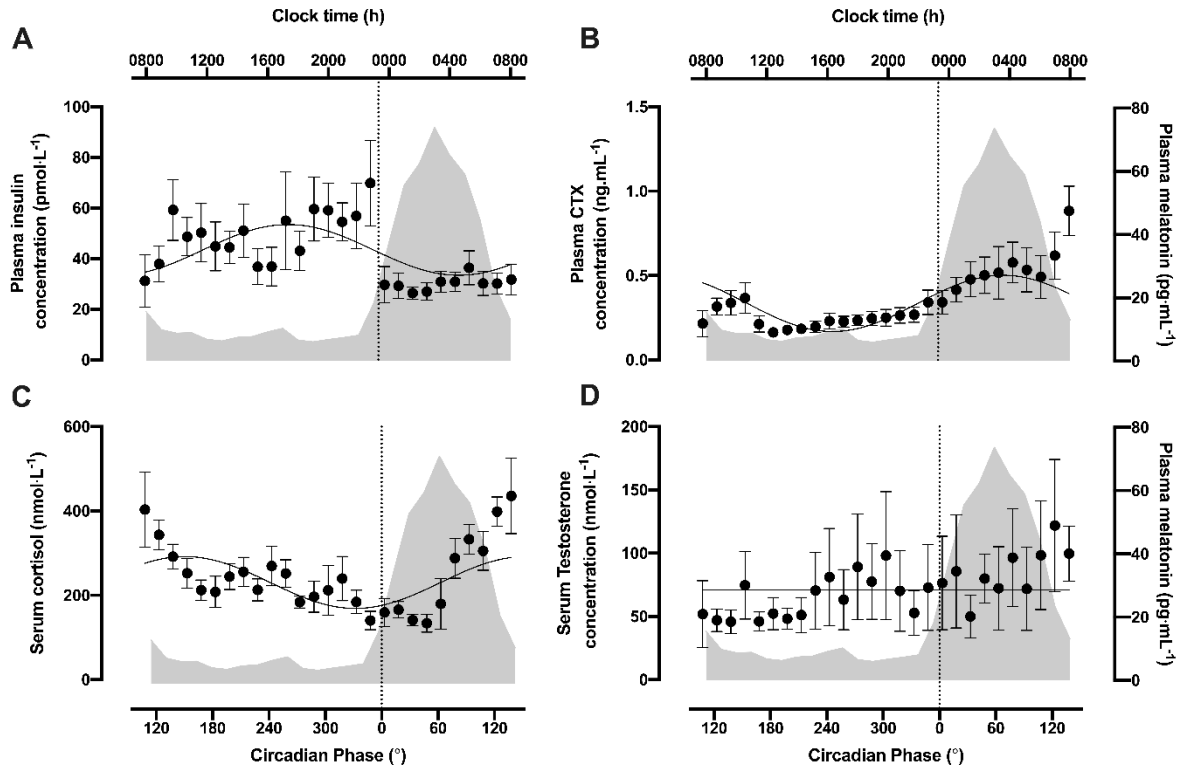
Figure 4.3 – 24-hour profile for dim light melatonin onset adjusted A) plasma insulin B) plasma cross-terminal telopeptide (CTX) C) serum cortisol D) serum testosterone. Solid lines denote the regression that best fits the data with the horizontal dotted line representing the 24-hour mean concentration used for the null comparison. The dotted vertical line denotes DLMO. The shaded areas represent 24-h melatonin profile.

4.3.4 Skeletal muscle gene expression

Of the 44 genes assessed, 26 displayed rhythmicity (all *p* < 0.05) (**Figure 4.4**). This diurnal rhythmicity was evident for all core clock genes: *ARNTL* (Acrophase 2218 h, Amplitude: 70%)*, CLOCK* (Acrophase 2329 h, Amplitude: 11%)*, CRY2* (Acrophase 1308 h, Amplitude: 23%)*, NPAS2* (Acrophase 0012 h, Amplitude: 37%)*, NR1D1* (Acrophase 0404 h, Amplitude: 63%)*, NR1D2* (Acrophase 0804 h, Amplitude: 36%)*, PER1* (Acrophase 1021 h, Amplitude: 48%)*, PER2* (Acrophase 0821 h, Amplitude: 41%)*, PER3* (Acrophase 0930 h, Amplitude: 57%), and *TP53* (Acrophase 0500 h, Amplitude: 20%). Genes relating to autophagy and protein metabolism were also diurnal: *MYOD1* (Acrophase 1914 h, Amplitude: 41%), *FOXO3* (Acrophase 0900 h, Amplitude: 26%), *FBXO32* (Acrophase 0716 h, Amplitude: 39%). Diurnal oscillations were also present in the expression of genes involved in glucose and lipid metabolism; *GLUT4* (Acrophase 1440 h, Amplitude: 25%), HK2 (Acrophase 1824 h, Amplitude: 20%), *FABP3* (Acrophase 1237 h, Amplitude: 15%)*, PDK4* (Acrophase 0530 h, Amplitude: 133%) and *CPT1B* (Acrophase 1258 h, Amplitude: 14%). Finally, diurnal variation was apparent in genes involved in mitochondrial signalling; *PPARGC1A* (Acrophase: 1613 h, Amplitude: 15%) and *UCP3* (Acrophase: 0659 h, Amplitude: 58%), as well as transcriptional/translational regulation; *CREB5* (Acrophase 0357 h, Amplitude: 19%)*, EIF4EBP1* (Acrophase 0741 h, Amplitude: 11%)*,* and *HNRNPDL* (Acrophase: 1317 h, Amplitude: 35%).
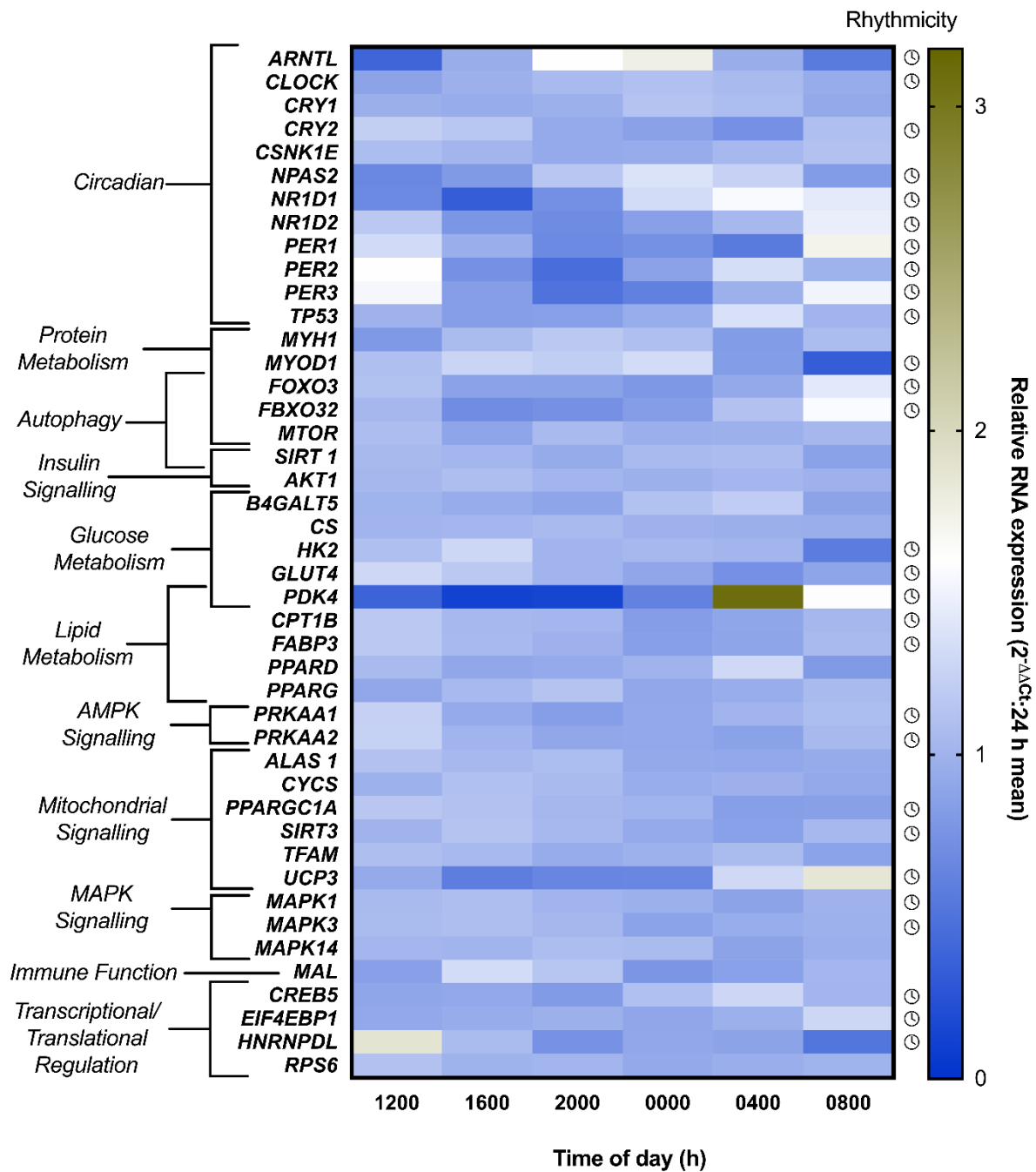
Figure 4.4. Relative changes in skeletal muscle RNA expression across the 24-h semi-constant routine. Diurnal rhythmicity (as determined by cosinor analysis) are denoted by a clock symbol.

4.4 Discussion

This is the first study to collect serial measures of human skeletal muscle alongside systemic markers of metabolic regulation under controlled diurnal conditions. Diurnal rhythms were apparent in skeletal muscle genes relating to carbohydrate, lipid and protein metabolism, as well as autophagy and mitochondrial signalling. Equally glucose, insulin, NEFA, glycerol, triglycerides, cortisol, and c-terminal telopeptide all displayed diurnal rhythmicity.

Rhythms in plasma glucose are driven by the endogenous clock. Plasma glucose concentrations displayed rhythmicity with an amplitude of 0.17 mmol·L^{-1} peaking at ~0030 h after oral nutrition was withdrawn. However, this is consistent with studies of circadian misalignment, constant routine, and forced desynchrony and further provides evidence of robust regulation of rhythms in plasma glucose by the endogenous clock (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Qian & Scheer, 2016). The amplitude of this rhythm whilst small perhaps demonstrates tight regulation of glucose homeostasis to maintain constant delivery of fuel to the brain (Fery *et al.*, 1990; Wasserman, 2009; Hengist *et al.*, 2020). Removal of a "large" meal stimulus (i.e., on average isocaloric feeds provided ~ 114 kcal each hour) may also explain the amplitude of the rhythm, however doing so was necessary to establish rhythmicity in the context of multiple diurnal factors (Qian & Scheer, 2016). Briefly, it must be acknowledged that adjustment of data points to DLMO resulted in a higher baseline blood glucose concentration relative to the remainder of the morning (**Figure 4.2A**). However, the mean value was 5.64 mmol·L^{-1} which is still within the range of normal fasting glucose concentrations (NICE, 2012).

Likewise, plasma insulin was rhythmic peaking in the evening (~1900 h) and falling to nadir overnight (~0400 h). This is similar to the rhythm reported during a 68-h glucose infusion in which insulin concentration peaked between 1200-1800 h and was lowest between 0000-0600 h as well as diurnal studies showing that insulin sensitivity is comparatively poorer in the evening than the morning (Boden *et al.*, 1996; Saad *et al.*, 2012).Given the contrasting pattern of nutrient delivery between the current study and the latter, it is likely that this rhythm occurs independent of food intake and are therefore endogenously driven (Ramracheya *et al.*, 2008; Stenvers *et al.*, 2019). However, insulin is highly responsive to nutrient intake, and therefore direct

comparison of nutrient feeding patterns is required to establish whether rhythms in insulin are under direct control of the endogenous system (Hengist *et al.*, 2020).

The diurnal profile of skeletal muscle gene expression relating to glucose metabolism was consistent with the observed rhythm in systemic insulin (Schalin-Jäntti *et al.*, 1994). Expression of *GLUT4* was rhythmic, with peak expression occurring at ~1500 h (i.e., when insulin was rising) and lowest expression at ~0400 h (i.e., when insulin was lowest). *PPARGC1A* followed a similar pattern with peak levels for the 24-h period occurring at ~1600 h and lowest levels at ~0400 h. Finally, peak *HK2* expression occurred at ~1830 h, in line with the regulatory effects of insulin on hexokinase activity (Mandarino *et al.*, 1995; Dimitriadis *et al.*, 2011). The broad alignment of the expression of these genes with the rhythm of plasma insulin reflects their involvement in glucose uptake and their potential to influence diurnal glucose metabolism (Watkins *et al.*, 1997; Michael *et al.*, 2001).

The diurnal profiles of NEFA and glycerol were also broadly anti-phasic to the 24-h profile of insulin. Circulating non-esterified fatty acids and glycerol were generally supressed during waking hours, before rising to peak at ~0400-0500 h, consistent with previous literature (Hagström-Toft *et al.*, 1997). The peak in lipids liberated from the adipose tissue coincided with when systemic insulin was lowest, consistent with the inhibitory effects of insulin on adipose tissue lipolysis (Mahler *et al.*, 1964; Arner *et al.*, 1981; Lafontan & Langin, 2009). Whilst the rhythm in insulin is likely endogenously driven, the opposing rhythms in NEFA and glycerol *could* also be reflective of feeding status and the subsequent changes in adipose tissue lipolysis in the overnight fasted state (Ruge *et al.*, 2009b; Chowdhury *et al.*, 2015, 2016b). However further work providing nutrition through the night is required to establish this.

From a regulatory perspective, the 24-h profile of muscle *PDK4* expression aligns with the observed rhythm in systemic insulin, peaking at ~0530 h when insulin was low (Lee *et al.*, 2004). Peak in expression of *PDK4* occurred approximate to the peak in systemic NEFA and is thus consistent with the role of this gene in stimulating fatty acid utilisation (Lee *et al.*, 2004; Zhang *et al.*, 2014b; Pettersen *et al.*, 2019). However, following peak mRNA levels, *PDK4* declined at ~0800 h, despite the continual fast state and resultant elevated NEFA availability, suggesting observed effects were not

solely due to the imposed fasting-feeding pattern. The profile of genes involved in the regulation of solubility, mobility, and transport of fatty acids (e.g., *CPT1B* and *FABP3*) did not align with systemic concentrations of NEFA (Bonnefont *et al.*, 2004; Furuhashi & Hotamisligil, 2008). mRNA doesn't necessarily correlate with protein levels, which may be explained by post-translational modifications, or autoregulation of protein levels (Buccitelli & Selbach, 2020). However, alignment between *UCP3* expression with the profile of systemic NEFA is consistent with the involvement of this gene in mitochondrial fatty acid oxidation (Samec *et al.*, 1999; Chrzanowski-Smith *et al.*, 2021). Circulating melatonin may also contribute towards diurnal rhythms in lipid metabolism; supplementation of endogenous melatonin maintains rhythmic patterns in lipid metabolism transcripts, upregulates lipolysis of intramuscular stores of lipids, reduces expression of genes involved in lipogenesis, and maintains normal absorption and excretion of lipids in the gut (Liu *et al.*, 2019; Hong *et al.*, 2020). In the current study, melatonin peaked at ~0330 h, shortly before peak systemic concentrations of NEFA and glycerol.

Plasma triglycerides were also rhythmic under controlled diurnal conditions, whereby systemic concentrations were low during the morning before rising to a peak at ~2330 h (**Figure 4.2D**). Previous studies have reported a similar rhythm in daily plasma triglycerides with lowest values during the day and peaking in approximation with DLMO (Brøns *et al.*, 2016; Yuan *et al.*, 2020). The rhythm observed in the current study is consistent in studies of constant routine and forced desynchrony, suggesting that it occurs independently of feeding patterns (Dallmann *et al.*, 2012; Kasukawa *et al.*, 2012; Chua *et al.*, 2013; Wehrens *et al.*, 2017a; Yuan *et al.*, 2020)

Numerous endocrine responses show rhythms and are relevant to tissue turnover. Cortisol, and testosterone for example contribute to daily balance in anabolic/catabolic processes whereas cross-terminal telopeptide reflects bone turnover. Cortisol, known for its largely catabolic action, displayed the expected rhythm over 24-h under tightly controlled diurnal conditions. Broadly, this response was anti-phasic to that of melatonin, which is unsurprising considering the contrasting effects of these hormones. Serum cortisol peaked in the morning (~1100 h) before falling to its lowest value in the evening, approximately coinciding with DLMO, consistent with previous literature (Wehrens *et al.*, 2017a). Cortisol typically results in the breakdown of contractile protein and the mobilisation of amino acids (Hoberman, 1950; Wing &

Goldberg, 1993). Peak expression of skeletal muscle *FBXO32* occurred during the morning period while cortisol was rising; consistent with the related action of this gene and hormone both playing role in catabolic processes (Bodine *et al.*, 2001; Gomes *et al.*, 2001). Following muscle breakdown, autophagy is a vital process to stimulate muscle regeneration (Luk *et al.*, 2021). Expression of *FOXO3*, which promotes expression of downstream targeted autophagy-related proteins, also peaked in the morning when cortisol is rising, therefore suggesting the morning rise in cortisol is likely to stimulate increased autophagic flux in skeletal muscle (Mammucari *et al.*, 2007; Shimizu *et al.*, 2011).

Conversely, serum testosterone did not display diurnal rhythmicity. Previous studies have demonstrated clear rhythmicity with highest values early in the morning (~0800 h) and corresponding lowest values ~12 h later (Resko & Eik-nes, 1966; Marrama *et al.*, 1982; Bremner *et al.*, 1983; Plymate *et al.*, 1989). This typical rhythm was not observed in the current study, and this could be explained by several mechanisms. Mixed-meal feeding reliably lowers circulating testosterone, so it is conceivable that, daytime nutrition resulted in suppression of serum testosterone (Lehtihet *et al.*, 2012; Gagliano-Jucá *et al.*, 2019). Sleep fragmentation disrupts the normal nocturnal rhythm in systemic testosterone, however sleep disruption was minimized in the current study when collecting night-time samples, and this is perhaps reflected by the rhythms observed in other assessed metabolites and hormones presently (Luboshitzky *et al.*, 2001). Small rises in cortisol across the day (i.e., from muscle biopsies) may have also dampened the 24-h profile of testosterone (Cumming *et al.*, 1983). Equally, age and biological sex also affect circulating levels of testosterone, with an age-related loss of rhythmicity observed in older males (Bremner *et al.*, 1983). However, participants in the current study were relatively young, with only 1 female participant so neither factor is likely to explain the lack of rhythmicity in the current study. The lack of rhythmicity could also be due to the use of commercial enzyme-based immuno-assays rather than gold standard measurement by liquid chromatography mass spectrometry (Handelsman & Wartofsky, 2013; Handelsman *et al.*, 2018).

Plasma cross-terminal telopeptide (CTX) is a marker of bone turnover that displays clear diurnal rhythmicity. Plasma CTX peaked during the biological night (~0500 h) in a remarkably similar rhythm to those reported in both 24-h fasted and 24-h fed literature, with a similar amplitude also (Aoshima *et al.*, 1998; Schlemmer & Hassager,

1999; Redmond *et al.*, 2016). This suggests that bone turnover is a rhythmic process robustly driven by the endogenous circadian system. However, diurnal factors such as feeding-fasting may modulate the magnitude of the rhythm, with Chubb (2012) noting that fasting values of CTX are generally lower than fed values. Nonetheless, it is evident that bone turnover is typically higher during sleep than wake, which further supports the importance of sleep for bone health (Swanson *et al.*, 2018; Ochs-Balcom *et al.*, 2020).

The rhythms reported support previous evidence demonstrating that metabolic control is poorer in the evening relative to the morning (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Van Cauter *et al.*, 1997; Gu *et al.*, 2020). Clinicians and researchers alike may also benefit from the daily rhythmicity reported in glucose and lipid metabolism and should seek to implement appropriate pre-sample controls (e.g., standardised time of day and/or nutritional status for sampling) for static blood sampling. Nonetheless, the current study demonstrates that semi-constant routine is a feasible method of assessing rhythmicity in the presence of diurnal factors (i.e., alignment of feed-fast with wake-sleep). However, the lack of a comparator group receiving nutrition through the night makes it difficult to fully establish whether the rhythms observed are driven by endogenous or exogenous factors. Further work should seek to adapt this protocol to explore whether the current reported rhythms persist when nutrition is provided continuously through sleep.

Participants were fed relative to individualised requirements, to account for the role of resting metabolic rate as a driver of energy intake and appetite (Blundell *et al.*, 2012; Blundell *et al.*, 2015). However, it must be recognised that 24 h bed rest eliminates the influence of physical activity and/or exercise on circadian clocks, glucose, lipid, and protein metabolism in skeletal muscle (van Loon *et al.*, 2003; Sylow *et al.*, 2017; Saracino *et al.*, 2019; Silva *et al.*, 2021). This is especially pertinent given that muscle samples were collected from the legs, which typically sustain greater load bearing than upper limbs so bed rest may elicit greater metabolic perturbation (Crossland *et al.*, 2019). Finally, the macronutrient composition of the feed provided to participants provided relatively less carbohydrate (45%) but more protein (30%) than typical UK daily intakes (~58 and ~20% respectively) (PHE, 2020). However macronutrient composition across the day tends to follow a diurnal distribution whereby the most carbohydrate and least protein are consumed in the morning and most protein

ingested in the evening (NHANES, 2016). Consequently, the feed provided an even distribution of macronutrients across the day.

As one female participant was recruited it must also be considered that the average intrinsic circadian periodicity is shorter in females than males, and rhythms in melatonin and body temperature are set an hour earlier when sleep/wake times are identical between sexes (Cain *et al.*, 2010; Duffy *et al.*, 2011). Consistent with this, this participant had the earliest DLMO of all participants, and displayed rhythmicity for circulating glucose and NEFA only. Due to small sample size the study is not sufficiently powered to detect sex differences in rhythmic metabolism, yet the pattern of the rhythms in glucose NEFA were similar to those seen in other participants.

In summary, this was the first study to demonstrate diurnal rhythms in human skeletal muscle genes relating to carbohydrate, lipid and protein metabolism, autophagy and mitochondrial signalling, alongside systemic concentrations of glucose, NEFA, glycerol, triglycerides, insulin, cortisol, and CTX under controlled diurnal conditions. These rhythms were consistent with feeding status (i.e., fed during waking hours only) and the resultant systemic insulin profile. This study provides novel context for metabolic regulation at both the tissue and systemic level.

Chapter 5 Influence of nutrient delivery pattern on 24-h human metabolism

5.1 Introduction

Metabolic regulation describes the mechanisms through which periodic delivery of dietary nutrients is channelled within and between tissues to meet variable physiological requirements (Frayn, 2010a). Temporal variance in nutrient flux poses a daily challenge to homeostasis, such that human physiology is heavily influenced by the alignment between daily light-dark, wake-sleep and fed-fasted cycles (Ekmekcioglu & Touitou, 2011; Dibner & Schibler, 2018). These states are anticipated by endogenous circadian rhythms to synchronise physiology and behaviour - with wakefulness, activity and feeding ideally scheduled to coincide during daylight in humans (Johnston, 2014a, b; Gerhart-Hines & Lazar, 2015; Longo & Panda, 2016; McGinnis & Young, 2016).

The presence and rhythmicity of this core clock machinery in muscle contributes towards the coordinated disposal, degradation, synthesis and recycling of metabolic substrates during diurnal fed-fasted cycles (DeFronzo *et al.*, 1981b; Ruge *et al.*, 2009b; Vollmers *et al.*, 2009; Frayn, 2010a). Previous work has verified the existence robust rhythmicity in core clock machinery at the transcript level in human skeletal muscle (Perrin *et al.*, 2015; Loizides-Mangold *et al.*, 2017; Perrin *et al.*, 2018a). Notably, feeding-responsive hormones such as insulin, glucagon, and IGF-1 are potent modulators of clock gene and/or protein expression in multiple tissues (Asher *et al.*, 2008; Tahara *et al.*, 2010; Hardie *et al.*, 2012; Mukherji *et al.*, 2015; Sun *et al.*, 2015; Ikeda *et al.*, 2018; Crosby *et al.*, 2019; Tuvia *et al.*, 2021). This suggests potential for feeding patterns to drive rhythmicity in peripheral tissue transcripts, which appeared to be modulated directly by the circadian clock in mice (Asher *et al.*, 2008; Lamia *et al.*, 2009; Vollmers *et al.*, 2009). The data reported in Chapter 4 provide the first *human* evidence of a rhythmicity in skeletal muscle RNA responding to cycles of fasting-feeding. In particular rhythmicity was observed in upstream regulators of skeletal muscle autophagy. It is now important to establish whether these rhythms occur independent of or are driven by feeding pattern (i.e., whether they are driven by endogenous or exogenous clocks, respectively) and the role of autophagy in doing so.

Autophagy is a vital component of the adaptive response of skeletal muscle to variable nutrient supply, exhibiting diurnal rhythmicity in mice such that catabolic processes mediated by autophagy primarily occur during the inactive phase (i.e., when food intake is low) (Kim & Lee, 2014; Yin *et al.*, 2016; Martinez-Lopez *et al.*, 2017; Ryzhikov *et al.*, 2019). Diurnal rhythmicity of autophagy therefore appears to be more aligned with intermittent rather than continuous feeding, yet research has neither examined the daily rhythm of these processes in human skeletal muscle *in vivo* nor the effects of nutrient delivery pattern on autophagy.

Beyond the need to better understand the fundamental physiological effects of typical daily meal patterns, studying enteral feeding patterns also holds important clinical applications to the pathologic state. At present, continuous administration of nutrition is the default approach for critically ill patients in intensive care, whereas intermittent or cyclic administration is favoured for short-term in-patient admissions. Current practices are based mainly on convenience, rather than replicating the physiological norm of intermittent feeding. There is therefore an urgent need for further trials to enable evidence-based approaches (Ichimaru, 2018). . In particular, insulin resistance occurs in ~33% of enterally fed patients, which could be explained by muscle disuse, injury/illness associated inflammation, and/or endocrine disruption resulting from an inappropriate enteral nutrition delivery pattern (Woolfson *et al.*, 1976; Dirks *et al.*, 2016; Luttikhold *et al.*, 2016). It has been shown that intermittent enteral feeding lowered fasting plasma glucose and GLP-1 concentrations relative to a continuous feeding pattern, which was associated with maintenance of peripheral insulin sensitivity during a 7-d bed rest (Gonzalez *et al.*, 2020). Furthermore, disruption of circadian clocks as a result of enteral feeding pattern may also lead to insulin resistance, yet no studies to date have examined skeletal muscle clocks in response to divergent feeding patterns (Shi *et al.*, 2013; Perrin *et al.*, 2018a). Given the critical role of skeletal muscle in postprandial metabolic regulation, it is important to establish the temporal responses of this tissue to enteral nutrition delivery pattern. This is especially important considering the potential of nutrients to entrain peripheral clocks and therefore influence circadian alignment (Leproult *et al.*, 2014; Javeed & Matveyenko, 2018).

Beyond the above relevance to enteral feeding in critically ill patients, the comparison of feeding patterns under such tightly controlled conditions may also help inform

research and dietary guidelines on eating patterns in non-critically ill patients receiving enteral nutrition as well as individuals outside of a clinical environment. For example, current guidelines are disproportionately informed by epidemiological evidence, partly because RCTs often struggle to attain sufficient statistical power to overcome the associated noise of free-living studies (Fabry *et al.*, 1964b; Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Therefore, whilst the tube feeding protocol adopted in this experiment represents a more extreme and artificial model than typical daily eating patterns, enteral feeding is the only method of achieving true continuous feeding. Furthermore, the insights gained may nonetheless complement existing epidemiological studies and clinical trials of free-living participants to advance understanding of physiological responses to varied eating patterns.

To this end, the aim of this study was to investigate the responses of skeletal muscle transcripts, autophagic flux, systemic metabolites, hormones, and telopeptides to enteral feeding, either delivered continuously across 24-h (*Continuous*), or in 2 evenly distributed boluses (*Bolus*) separated by 12 hours.

5.2 Methods and Materials

5.2.1 Approach to the research question

In Chapter 4 feeding was restricted to waking hours only (i.e., a semi-constant routine). The use of enteral (nasogastric) feeding in the current study therefore allowed for continuous nutrient delivery over 24-h (including during sleep). In the *Bolus* condition two 30-min feeds were chosen to achieve the minimum frequency of feeding whilst still maintaining an evenly balanced delivery of nutrients across 24 h, therefore providing a polarised contrast to the continuous delivery of nutrients (i.e., the maximal possible frequency of nutrient delivery). Furthermore, the *Bolus* feeding pattern selected allowed for assessment of time-of-day effects of feeding as seen in previous studies of diurnal metabolic regulation (Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Van Cauter *et al.*, 1997).

As per the data reported in Chapter 4, hourly resolution was deemed sufficient for assessing rhythms in blood metabolites (Templeman *et al.*, 2021b). Likewise, four-hourly resolution was deemed practical and sufficient for detecting rhythmicity *in vivo,* in skeletal muscle (Loizides-Mangold *et al.*, 2017; Perrin *et al.*, 2018a).

Autophagic flux was assessed in isolated neutrophils at 2000 and 0000 h hours. These time-points were chosen to contrast measures of autophagic flux 1) between individuals in a fasted (i.e., 12-h since last feed) state (i.e., *Bolus*) *versus* 12 hours continual nutrition (2000 h) and b) In the postprandial (i.e., 4-h since last feed) state (*Bolus*) *versus* 16 hours continuous nutrition (0000 h).

5.2.2 Research Design

A randomised parallel group design was employed to investigate the response of temporal rhythms in skeletal muscle transcripts, plasma glucose, non-esterified fatty acids, insulin, glycerol, triglycerides, urea, GLP-1, and melatonin, as well as serum testosterone to two distinct enteral feeding patterns. Following 7 days of lifestyle standardisation (**Figure 5.1**), participants underwent a 37-hour in-patient visit to the resting laboratory at the University of Bath. During the final 24 hours of this visit, participants were randomised based on biological sex and age to receive all nutrition enterally, either continuously or as two bolus2 boluses (30-min infusion) separated by 12 hours. Participants had a designated sleeping opportunity to preserve the diurnal

influence of usual sleep-wake patterns. Hourly blood samples were collected throughout day and night during sleep for assessment of systemic concentrations of glucose, non-esterified fatty acids, insulin, glycerol, triglycerides (TG), urea, GLP-1, and serum testosterone, as well as melatonin to provide a validated internal phase marker. Ethical approval for the experimental protocol was obtained from the NHS research ethics committee (reference: 18/SW/0176).

5.2.3 Participants

Eighteen healthy men and women (16M;2F, Table 1) were recruited via local advertisement. Participant screening was undertaken through completion of general health and validated chronotype questionnaires to assess habitual sleep patterns and diurnal preferences (Horne & Ostberg, 1976; Buysse *et al.*, 1989; Roenneberg *et al.*, 2003). Volunteers were fully briefed on the requirements of the study prior to provision of written informed consent.

Table 5.1: Participant characteristics of the study cohort. Data are presented as mean ± SD.

Characteristic	Continuous	Bolus	Combined
Sex (M/F)	8/1	8/1	16/2
Age (y)	26 ± 8	26 ± 7	26 ± 8
Height (m)	1.81 ± 0.10	1.80 ± 0.08	1.80 ± 0.09
Body Mass (kg)	75.6 ± 7.9	77.4 ± 10.9	76.5 ± 9.3
Body Mass Index (kg·m^{-2})	23.2 ± 2.4	23.8 ± 2.3	23.5 ± 2.3
Resting Metabolic Rate (kcal·day^{-1})	1820 ± 182	1865 ± 278	1842 ± 229
Average sleep duration (hh:mm)*	08:02 ± 00:59	08:02 ± 00:37	08:02 ± 00:48
Midsleep time (hh:mm)*	03:43 ± 00:36	03:40 ± 00:35	03:42 ± 00:34
Horne-Östberg Score	61 ± 7	60 ± 8	60 ± 7
Pittsburgh Sleep Quality Index	5 ± 2	3 ± 2	4 ± 2
Epworth Sleepiness Scale	4 ± 3	5 ± 4	4 ± 3

*Determined from the Munich Chronotype Questionnaire (Roenneberg *et al.*, 2003)

5.2.4 Pre-experimental standardisation week

Participants adhered to a strict routine of feeding, sleeping, light-exposure and physical activity timing in the 7-days prior to entering the laboratory. Specifically, participants woke between 0600 and 0700 h and went to sleep between 2200 and

2300 h, confirmed by a time-stamped voicemail. Upon waking, participants ensured at least 15 minutes of natural light exposure was achieved within 1.5 hours of waking, confirmed by wrist actigraphy using a light sensor, further confirming standardisation of sleep-wake patterns (ActiwatchTM, Cambridge Neurotechnology; Cambridge, UK). Self-selected meals were scheduled at 0800, 1200 and 1800 h, with assigned snacking at 1000, 1500 and 2000 h. Participants also completed a weighed record of all food and fluid intake on the final two days of this 7-day standardisation period (**Table 5.2**). On the final day of standardisation (experimental day 1), participants consumed a standardised snack shortly after waking (0800 h) (Frusli, Jordans, UK), prior to being provided with a within-participant self-selected standardised breakfast (0900 h), lunch (1200 h) and mid-afternoon snack (1500 h) (**Table 5.2**).

Table 5.2 – Macronutrient composition of standardised meals.

	Continuous	Bolus
AM Snack (kcal)	239	
Carbohydrate (g)	46	
Fat (g)	4	
Protein (g)	3	
Breakfast (kcal)	995 ± 358	1301 ± 468
Carbohydrate (g)	71 ± 33	105 ± 58
Fat (g)	57 ± 26	74 ± 22
Protein (g)	52 ± 19	53 ± 16
Lunch (kcal)	546 ± 214	483 ± 176
Carbohydrate (g)	59 ± 18	59 ± 20
Fat (g)	23 ± 18	15 ± 10
Protein (g)	26 ± 6	23 ± 6
PM Snack (kcal)	89 ± 4	80 ± 25
Carbohydrate (g)	20 ± 0	17 ± 5
Fat (g)	0.6 ± 0.4	0.8 ± 0.2
Protein (g)	4 ± 1	2 ± 2
Dinner (kcal)	1017	
Carbohydrate (g)	124	
Fat (g)	39	

Protein (g)	42

5.2.5 Experimental Protocol

Following standardisation, participants reported to the laboratory at the University of Bath at 1900 h on experimental day 1 to acclimatise to the laboratory environment (**Figure 5.1**). Ambient conditions were standardised for the duration of their stay, with blackout-blinds to prevent natural light and room temperature maintained at 20-25°C. Artificial lighting was set at 800 lux in the direction of gaze for waking hours (0700-2200 h) and turned off (0 lux) during sleeping hours (2200-0700 h), during which time participants also wore an eye mask. Participants remained in a semi-recumbent position throughout (i.e., head-end of bed elevated to ~30°). Upon arrival, participants were shown to their bed and provided with a prescribed meal composed of a baked potato with butter and cheese, steamed vegetables (broccoli and mini corn), followed by a bowl of fresh raspberries and blueberries. An instant hot chocolate made with whole-milk was then provided at 2130 h before lights out at 2200 h (**Table 5.2**).

Participants were woken up at 0700 h and resting metabolic rate was immediately measured over 20 minutes using indirect calorimetry via the Douglas bag technique (Compher *et al.*, 2006). The nasogastric tube (10 Fr Length 91 cm; with stylet and ENFit® connector, Corflo, UK) was then fitted (position at nostril 59 ± 2 cm), and the position confirmed by pH aspirate prior to insertion of the intravenous cannula into an antecubital vein to allow for hourly 10 mL blood draws from 0800 h. Visual analogue scales were provided to participants hourly during waking hours for assessment of subjective appetite and mood. Muscle biopsies were collected every 4 hours from 1200 h on day 2 through to 0800 h on day 3 as described in Chapter 3.5.

Throughout the experimental 24-h, participants received enteral nutrition standardised to individual resting metabolic rate, either in a 24-h continuous nutrient delivery pattern from 0800 h (1.3 ± 0.1 kcal·min^{-1}; *Continuous*) or in two 30-min bolus infusions at the start (0800 h) and mid-point (2000 h) of the monitoring period (31 ± 5 kcal·min^{-1}; *Bolus*) (**Table 5.3**). The enteral meal-replacement solution was a standard commercially available product used in healthcare, providing 100 kcal per 100 ml (7.6 g carbohydrate, 3.8 g fat, 9.2 g protein, plus vitamins and minerals; Nestlé Peptamen).

Enteral infusion patterns were matched for volume through bolus feeding of water at 0800 and 2000 h in the *Continuous* condition (37 ± 21 mL·min^{-1}) and continuous water infusion in the *Bolus* condition (1.2 ± 0.3 mL·min^{-1}). Plain water was consumed *ad libitum* and participants had access to mobile devices, on-demand entertainment, music and reading material throughout waking hours only. Toilet breaks were permitted in the first half of each hour as required.

The final set of waking measurements were collected at 2200 h, before lights were switched-off. Blood samples continued throughout the night at hourly intervals without intentionally waking the participants. Lights were switched on at 0700 h, participants were woken up and immediately completed a set of VAS prior to drawing a blood sample. The final set of measurements were made at 0800 h.

Table 5.3 – Macronutrient composition of the enteral feed.

	Continuous	Bolus
Total volume of enteral feed (mL)	1871 ± 209	1864 ± 278
Total energy of enteral feed (kcal)	1871 ± 209	1864 ± 278
Bolus Volume (mL)	910 ± 91	932 ± 139
Bolus Energy (mL)	N/A	932 ± 139
Carbohydrate (g)	137 ± 15	136 ± 20
Carbohydrate (kcal)	547 ± 61	544 ± 81
Fat (g)	69 ± 8	69 ± 10
Fat (kcal)	623 ± 69	620 ± 92
Protein (g)	174 ± 19	173 ± 26
Protein (kcal)	696 ± 78	694 ± 103

N.B. Bolus values represent total amount fed across 2 equal boluses. I.e., divide all values by 2.
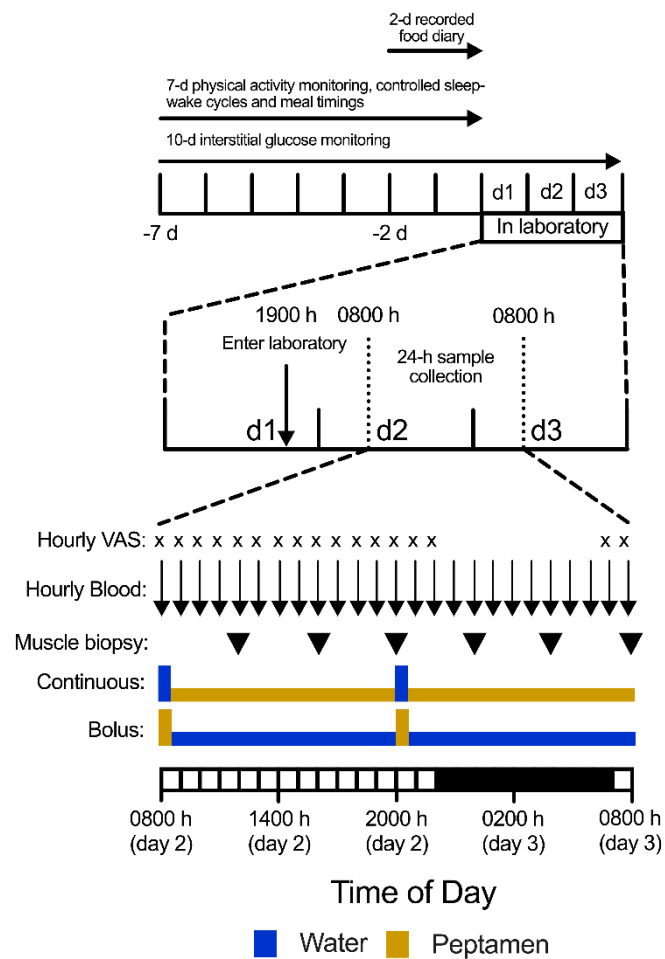
Figure 5.1: Schematic representation of the study protocol. White boxes represent waking hours and shaded boxes represent sleep opportunity. The pattern of enteral feed is depicted with blue and gold boxes for water and enteral feed respectively.

5.2.6 Outcome Measures

Blood Sampling and Analysis –As described in **Chapter 3.3.4**, 10 mL of whole venous blood was drawn on the hour, each hour, and immediately distributed into tubes treated with lithium heparin (for melatonin), ethylenediaminetetraacetic acid (EDTA; plasma for glucose, NEFA, glycerol, triglycerides, insulin, GLP-1, CTX) or left to clot at room temperature for 15 minutes (serum for cortisol and testosterone). Both tubes were immediately centrifuged for 10 minutes (3466 x *g*, 4°C), after which the supernatants were removed and stored at -80°C.

Plasma Insulin (Mercodia), GLP-1 (Merck-Millipore, USA), C-Terminal telopeptide (CTX) (ISD, UK), glucose, non-esterified fatty acids (NEFA), glycerol, and triglycerides (Randox, UK) were quantified in EDTA-treated plasma with testosterone (R&D Systems, Bio-Techne, USA) quantified in serum using commercially available enzyme-linked immunosorbent assays. Assay performance is reported in **Chapter 3.5.3.**

5.2.7 Skeletal muscle sampling

Skeletal muscle samples were collected from the *vastus lateralis* under local anaesthesia (1% lidocaine: Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples were collected at 4-hourly intervals from 1200 until 0800 h (i.e., 6 in total) from a 3-5 mm incision in the anterior aspect of the thigh using a Bergstrom needle adapted for suction (Bergstrom, 1962b; Tarnopolsky *et al.*, 2011). Samples were taken from each leg in a randomly determined alternating order between dominant and non-dominant leg, ascending up the leg with skin incisions separated by 2–3 cm. Daytime biopsies

were taken following the VAS and blood sample but before the prescribed feed. Prior to sleep (2100 h), incisions for the night-time biopsies were made to minimise disruption to participants sleep. For night-time tissue biopsies (i.e., 0000 and 0400 h) participants were woken up briefly but continued to wear the eye mask while samples were taken by torch light. Samples were immediately snap-frozen in liquid nitrogen for subsequent storage at -80°C.

5.2.8 Autophagic flux assessed by flow cytometry

At 2000 and 0000 h, 9 mL of venous blood was immediately distributed into sodium heparin tubes (BD, USA) for assessment of autophagic flux in neutrophils by flow cytometry using a commercially available kit (FlowCellect Autophagy LC3 antibody-based kit, Appendix M). This kit allows for the selective measurement of autophagosome-associated LC3-II protein and autophagic flux. Flux refers to the accumulation of autophagosome-associated LC3-II (i.e., median fluorescence intensity of an antibody-fluorophore conjugate that binds to LC3) between neutrophils treated with an autophagosome/lysosomal degradation inhibitor compared to untreated cells. Due to permeabilization, cytosolic LC3-I is washed away, therefore fluorescence represents autophagosome associated LC3-II accumulation. The plasma portion of samples containing untouched neutrophils was harvested and diluted in a 1:1 ratio with sterile PBS (free from magnesium and calcium) and counted manually using a light microscope and haemocytometer before centrifugation for 5 min (200 x g, 21°C). Neutrophils were then resuspended in a volume of RPMI solution containing 10% human serum to provide 500 neutrophils·μL^{-1}. 1 mL of this cell suspension was added to 8 sterile tubes. The first 4 tubes were treated with 50 µL of an autophagosome/lysosome degradation inhibitor for 30 minutes to causing LC3-II to accumulate (i.e., a measure of autophagic flux over 30 minutes). The other 4 tubes were treated with 50 µL of additional RPMI-10% human serum. All tubes were incubated for 30 min at 37°C/5 % CO_2. Cells were recovered following centrifugation for 5 min (200 x g, 21°C) and transferred to a 96-well V-well plate before washing with 100 µL sterile PBS (200 x g for 5 min). Cells were incubated for 10 min in the dark at room temperature with 50 µL fluorophore-conjugated antibody mix in each well (1 µL CD45 Pacific Blue, J33, Beckman Coulter; 1 µL aCD16 PE, 3G8, Beckman Coulter; 0.5 µL CD66b A647, 6/40c, Biolegend in 50ul PBS/BSA 1 % per well). Cells were then

washed with 100 µL sterile PBS and centrifuged (200 x *g* for 5 min). 100 µL of a cell permeabilization buffer containing Benzonase (50 units/mL) was added before immediately centrifuging (200 x *g* for 5 min). Cells were then incubated with an anti-LC3 antibody conjugated to FITC (clone: 4E12; Millipore) for 20 min at room temperature in the dark. Following incubation cells were washed with 100 µL of assay buffer and resuspended in 300 µL of the same buffer prior to flow cytometry. Cells were assessed using a FACSAria III (BD Biosciences) immediately and results were analysed in FlowJo (v10; FlowJo LLC, USA). The gating strategy is shown in Figure **5.2** and autophagic flux is presented as Median Fluorescence Intensity (MFI) of the anti-LC3 antibody conjugated to FITC.
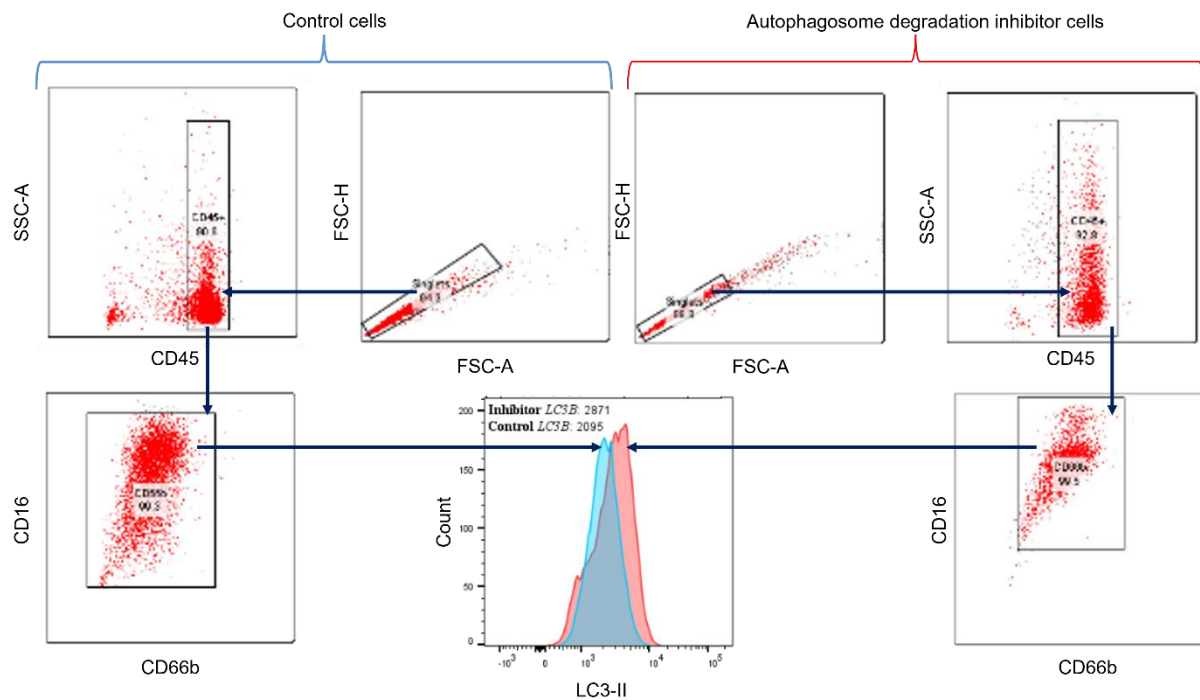
Figure 5.2 – Representative flow cytometry gating profile for neutrophils in peripheral blood. FSC-H/A = forward scatter height/area. SSC-H/A = Side scatter height/area: LC3 = autophagosomal autophagy protein.

5.2.9 Statistical Analysis

Time-series data were analysed by repeated measures ANOVA (Condition x Time) with condition as the between-subjects factor to identify differences between

experimental conditions; the Greenhouse-Geisser correction was employed for Greenhouse-Geisser epsilon <0.75 and the Huynh-Feldt correction adopted for less severe asphericity (Greenhouse & Geisser, 1959). Interaction effects were followed by Ryan-Holm-Bonferroni stepwise correction using unpaired t-tests to adjust the resulting p-values for multiple comparisons and thus avoid inflation of type I error rate (Ludbrook, 1998; Atkinson, 2002). Time-series data were separated in to 2 12-h periods that followed each bolus infusion (i.e., Period 1 and Period 2). Area under the curve data were calculated using the time-series response analyser (Narang *et al.*, 2020) and was analysed using a 2-way ANOVA (Condition x Period) with condition as the between-subjects factor.

Analysis of 24-h rhythmicity was performed using the cosine method at the group level for mean concentrations (as describe in **Chapter 3.8**) (Prism 9, Graphpad; CA, USA) (Refinetti *et al.*, 2007; Cornelissen, 2014). All data are presented as mean ± SD unless otherwise stated (e.g., figures are mean ± 95 % Confidence Intervals).

Based on the observation of rhythmicity in circulating metabolites and tissue metabolism in **Chapter 4**, as well as similar studies examining feeding patterns, it was estimated that a sample size of 18-20 (9/10 per group with a 1:1 allocation) would be sufficient to detect differences in the metabolic, hormonal, and tissue responses to enteral feeding pattern.

5.3 Results

5.3.1 Autophagic flux in neutrophils

Despite markedly higher mean values for some participants in the Continuous feeding condition (and thus a higher group mean), autophagic flux in neutrophils was not different between *Continuous* and *Bolus* delivery patterns (Condition p = 0.07) and also did not differ between 2000 h and 0000 h (Time p = 0.77). Nutrient delivery pattern did not affect autophagic flux over time (Continuous: 2000 h: 1666 ± 1716 *vs* 0000 h: 2201 ± 1746 LC3-B MFI·30 min^{-1}; *Bolus*: 2000 h: 845 ± 843 vs 1256 ± 844 LC3-B MFI·30 min^{-1}) (Condition x Time p = 0.93) (**Figure 5.3**).
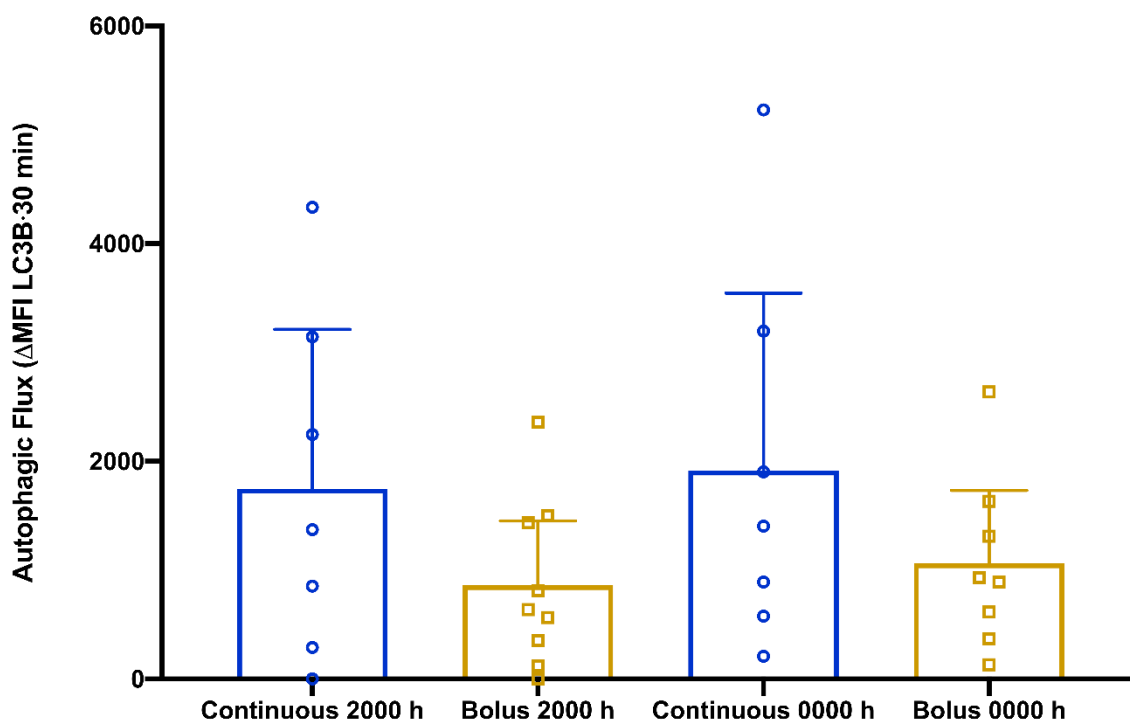
Figure 5.3 - Autophagic flux in neutrophils. Flux refers to the difference in FITC fluorescence (i.e., an anti-LC3 antibody) between neutrophils treated with an autophagosome/lysosomal degradation inhibitor compared to untreated cells. Due to permeabilization, cytosolic LC3-I is washed away, therefore fluorescence represents autophagosome associated LC3-II accumulation. Bars that share letters were not different from one another (i.e., p > 0.05).

5.3.3 Metabolites

Bolus feeding resulted in a different plasma glucose response across 24-h relative to the Continuous group (time x condition $p < 0.001$). In particular, *Bolus* provision of nutrients resulted in a spike in plasma glucose at 2100 h relative to the continuous condition, such that peak concentration across the period was higher when participants were fed in a dual bolus pattern (*Continuous:* 6.53 ± 0.51 *vs Bolus:* 8.19 ± 2.28 mmol·L^{-1}; $p = 0.05$) (**Figure 5.4A**). Systemic exposure to glucose above baseline was similar whether participants received nutrients in a *Continuous* or *Bolus* pattern (Condition $p = 0.10$), with exposure highest in period 2 compared to period 1 (Period $p < 0.01$). The temporal response of glucose exposure was modulated by enteral feeding pattern such that the increase from period 1 to period 2 was greater in the *Bolus* condition than in the *Continuous* condition (Condition x Period $p = 0.02$) (**Figure 5.4B**). Both continuous feeding (Acrophase: 0114 h Amplitude: 0.27 mmol·L^{-1}, Mesor: 5.50 mmol·L^{-1}; $p < 0.01$) and bolus feeding (Acrophase: 2305 h, Amplitude: 0.50 mmol·L^{-1}, Mesor: 5.30 mmol·L^{-1}; $p < 0.01$) resulted in rhythmicity in plasma glucose concentration (**Figure 5.6A**).

Serial plasma NEFA responses also differed between feeding patterns (Time x Condition $p < 0.001$) (**Figure 5.4C**). Despite an initial feeding-induced suppression, exposure to circulating NEFA was greater across the *Bolus* condition (11.3 ± 2.0 mmol·L^{-1}·24 h) relative to *Continuous* feeding (7.0 ± 2.1 mmol·L^{-1}·24 h) (Condition $p = 0.0004$) with greater total exposure in Period 1 than Period 2 (Period $p = 0.002$). However, the higher exposure to systemic NEFA in the *Bolus* condition relative to *Continuous* was consistent between period 1 and period 2 (Condition x Period $p = 0.17$) (**Figure 5.4D**). Overall, the profile of systemic NEFA was rhythmic when fed continuously (Acrophase: 1746 h, Amplitude: 0.10 mmol·L^{-1}, Mesor: 0.29 mmol·L^{-1}; $p < 0.01$) or in 2 bolus feedings (Acrophase: 1646 h, Amplitude: 0.10 mmol·L^{-1}, Mesor: 0.48 mmol·L^{-1}; $p < 0.01$) (**Figure 5.4B**).

Glycerol profile did not differ across 24-h whether participants were fed 2 Boluses or continuously (Time x Condition $p = 0.48$). Whilst exposure to plasma glycerol was greater in Period 1 than Period 2 (Period $p = 0.004$), *Continuous* nutrient delivery resulted in similar circulating glycerol exposure (1.64 ± 1.17 mmol·L^{-1}·24 h) to a dual *Bolus* delivery (1.58 ± 1.11 mmol·L^{-1}·24 h) (Condition $p = 0.89$). Equally, the pattern of

nutrient delivery did not alter the exposure to glycerol over each period (Condition x Period p = 0.81). Plasma glycerol profiles across the period were however rhythmic whether participants were fed in a *Continuous* (Acrophase: 1729 h Amplitude: 0.03 mmol·L^{-1}, Mesor: 0.07 mmol·L^{-1}; p < 0.01) or *Bolus* pattern (Acrophase: 1634 Amplitude: 0.02 mmol·L^{-1}, Mesor: 0.07 mmol·L^{-1}; p < 0.01).

Plasma triglycerides displayed differing profiles over 24 h between *Bolus* and *Continuous* feeding (Time x Condition p < 0.001). When fed continuously plasma triglycerides gradually increased across 24 h, whereas Bolus feeding resulted in a more stable concentration of plasma triglycerides over 24 hours (**Figure 5.4G**). Total exposure to systemic triglycerides (iAUC) was similar between *Continuous* (5.11 ± 3.24 mmol·L^{-1}·24 h) and *Bolus* feeding patterns (2.60 ± 1.81 mmol·L^{-1}·24 h) (Condition p = 0.97) and did not differ between period 1 and period 2 (Period p = 0.42). Likewise, the temporal pattern of systemic triglyceride exposure did not differ as a result of enteral feeding pattern (Condition x Period p = 0.35) (**Figure 5.4H**). Furthermore, plasma triglycerides were not rhythmic in either the *Continuous* (p = 0.07) or *Bolus* conditions (p = 0.46).

Finally, two bolus feedings distinctly altered the pattern of plasma urea concentrations compared to Continuous enteral feeding (Time x Condition p < 0.001) (**Figure 5.4I**). Total plasma urea exposure (iAUC) was greater in the *Bolus* condition (29.3 ± 7.4 mmol·L^{-1}·24 h) relative to *Continuous* feeding (17.4 ± 10.0 mmol·L^{-1}·24 h) (Condition p < 0.01) primarily due to greater exposure in Period 2 than Period 1 (Period p = 0.003). However, the temporal response was similar between enteral feeding patterns (Condition x Period p = 0.16) (**Figure 5.4J**). Systemic urea was rhythmic whether participants were fed continuously (Acrophase: 0240 h, Amplitude: 0.72 mmol·L^{-1}, Mesor: 6.18 mmol·L^{-1}; p < 0.01) or in boluses (Acrophase: 0219 h, Amplitude: 0.54 mmol·L^{-1}, Mesor: 6.74 mmol·L^{-1}; p < 0.01).
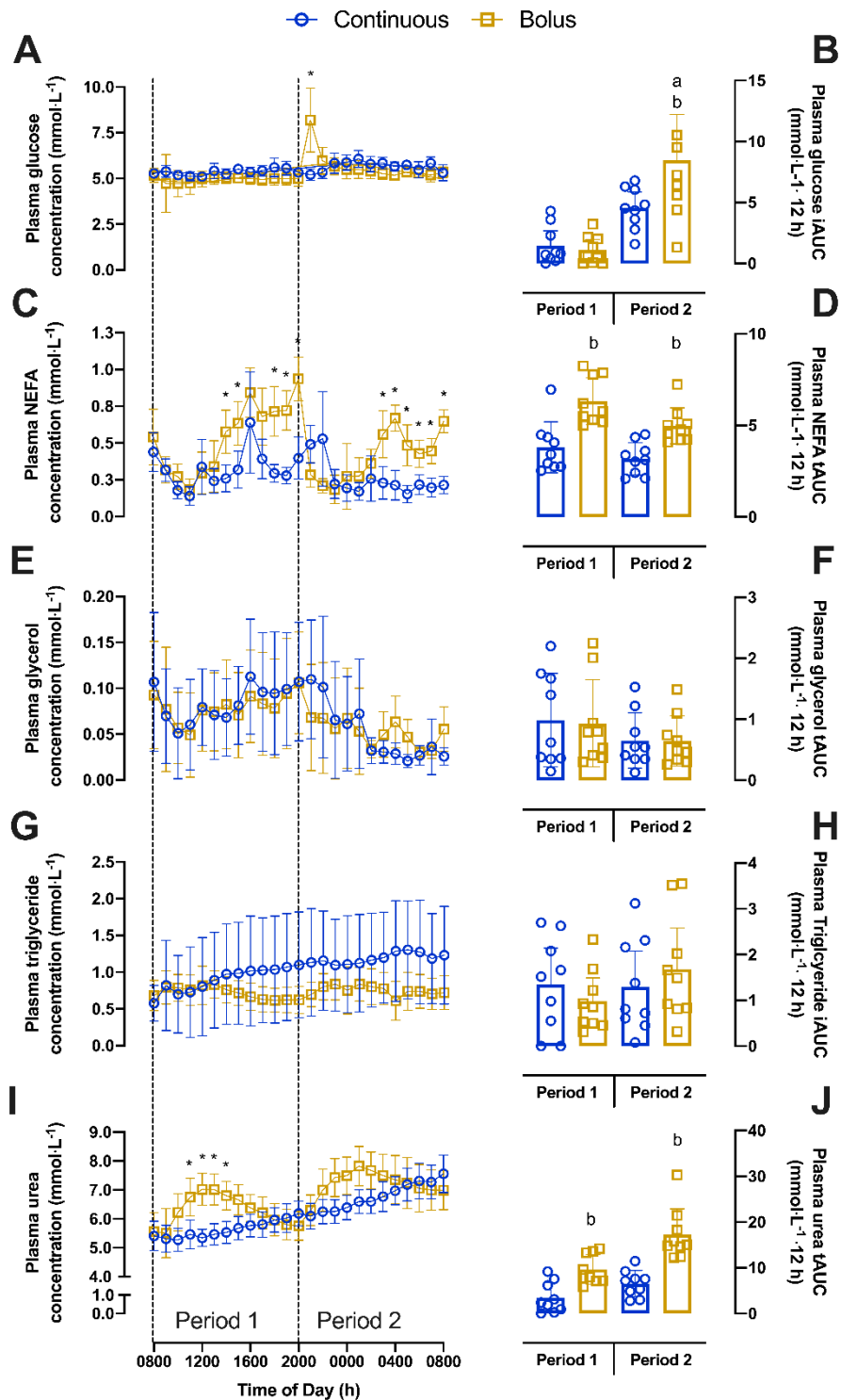
Figure 5.4 – Responses of plasma A) glucose B) glucose iAUC C) NEFA D) NEFA tAUC E) glycerol F) glycerol tAUC G) triglyceride H) triglyceride iAUC I) urea J) urea iAUC to *Continuous* and *Bolus* enteral nutrient delivery patterns. *Denotes difference between conditions ($p \leq 0.05$). "a" denotes between period, with condition difference in AUC ($p \leq 0.05$). "b" refers to within period, between condition differences ($p \leq 0.05$).

5.3.4 Hormones and telopeptides

Bolus feeding resulted in an altered pattern of systemic insulin compared to Continuous feeding (Time x Condition $p < 0.001$). Two evenly distributed Bolus feedings resulted in distinct spikes in plasma insulin concentrations between 0800-1200 h and 2000-0000 h, whereas insulin concentrations were relatively stable under continuous feeding conditions (**Figure 5.5A**). Accordingly, total exposure to insulin across the study was greater in the *Bolus* condition (2.48 ± 1.47 nmol·L^{-1}·24 h) compared to *Continuous* (0.56 ± 0.36 nmol·L^{-1}·24 h) (Condition $p = 0.001$). Exposure was evenly distributed across period 1 and period 2 (Period $p = 0.26$) and the temporal response was not modulated by nutrient delivery pattern (Condition x Period $p = 0.69$) (**Figure 5.5B**). Interestingly rhythmicity in plasma insulin concentration was only apparent in the continuous feeding condition (Acrophase: 0343 h, Amplitude: 10.3 pmol·L^{-1}, Mesor: 53.4 pmol·L^{-1}) (**Figure 5.6E**).

Feeding pattern also determined plasma GLP-1 response (Time x Condition $p < 0.001$). Similar to insulin, *Bolus* feeding resulted in 2 distinct increases in plasma total GLP-1 across the period relative to smaller temporal fluctuations in the Continuous feeding condition (**Figure 5.5C**). Consequently, 24 h exposure to total GLP-1 was greater in the *Bolus* feeding (336 ± 75 pmol·L^{-1}·24 h) condition relative to the *Continuous* feeding (154 ± 93 pmol·L^{-1}·24 h) (Condition $p < 0.01$), with greater exposure in period 1 (Period $p = 0.008$) (**Figure 5.5D**). However, the greater exposure to total GLP-1 with *Bolus* delivery was consistent across period 1 and period 2 (Condition x Period $p = 0.74$). Furthermore, plasma total GLP-1 was not rhythmic over the total study period in either *Continuous* ($p = 0.93$) or *Bolus* ($p = 0.46$) conditions.

Plasma CTX was modulated by feeding patterns (Condition x Time $p = 0.006$). Bolus feeding resulted in a suppression of plasma CTX after both 0800 and 2000 h, followed by a rise in concentration throughout the remainder of each post-bolus period (**Figure 5.5E**). Total exposure to plasma CTX was similar between *Continuous* (9.59 ± 6.42 ng·mL^{-1}·24 h) and *Bolus* (10.2 ± 6.10 ng·mL^{-1}·24 h) enteral nutrient delivery patterns (Condition $p < 0.83$), with markedly higher exposure occurring during period 2 relative to period 1 (Period $p < 0.01$). Nutrient delivery pattern modulated temporal responses to systemic CTX; whereas exposure was similar between conditions during period 1, CTX exposure during period 2 was higher in the *Continuous* condition (41.6 ± 13.2 ng·mL^{-1}·24 h) compared to Bolus (33.6 ± 17.6 ng·mL^{-1}·24 h) (Condition x Period $p =$

0.05) (**Figure 5.5F**). Plasma CTX was rhythmic when nutrients were delivered in either *Bolus* (Acrophase: 0520 h Amplitude: 0.08 ng·mL^{-1}, Mesor: 0.40 ng·mL^{-1}) or *Continuous* patterns (Acrophase: 0248 h, Amplitude: 0.16 ng·mL^{-1}, Mesor: 0.43 ng·mL^{-1}).

The 24-h profile of Serum testosterone did not differ between Continuous or Bolus delivery (condition x time p = 0.37) (**Figure 5.5G**). Accordingly, total exposure to systemic serum testosterone did not differ between *Continuous* (1111 ± 616 nmol·L^{-1}·24 h) or *Bolus* (1905 ± 2117 nmol·L^{-1}·24 h) enteral feeding (Condition p = 0.30) or between period 1 and 2 (Period p = 0.08). Likewise, the pattern of nutrient delivery did not alter the temporal profile of serum testosterone exposure (Condition x Period p = 0.54) (**Figure 5.5H**). Serum testosterone did not display rhythmicity when fed continuously (p = 0.89) or as two bolus feeds (p = 0.81).
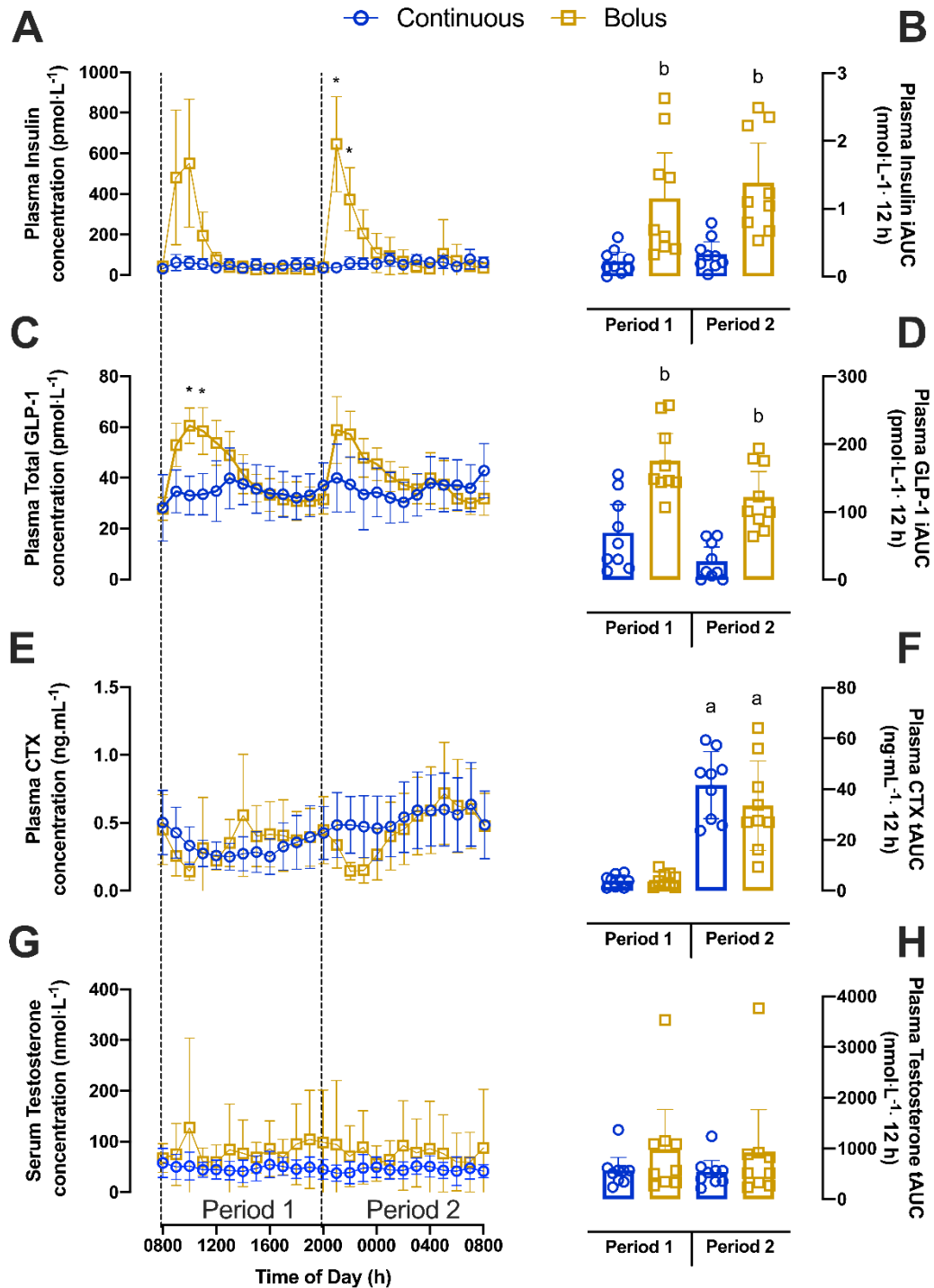
Figure 5.5 – Responses of plasma A) insulin B) insulin iAUC C) Total GLP-1 D) Total GLP-1 iAUC E) C-Terminal Telopeptide (CTX) F) CTX tAUC and G) Serum testosterone H) testosterone iAUC to *Continuous* and *Bolus* enteral nutrient delivery patterns. *Denotes difference between conditions ($p \leq 0.05$). "a" denotes between period, with condition difference in AUC ($p \leq 0.05$). "b" refers to within period, between condition differences ($p \leq 0.05$).
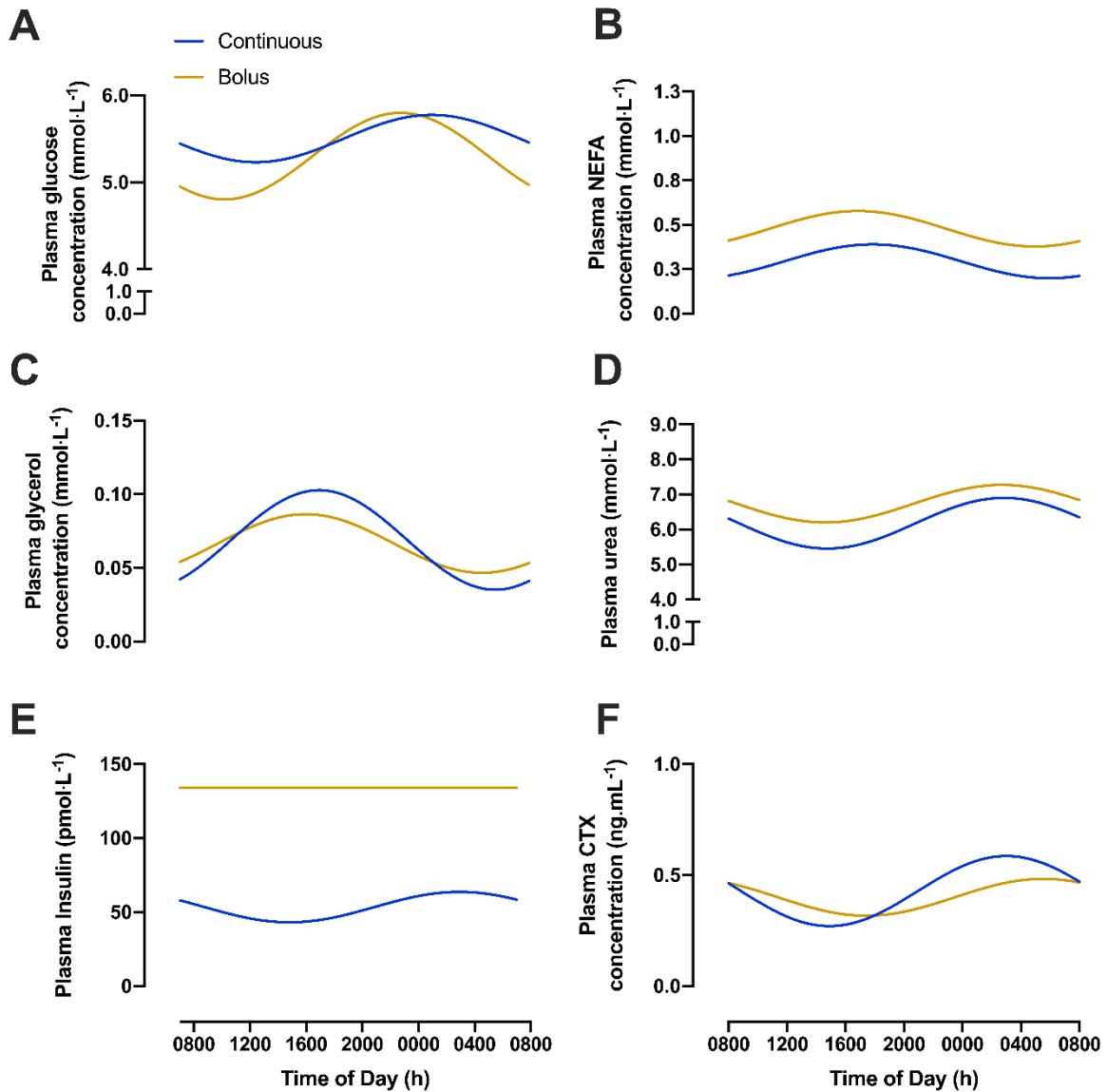
Figure 5.6 – 24-hour rhythms for A) mean plasma glucose B) mean plasma non-esterified fatty acids C) mean plasma glycerol D) mean plasma urea E) mean plasma insulin F) mean plasma CTX. Solid lines denote the regression that best fits the data.

5.2.5 Interstitial glucose responses

Within-laboratory interstitial glucose concentrations did not differ between nutrient delivery patterns (Condition $p = 0.35$) but did vary over time in both conditions (Time $p = 0.01$). Importantly, the pattern in which nutrients were delivered altered the interstitial glucose response over time (Condition x Time $p < 0.01$), such that 2 distinct spikes in concentration were observed in the *Bolus* condition between 0800 – 1100 h and 2000 – 0200 h relative to *Continuous* nutrient delivery (**Figure 5.7A**).

Interstitial glucose exposure in response to standardised meals on day 1 and 3 was higher in the *Bolus* group relative to the *Continuous* group (Condition $p = 0.006$) (**Figure 5.7B**). However, there was no difference in exposure between pre- and post-trial day (Time $p = 0.63$) nor did within laboratory nutrient delivery pattern result in differences in interstitial glucose exposure (Condition x Time $p = 0.61$) (**Figure 5.7C**).
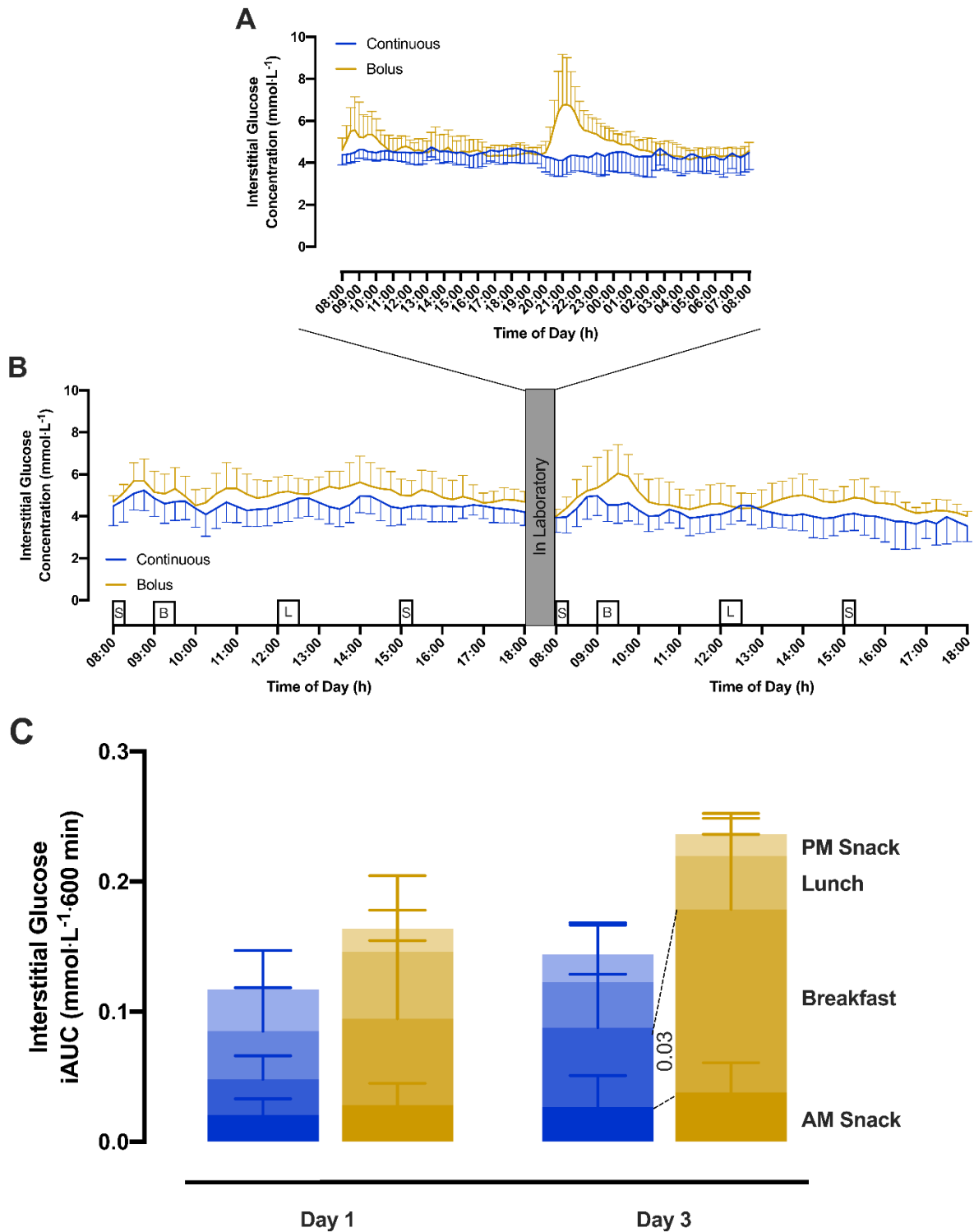
Figure 5.7 – Interstitial glucose responses to A) Within laboratory enteral feeding patterns B) Standardised meals on Days 1 and 3 C) iAUC responses of day 1 and day 3. Bars are labelled with the respective meal on the right-hand side of the figure. $p = 0.03$ represents significant difference between breakfast response between conditions on day 3.

5.2.6 Appetite

Composite appetite profile over 24-h was different between feeding patterns (time x condition $p < 0.001$). Subjective appetite was suppressed following morning and evening Bolus feeds relative to continuous feeding (**Figure 5.8A**). Despite this total composite appetite response was not different between C*ontinuous* or *Bolus* delivery patterns (Condition $p =0.82$) but was greater in period 1 than period 2 (Time $p = 0.03$). Enteral nutrient delivery pattern did not alter temporal appetite responses (Condition x Time $p = 0.77$). Interestingly Bolus delivery, resulted in rhythmicity of subjective appetite (Acrophase: 1601 h, Amplitude: 9 mm, Mesor: 52 mm, $p = 0.01$) that was not apparent when participants were fed continuously ($p = 0.54$).
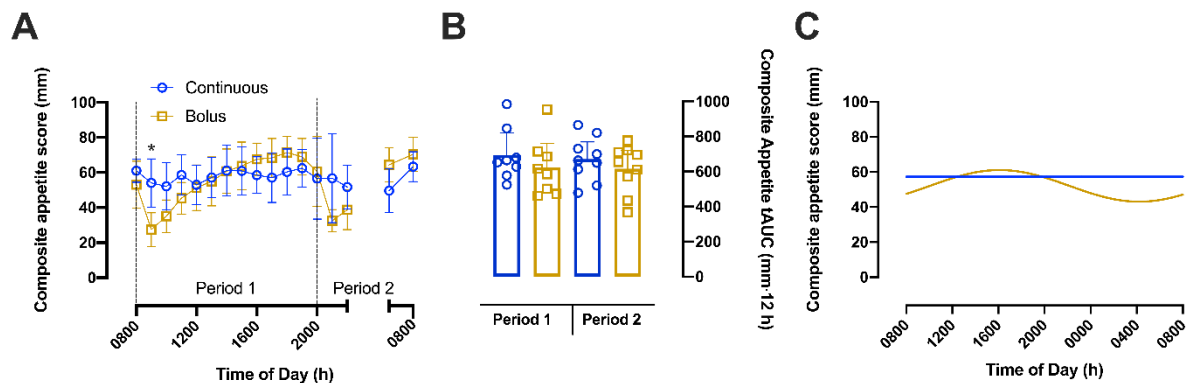
Figure 5.8 – A) Time course response of subjective composite appetite as assessed through visual analogue scale B) total area under the curve for composite appetite split between period 1 (0800-2000 h) and period 2 (2000-0800 h) C) Composite appetite rhythms to *Continuous* and *Bolus* enteral nutrient delivery patterns as determined by Cosinor analysis. *Denotes difference between conditions ($p \leq 0.05$).

5.4 Discussion

This is the first study to compare 24-h metabolic, endocrine, and skeletal muscle responses to two different isocaloric patterns of enteral nutrient delivery. The default clinical approach of continuous nutrient delivery resulted in very different metabolic responses of systemic glucose, NEFA, triglycerides, urea, insulin, GLP-1 and CTX – relative to the more physiologically typical 'meal' responses to defined bolus infusions during waking hours.

Interestingly, in both the current and previous studies, bolus delivery of nutrients in the morning did not result in an observable "spike" in plasma glucose concentration. This is initially surprising given the energy content and macronutrient composition of the Bolus provided. However, glucose control is relatively better in the morning than the evening and hourly blood sampling does not provide sufficient resolution to capture the acutely responsive nature of plasma glucose to a feeding stimulus (Van Cauter *et al.*, 1992; Chowdhury *et al.*, 2015; Qian & Scheer, 2016). Taken together it is plausible that plasma glucose had already peaked and returned to baseline by the sampling time point at 1000 h and is therefore not apparent on the time series trace reported in Gonzalez *et al.* (2020). This is confirmed by within laboratory interstitial glucose traces, showing a plasma glucose response to the morning bolus that was near complete by 1000 h. Moreover, the morning interstitial glucose response was lesser than the response to the evening Bolus, consistent with the established diurnal rhythm in postprandial glucose metabolism (Saad *et al.*, 2012). This was supported by pattern of plasma insulin responses to bolus enteral feeding with greater exposure to insulin across 24-h relative to continuous nutrient delivery. Plasma insulin responses were similar between each bolus feeding, yet this is not to say there is no diurnal control over plasma insulin action. *β*-cell responsivity to glucose and disposition index tends to be higher in response to breakfast relative to dinner. Equally, hepatic insulin extraction and suppression of endogenous glucose production tend to be lower at breakfast such that insulin sensitivity is highest in the morning Saad *et al.* (2012). Alongside the high-protein content (9.3 g·100 mL) of the enteral feed, time-of-day differences in insulin sensitivity may have accentuated the action of insulin action on postprandial glycaemia seen following the morning bolus. The temporal response in systemic insulin is also consistent with the contrast in plasma insulin responses across

24-h when participants were fed intermittently or continuously (Gonzalez *et al.*, 2020). Again, this reflects the acutely responsive nature of insulin to changes in nutrient availability (Chowdhury *et al.*, 2015; Hengist *et al.*, 2020).

Enteral nutrient delivery pattern affected the profile of 24-h rhythmicity in plasma glucose and insulin. Rhythmicity in plasma glucose was apparent regardless of whether participants were provided enteral nutrition continuously or in two evenly distributed boluses across the 24-h laboratory visit. Interestingly, bolus provision resulted in an earlier peak (~2300 h *vs* ~ 0115 h) and higher amplitude (0.50 *vs* 0.27 mmol·L^{-1}) of glycaemia relative to continuous provision of nutrients. Nonetheless, the overall rhythm of glucose in both nutrient delivery patterns is consistent with previous studies employing circadian misalignment, constant routine, and forced desynchrony - therefore providing direct evidence that whilst plasma glucose is acutely responsive to meal intake, daily rhythmicity in plasma glucose is robustly directed by the endogenous clock (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Qian & Scheer, 2016). The pattern of rhythmicity in plasma insulin during continuous provision of nutrition is similar to glucose, with the nocturnal rhythmic peak being explained by relatively poorer insulin sensitivity during the night than the day (Boden *et al.*, 1997; Saad *et al.*, 2012). On the other hand, rhythmicity in plasma insulin was not detected when nutrients were provided in a dual bolus pattern. however, this is not to say that feeding intermittently does not result in a rhythmic profile of insulin. Bolus provision of nutrients resulted in equal insulin responses in both period 1 and period 2, such that the visual profile of this response could almost be considered "rhythmic". Whereas cosinor is a useful tool for assessing rhythmicity across a 24 h period, when the frequency of an event (i.e., peaks in insulin) increases they can be considered diurnal rather than circadian and therefore it is more appropriate to consider the outcomes of ANOVA rather than cosinor analysis. The effects of enteral feeding pattern on plasma insulin responses is an important clinical consideration for critically ill patients, who often develop insulin resistance (Woolfson *et al.*, 1976). The episodic nature of insulin in response to a meal suggests a propensity for intermittent delivery of nutrients. Certainly, over a longer period of enteral feeding continuous provision of nutrients increases insulin requirements to maintain euglycemia (Gonzalez *et al.*, 2020). The difference in insulin responses over 24 h may also hold

important implications for clock function in peripheral circadian clocks such as the skeletal muscle (Perrin *et al.*, 2018b; Tuvia *et al.*, 2021).The "typical meal response" seen with Bolus feeding in the current study may serve to maintain regular fluctuations of circadian clocks in skeletal muscle, which play an important regulatory role for insulin sensitivity in this tissue (Shi *et al.*, 2013; Perrin *et al.*, 2018b).

Similarly, total GLP-1 exposure was greater when participants received nutrients in two equally distributed bolus feeds relative to continuous enteral nutrition. The postprandial increases in total GLP-1 reflect the role of this peptide in potentiating insulin sensitivity and acutely suppressing appetite (van Bloemendaal *et al.*, 2014; Drucker, 2018). Furthermore, the patterns in total plasma GLP-1 in response to divergent feeding patterns in the current study agree with Gonzalez *et al.* (2020). Total plasma GLP-1 was not rhythmic under *Continuous* or *Bolus* enteral nutrient delivery conditions. Whilst this contradicts previous studies demonstrating a clear diurnal rhythm in plasma GLP-1 concentrations, the more episodic pattern of response in total GLP-1 may have meant that rhythmicity was not detected by cosinor analysis (Galindo Munoz *et al.*, 2015). Notably, the latter study did report that effects of time on GLP-1 patterns may have been due to the proximity of sampling to main meals as well as the fasting time between provided meals, suggesting that the availability of nutrients throughout the day might alter temporal GLP-1 patterns. This could also be the case in the current study

Plasma NEFA responses diverged between enteral nutrient delivery patterns. Briefly, exposure to circulating NEFA across the total period was greater when nutrients were delivered as 2 bolus feeds compared to continuously. Conversely, the 24-h profile of circulating glycerol was similar between feeding patterns. Following an initial suppression, plasma glycerol gradually increased across the day before falling across the night until wake. Interestingly both enteral feeding patterns initially resulted in suppression of plasma NEFA, consistent with the regulatory effects of insulin on adipose tissue lipolysis (Mahler *et al.*, 1964; Arner *et al.*, 1981; Lafontan & Langin, 2009). However, greater rebound of plasma NEFA in the bolus trial in both period 1 and period 2 resulted in greater NEFA exposure across 24-h. This rebound

phenomenon coincided with the return of plasma insulin to postabsorptive levels, which is associated with the restoration of lipolysis, decreased adipose tissue NEFA re-esterification, and an increased LPL action (Jelic *et al.*, 2009).

Enteral nutrient delivery pattern results in a phase shift in rhythmic plasma NEFA and glycerol. Both plasma NEFA and glycerol were rhythmic under conditions of bolus or continuous enteral feeding. The rhythms in these biomarkers were very similar in their acrophase under both conditions, with peak values occurring at ~1730h and ~1630h for continuous and bolus delivery, respectively. Importantly the rhythms in NEFA and glycerol opposed the rhythm in plasma insulin observed in the continuous trial (Lafontan & Langin, 2009). However, these rhythms represent conflicts with previous literature, that shows rhythmic peaks in circulating NEFA and glycerol at ~0400-0500 h (Hagström-Toft *et al.*, 1997; Dallmann *et al.*, 2012). Whilst lipid metabolism is directly regulated by the circadian clock, the current data suggest that nutrient feeding patterns may alter the phase of rhythmic lipids such as NEFA and glycerol (Dallmann *et al.*, 2012; Chua *et al.*, 2013). Specifically, delivery of nutrients in two evenly distributed bolus feeds across the day resulted in a 1-hour phase shift in the peak timing of both NEFA and glycerol compared to continuous nutrient provision. The relatively earlier rhythmic peak in both lipids with *Bolus* feeding could plausibly be explained by the greater rebound in plasma NEFA as a result of insulin returning to postabsorptive levels (Jelic *et al.*, 2009).

This was the first study to contrast serial plasma triglyceride responses to divergent patterns of enteral nutrition delivery. Continuous provision of nutrients resulted in a gradual increase in plasma triglycerides across 24 h, whereas Bolus feeding resulted in a "typical meal response" within each 12 h period. Speculatively, the steady increase in plasma triglycerides under conditions of continuous nutrient provision may reflect a state of sustained postprandial triglyceridaemia across 24-h relative to the bolus trial in which a more typical postprandial triglyceride response was observed (i.e., elevation in concentrations for ~5-h). With such large insulin responses in the bolus condition, it could be that there is less suppression of VLDL-TAG production and/or adipose LPL activity with continuous compared to bolus (Malmström *et al.*, 1997; Jelic *et al.*, 2009).

Interestingly, plasma triglycerides were not deemed to be rhythmic by cosinor analysis in either condition. This was surprising as previous studies report robust endogenously driven rhythmicity in plasma triglycerides (Brøns *et al.*, 2016; Yuan *et al.*, 2020). This again could be explained by the aforementioned limitations of cosinor analysis. An increase in triglycerides is common for in-patient stays at hospitals and is associated with worse clinical outcomes and longer-term hospitalisation in the ICU (Wu *et al.*, 2018). It is surprising that no research has studied the effects of nutrient feeding pattern on circulating triglycerides. The responses reported here currently suggest that intermittent provision of nutrition could be an effective method of attenuating rises in triglycerides in patients required to be fed enterally while in ICU.

With regards to the metabolic fate of ingested protein, provision of enteral nutrition in 2 bolus feedings distinctly altered the pattern of plasma urea concentrations compared to continuous enteral feeding. Despite a matched quantity of protein infused and therefore mass of nitrogen to handle, exposure to plasma urea (iAUC) was greater in the *Bolus* condition relative to *Continuous*. Each individual bolus in the current study provided ~88 g protein, which vastly exceeds the >20 g threshold for stimulating amino acid oxidation and ureagenesis (Witard *et al.*, 2014). In contrast, plasma urea concentration increased linearly through the continuous condition, so may reflect a lower rate of ureagenesis and thus nitrogen excretion when protein is delivered more gradually over time. Interestingly, despite differing patterns of urea, the response in each period concentrations at the start and end of each period where similar between trials, this may have implications for assessment of protein oxidation through measurement of urea in plasma and urine. In particular, assessment of plasma urea at the beginning and end of a given period (as is typically advocated to calculate non-protein RER) may not give a true reflection of urea exposure in response to any given nutritional intervention. Furthermore, systemic urea was rhythmic whether participants were fed continuously (peak ~0245 h) or in bolus feeds (peak ~0215 h). Urea has been shown to rise throughout the day and then decrease overnight, whereas the current study showed the opposite (Mackay & Mackay, 1927). Differences between the current and previous study are likely a reflection of differences between patterns of nutrient provision. Whereas Mackay and Mackay (1927) limited caloric intake to the waking period only, the evening bolus and provision of nutrition through the night result

in the current study resulted in nocturnal rhythmic peaks for plasma urea (i.e., reflecting greater breakdown of dietary protein overnight).

The lack of temporal or feeding pattern induced response in serum testosterone, could be explained by several factors. Small rises in cortisol across the day from muscle biopsies may dampen any temporal variation in testosterone (Cumming *et al.*, 1983). Assessment by commercial enzyme-based immuno-assays rather than gold standard measurement by liquid chromatography mass spectrometry may also influence outcomes (Handelsman & Wartofsky, 2013; Handelsman *et al.*, 2018). However, mixed-meal feeding reliably lowers circulating testosterone, so it is conceivable that, regardless of delivery pattern, enteral nutrition resulted in 24-h suppression of serum testosterone (Lehtihet *et al.*, 2012; Gagliano-Jucá *et al.*, 2019). This is likely due to increased amino acid availability in response to enteral nutrition, which is more potent for initiating tissue turnover than physiological ranges of testosterone (West *et al.*, 2009; West *et al.*, 2010).

The suppression of plasma CTX following each bolus in the current study aligns with literature showing that feeding acutely suppresses circulating CTX, associated with postprandial increases in gut hormones such as GLP-1 (Iepsen *et al.*, 2015; Jensen *et al.*, 2021). This in turn may explain why bolus delivery of nutrients blunted rhythmic CTX such that amplitude is the continuous condition rhythm was twice that of the bolus condition and provides further evidence that food intake is capable of modulating the amplitude of rhythmic CTX (Schlemmer *et al.*, 1992; Aoshima *et al.*, 1998; Christgau *et al.*, 2000; Chubb, 2012; Redmond *et al.*, 2016). Circulating CTX appears to increase in critically ill patients, reflecting a greater rate of bone resorption. Consequently, critically ill patients experience a decrease in bone mineral density, which is an important predictor of clinical outcomes (Orford *et al.*, 2016; Schulze-Hagen *et al.*, 2021). Based on the plasma CTX response to *Bolus* enteral feeding it could be suggested that *Bolus* feeding may be an effective strategy of attenuating the rise in plasma CTX. However, feeding alone is unlikely to remedy changes in bone mineral density due to the associated de-loading of bones during bed rest (Beller *et al.*, 2011).

Nonetheless further work in critically ill patients is required to further explore this possibility.

At the point of the 2000 h measure, the bolus group had not 'ingested' anything for 12 hours, whereas the continuous group had been 'ingesting' nutrients non-stop for 12 hours. From this standpoint it was expected that the bolus group would exhibit characteristics of 'fasting' state (i.e., higher levels of autophagy). It was fascinating that (if anything) the exact opposite was true; it appears that absolute levels of autophagic flux may be higher under continuous feeding in contrast to bolus feeding – at least for some participants. Given the "typical" meal response observed with Bolus feeding, the apparent effect in autophagy is plausible as a sufficient meal stimulus triggers the mTOR pathway, which itself downregulates autophagy (Ganley *et al.*, 2009; Kim *et al.*, 2011; Khapre *et al.*, 2014; Kim & Guan, 2015; Tao *et al.*, 2022). Conversely, this may also suggest that continuous feeding did not provide a sufficient metabolic stimulus to inhibit autophagy and therefore has implications for cellular homeostasis, which is vital in both the physiological and pathophysiological state (Ravikumar *et al.*, 2010). This supports previous work suggesting that ≥16-19 h are needed to reach a "true" post-absorptive state (Cahill, 1970; Soeters *et al.*, 2012; Templeman *et al.*, 2020). Autophagy has been shown to exhibit diurnal rhythmicity in mice such that catabolic processes mediated by autophagy primarily occur when food intake is low (Kim & Lee, 2014; Yin *et al.*, 2016; Martinez-Lopez *et al.*, 2017; Ryzhikov *et al.*, 2019). Certainly the apparent patterns between conditions in the current study would support the notion that autophagy aligns with intermittent rather than continuous feeding, to best respond to fluctuating nutrient availability (Tao *et al.*, 2022). However, further work is necessary to provide a clearer picture of how different feeding patterns affect autophagic responses in different tissues and cell types as well as in critically ill patients.

Neutrophils were chosen to assess autophagic flux for their relative abundance in the circulation. However, it must be acknowledged that increases in stress potentially associated with sampling procedures may have increased neutrophil count, and altered neutrophil phenotype, in peripheral blood (Khanfer *et al.*, 2010; Tang *et al.*, 2022). Increased cortisol typically results in the breakdown of contractile protein and

the mobilisation of amino acids following which autophagy may be upregulated to stimulate muscle regeneration (Hoberman, 1950; Wing & Goldberg, 1993; Luk *et al.*, 2021). Nevertheless, in order to minimise the potential for stress and/or cortisol to influence neutrophils in blood samples collected for flow cytometry, samples were taken prior to muscle biopsies rather than following.

Enteral feeding pattern altered 24-h composite appetite responses. Subjective appetite was suppressed following morning and evening Bolus feeds relative to continuous feeding yet remained relatively stable across the day with continuous feeding (**Figure 5.9A**). The respective patterns for subjective appetite agree with previous research investigating the effects of continuous and bolus enteral feeding on appetite and appetite regulation (Stratton *et al.*, 2003, 2008). This was an entirely expected response given that Bolus feeding more closely mirrors typical feeding patterns in humans. Furthermore, the observed pattern in composite appetite certainly aligns with the temporal response of GLP-1 (**Figure 5.6B**) as well as the proposed function of this peptide to suppress appetite (Larsen *et al.*, 1997; van Bloemendaal *et al.*, 2014). The suppression of appetite resulting from bolus nutrient delivery resulted in rhythmicity of subjective appetite (peak ~1600 h) that was not apparent when participants were fed continuously (**Figure 5.9C**). Speculatively, maintaining pulsatile fluctuations in appetite regulatory hormones may help to maintain appetite regulation not only in healthy individuals but in critically ill patients post discharge also. However, further work is required to establish this in both healthy and critically ill populations.

Finally, the higher exposure to interstitial glucose on day 3 following breakfast in the *Bolus* condition relative to *Continuous* feeding further supports the idea that continuous feeding is physiologically alike to fasting. Consequently glycaemic control isn't altered after a single day in bed (i.e., the liver is more insulin sensitive to replenish glycogen stores in ana acutely fasted state) (Iwayama *et al.*, 2020). Whilst this contradicts the effects of 7 days of continuous feeding on insulin sensitivity seen in Gonzalez *et al.* (2020), the current study was only 24 h highlighting the need for similar trials in critically ill patients requiring enteral feeding.

Providing enteral nutrition in a bolus pattern certainly induces a more physiological typical response to nutrient delivery compared to continuous provision. Inferences for shift workers from this data are difficult to draw as individuals maintained a 24-h period with a typical sleep-wake cycle. Speculatively, continuous provision of nutrients through the night reveals higher glucose compared to daytime day so shift workers may need to account for higher glycaemic responses to ingested nutrients during nightshifts. However, this study was acute (24-h), and in healthy non-shift workers, so inferences on best practice for shift workers is better taken from other literature directly studying this population (Harding *et al.*, 2022).

Two females participated in the current study, with randomisation allocating these participants equally across the feeding conditions. Nonetheless, consideration of physiological differences in the average intrinsic circadian periodicity between sexes is important (Cain *et al.*, 2010; Duffy *et al.*, 2011). Furthermore, there are inherent differences between biological sexes in energy partitioning, substrate utilisation, insulin sensitivity and transcriptional regulation of resting skeletal muscle, all of which may alter postprandial handling of ingested nutrients (Maher *et al.*, 2009; Tramunt *et al.*, 2020). Despite inherent physiological sex differences, it was deemed necessary to include and recruit female participants to allow for greater translation of this mechanistic work to a clinical setting in which both men and women require enteral feeding. However, lack of uptake by female participants meant the study was not sufficiently powered to detect sex-differences to feeding pattern.

In summary, delivery of enteral nutrition in a bolus pattern resulted in postprandial metabolic and endocrine responses that more closely match typical feeding responses than continuous enteral nutrient delivery. Furthermore, divergent feeding patterns also alters rhythmic responses of metabolic and endocrine markers. Collectively, these results hint at the possibility of nutrient feeding patterns to affect longer term metabolic control which has important implications for critically ill patients receiving enteral feeding.

Chapter 6 – Effect of sleep fragmentation and morning caffeinated coffee on glucose control

6.1 Introduction

Sleep curtailment is a risk factor for obesity-associated metabolic diseases, possibly due to the important role of sleep in maintaining glucose homeostasis (Briancon-Marjollet *et al.*, 2015). Impaired glucose clearance and whole-body insulin sensitivity can occur following a single night of either sleep deprivation (e.g. limiting the habitual duration), broken sleep (i.e. 2 periods of sleep separated by an extended waking interval - e.g. 2300-0100 h & 0500-0730 h), or nonspecific sleep-fragmentation (e.g. random arousal stimuli throughout the night) (Donga *et al.*, 2010; Stamatakis & Punjabi, 2010; Wang *et al.*, 2016; Sweeney *et al.*, 2017). Studies to date have employed differing protocols to curtail sleep however, only one previous study has examined the influence of specific sleep-fragmentation on postprandial metabolism (Gonnissen *et al.*, 2013). In that study, postprandial insulin was lower following breakfast in the fragmentation condition relative to a normal night of sleep. However, whilst the use of metabolic units used in that study allowed for a highly controlled situation in which subjects are in a stable environment, it is important to consider the first night effect of an unfamiliar environment on sleep quality (Tamaki *et al.*, 2016), and therefore to see whether these findings translate into more ecologically valid contexts (i.e. participants sleep in their own beds).

From an ecological standpoint, coffee is a commonly used means of combatting feelings of lethargy and fatigue (Statista, 2017), therefore acting as a 'therapeutic tool' following disrupted sleep. However, whilst moderate habitual caffeinated coffee consumption is associated with reduced risk of cardiovascular mortality and cancer incidences (Poole *et al.*, 2017), studies by Moisey *et al.* (2010) (~62 mg caffeine per 100 mL coffee) and Robertson *et al.* (2015) (100-400 mg caffeine) demonstrate the potential for a single serving of caffeinated coffee to acutely impair postprandial glucose metabolism in both normal-weight and overweight individuals. This raises the possibility that coffee consumption could potentiate any negative effects of sleep disruption on glucose metabolism. Furthermore, this effect of caffeine upon postprandial glycaemia seems to be modulated by a single nucleotide polymorphism

in the CYP1A2 gene, which codes for an enzyme responsible for caffeine metabolism in the liver (Sachse *et al.*, 1999). Caffeine is primarily metabolised (>95%) through CYP1A2 activity (Thorn *et al.*, 2012). However, this modulating role has yet to be investigated under a variety of scenarios (Robertson *et al.*, 2018; Banks *et al.*, 2019).

It is remarkable therefore that no study to date has investigated the combined effects of sleep fragmentation and caffeine on glucose control upon waking, although one study has investigated the combined influence of caffeinated coffee (65 mg caffeine) *prior to* sleep deprivation on next-day glucose control. Higher fasting serum insulin and increased levels of glucose and insulin were observed after an oral glucose tolerance test (OGTT) following sleep deprivation (4 h in bed) with consumption of caffeinated coffee relative to decaffeinated coffee (Rasaei *et al.*, 2016). However, coffee was consumed prior to sleep, which is not an ecologically valid model of when coffee is usually consumed, especially as a remedy following disrupted sleep. In addition, caffeine is a fast-acting pharmacological agent, with metabolic effects that occur rapidly and may subside within hours (especially amongst habitual caffeine consumers; (Bell & McLellan, 1985)), so there is also a clear physiological rationale to examine the acute effects of caffeine intake upon waking immediately prior to the first intake of nutrients following the overnight fast.

The aim of this study, therefore, was to determine the effects of one night of sleep fragmentation with and without morning caffeinated coffee on glycaemic control relative to an undisturbed night of sleep in healthy young adults. An exploratory aim of the study was to examine whether individual responses were mediated by the CYP1A2 genotype of participants. It was hypothesised that sleep fragmentation *per se* would impair insulin sensitivity and that morning coffee would exacerbate this response, with the latter effect modulated by the relevant polymorphism of CYP1A2.

6.2 Methods and Materials

6.2.1 Approach to the research question

The novel aspect of this study relates to the sequence in which caffeinated coffee was consumed relative to the prescribed sleep opportunity (i.e., consumed as a morning remedy for poor sleep, rather than to prolong wakefulness prior to sleep). Whilst the dose of caffeine provided to participants (~300 mg) exceeds that typically consumed in one cup of coffee (~100-200 mg), however a higher dose was chosen on the basis that individuals are likely to make a stronger coffee following a poor night of sleep, especially if made using instant coffee (Desbrow *et al.*, 2007; Moisey *et al.*, 2010; Ludwig *et al.*, 2014).

Specific hourly sleep fragmentation was chosen rather than total/partial sleep disruption to align with design of the studies described in chapters 4 and 5 whereby hourly blood sampling and four hourly muscle sampling through the night could feasibly result in a similar pattern of sleep curtailment.

An oral glucose tolerance test (OGTT) was chosen to assess the influence of fragmented sleep and caffeinated coffee on glucose control independent from the effects of dietary lipids and proteins on glycaemia and insulinaemia.

6.2.2 Research Design

Participants underwent three trials in a randomised cross-over design, with a washout interval of 7-14 days. For 48 h prior to each trial, participants standardised diet and physical activity and refrained from consuming caffeine and alcohol. Main trials involved an oral glucose tolerance test (OGTT) following a habitual night of sleep (Control; in bed, lights-off trying to sleep 8 consecutive hours, waking <1 hour prior to arrival at the lab) and a night of sleep fragmentation (Fragmented*;* as control but waking hourly for 5 min – prompted and verified by repeated text messaging), with and without morning coffee (Fragmented+Coffee*;* 300 mg caffeine as black coffee 30 min prior to OGTT).

6.2.3 Participants

Twenty-nine healthy men and women (Age: 21 ± 1 years, BMI: 24.4 ± 3.3 kg·m^{-2}) participated in the study. Exclusion criteria included body mass index outside of the range of 18.5-29.9 kg·m^{-2}, any diagnosed metabolic disease (e.g. type 1 or type 2 diabetes), reported use of substances which may pose undue personal risk to the participants or introduce bias into the experiment, and non-standard sleep-wake cycle (e.g. shift worker). All were informed of any potential risks and discomfort involved in the study prior to providing written and oral informed consent. The study was given a favourable ethical opinion by the Research Ethics Approval Committee for Health (REACH) at the University of Bath (SES/HES: 18R1-019). The measurements of *CY1PA2* gene polymorphism were completed as part of a wider screening project for which ethical approval was granted by the National Health Service Research Ethics Committee (18/NW/0573). All procedures were performed in accordance with the Declaration of Helsinki.

Table 6.1 – Summary of Participant Characteristics.

	Male (*n*=16)	Female (*n*=13)	Combined (*n*=29)
Age (y)	22 ± 1	21 ± 1	21 ± 1
Height (m)	1.81 ± 0.07	1.69 ± 0.07	1.78 ± 0.09
Body mass (kg)	82.2 ± 10.5	66.3 ± 11.5	75.3 ± 13.47
Body Mass Index (kg·m^{-2})	25.0 ± 2.6	23.2 ± 3.8	24.2 ± 3.3
Waist circumference (cm)	84.0 ± 6.4	73.3 ± 8.2	80.4 ± 8.7
Hip circumference (cm)	100.7 ± 6.8	97.1 ± 12.9	99.1 ± 10.0
Waist:Height (cm)	0.46 ± 0.03	0.43 ± 0.05	0.45 ± 0.04

Values are Mean ± Standard Deviation.

6.2.4 Experimental Protocol

Participants arrived in the laboratory at between 0800 h and 1000 h (within 1-hour of waking) in an overnighted fasted state (~10 h). Height, body mass, waist:hip circumference were assessed before participants completed baseline subjective assessments of sleep quality, mood, and appetite on a 0-100 mm scale. Waist and hip circumferences were measured using a tape measure around the mid-point between

bottom rib and top of the iliac crest and at the largest circumference between the waist and thighs respectively (Rimm *et al.*, 1990). An intravenous cannula was placed into an antecubital vein and a baseline sample of 5 mL venous blood collected (BD Venflon Pro; BD, Eysins, Switzerland). Cannulae were kept patent throughout all trials by flushing with 0.9% NaCl infusion (B. Braun; Sheffield, UK). Participants would then either consume a cup of caffeinated coffee (8.8 g Nescafé Original, Nestlé, SUI with 300 mL water; ~300 mg caffeine, ~163 mg total caffeoylquinic acids) or a matched volume of hot water over a 10-min period, 30-minutes prior to undergoing an OGTT. At the end of this 30-minute period a 5 mL blood sample was obtained before ingesting a 75 g oral load of glucose (113 mL Polycal; Nutricia, UK with 87 mL water). Further blood samples were then taken at 15- and 30-minute intervals for the first and second hour of the protocol respectively, alongside hourly assessments of subjective mood and appetite.
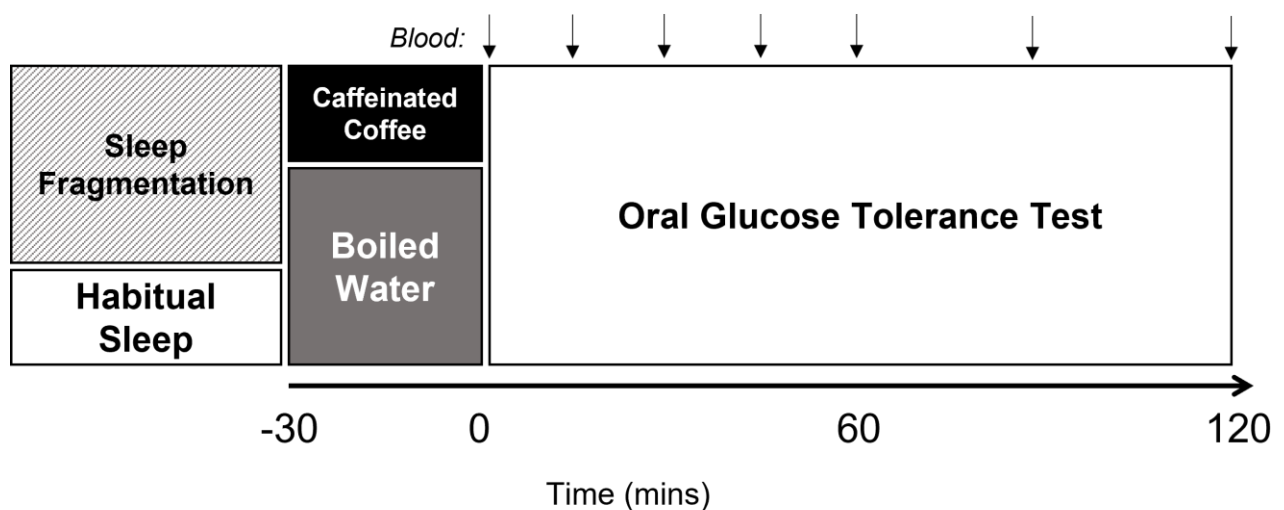
Figure 6.1 Schematic representation of the study protocol.

6.2.5 Sleep Fragmentation

Participants were asked to achieve 8-h time in bed, trying to sleep (~2300-0700 h – modifiable according to individual preference and time of testing the next day). Audible alarms were set on the hour every hour throughout the night. Upon waking, participants would receive a series of 10 text messages from a member of the research team, at a rate of 1 every 30 seconds, which required simple responses prior to being able to fall asleep again (e.g., simple arithmetic). This would be repeated until wake

time. Participants rated subjective sleep fragmentation using visual analogue scales upon entering the laboratory.

6.2.6 Blood analysis

All blood samples were immediately transferred into tubes treated with EDTA prior to centrifugation (10 min, 4000 x g, 4°C) before the plasma supernatant was aliquoted and stored at -80°C for subsequent metabolite analysis. In the control trial the buffy coat layer of the centrifuged bloods was removed and stored at -80°C for later genetic profiling. All plasma samples were later analysed for plasma glucose, and insulin as described in Chapter 3.6.1 & 3.6.2

6.2.7 DNA extraction and analysis

DNA was extracted from the buffy coat layer using QIAamp DNA Blood Mini Kit following manufacturer's instructions (Qiagen, Germany) and frozen at -80°C until analysis. Extracted DNA was then analysed for the rs762551 SNP using a 5'-nuclease allelic discrimination assay (Taqman drug metabolism genotyping assay SNP ID rs762551, C___8881221_40 [C/A], gene CYP1A2; ThermoFisher Scientific, US).

6.2.8 Statistical Analysis

Sample size estimations were performed using G*Power software v3.1.9.4. Based on differences in plasma insulin following sleep deprivation (D=1.58), and considering the multi-level (i.e., 3 condition) design of the study a sample size of 30 was deemed adequate to provide a 95% chance of detecting such an effect at α=0.05.

All in text values are reported as means ± standard deviations, unless otherwise stated. Normality of data was assessed using the Shapiro-Wilk test, with a paired t-test or Wilcoxon's test employed to analyse parametric data and non-parametric data respectively. A mixed model ANOVA (condition, time, and condition x time) was used to examine differences in blood glucose and insulin data, with *post-hoc* Bonferroni corrections applied in GraphPad Prism (GraphPad Software Inc., California, USA). Statistical significance was accepted at $p < 0.05$. Error bars shown on figures are also normalised confidence intervals (CI) corrected for between-participant variation, such

that the magnitude of these CI's therefore directly infers the contrast between paired means at each time-point rather than variance of individual values around the mean (Loftus & Masson, 1994). Using this approach, error bars not overlapping their respective comparison would typically be deemed significantly different according to conventional null hypothesis testing (i.e. $p<0.05$). Incremental area under the curve (iAUC – Trapezoid method (Wolever, 2004)) and Matsuda Insulin sensitivity index (Matsuda index; (Matsuda & DeFronzo, 1999)) were calculated from plasma glucose and insulin data using Microsoft excel (Version 16.04848.1000, Microsoft, Redmond, WA, USA). Updated homeostatic model of insulin resistance (HOMA2-IR; (Levy *et al.*, 1998)) was calculated using publicly available online software (https://www.dtu.ox.ac.uk/homacalculator/). Distribution of the rs762551 SNP was tested for fit against the global expected distribution using a Pearson's χ^2 test with 1 degree of freedom. All values for genotype data were taken from the Fragmented+Coffee condition, with ΔiAUC calculated as the difference between Fragmented+Coffee and Fragmented. As data for genotype was not paired, differences between means was compared using standard 95% confidence intervals. Effects of trial order were assessed using two-way ANOVA testing for effects of Condition, Sequence, or Sequence x Condition interactions (Wellek & Blettner, 2012; Betts *et al.*, 2020).

6.3 Results

6.3.1 Glycaemia, Insulinaemia, and Insulin Sensitivity

Ingestion of the caffeinated coffee did not influence systemic glucose prior to the OGTT but exaggerated postprandial glucose concentrations (condition x time $p < 0.01$) (**Figure 6.2A**). After ingestion of the oral glucose load, plasma glucose concentrations rose to a greater extent in the Fragmented+Coffee (8.96 ± 1.58 mmol·L^{-1}) relative to both the Fragmented (8.23 ± 1.46 mmol·L^{-1}; $p = 0.02$) and Control conditions (8.20 ± 1.55 mmol·L^{-1}; $p = 0.02$). Peak glucose concentrations did not differ between Control and Fragmented conditions ($p = 0.82$) (**Table 6.2**). Plasma glucose concentration remained higher in the Fragmented+Coffee condition relative to Control and Fragmented conditions at between 30-60 mins and at 120-mins ($p < 0.05$) such that plasma glucose iAUC was higher in the Fragmented+Coffee condition (197 ± 150 mmol·L^{-1}·120 min) relative to the Fragmented (130 ± 119 mmol·L^{-1}·120 min; $p = 0.01$) but not Control (153 ± 143 mmol·L^{-1}·120 min; $p = 0.15$) conditions.

Caffeinated coffee did not influence fasting or postprandial systemic insulin (condition x time $p = 0.053$) (**Figure 6.2B**). However, following ingestion of the glucose load, peak insulin was higher in the Fragmented+Coffee condition (310 ± 145 pmol·L^{-1}), relative to both Control (265 ± 116 pmol·L^{-1}) and Fragmented conditions (235 ± 90 pmol·L^{-1}). Despite this, plasma insulin incremental area under the curve (iAUC) was not different in the Fragmented+Coffee condition (10.0 ± 6.09 nmol·L^{-1}·120min) relative to the Fragmented (7.84 ± 3.70 nmol·L^{-1}·120min; $p = 0.06$) and Control (8.42 ± 4.27 nmol·L^{-1}·120min; $p = 0.08$) Conditions. Time to peak insulin, HOMA2-IR and Matsuda insulin sensitivity index were not different between conditions (**Table 6.2**). There was no effect of biological sex on postprandial glucose (condition x time x sex $p = 0.84$) or postprandial insulin (condition x time x sex $p = 0.31$).
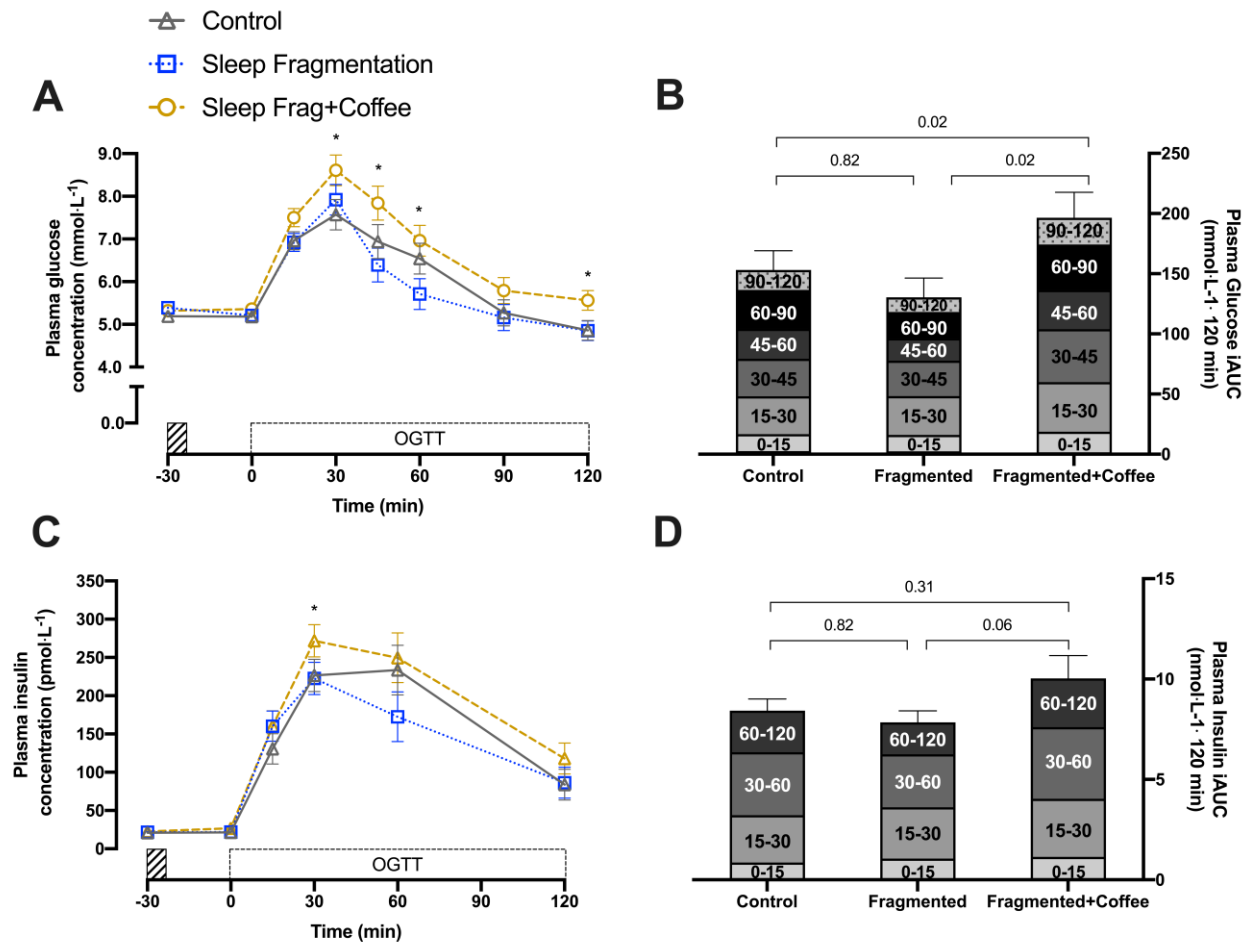
Figure 6.2 - A) Time course plasma glucose B) Plasma glucose iAUC C) time course plasma insulin D) Plasma insulin iAUC. *$p < 0.05$. ▨caffeinated coffee/boiled water. OGTT; Oral glucose tolerance test.

Table 6.2 Peak and time to peak glucose and insulin values in each condition alongside Matsuda insulin sensitivity index. Values are mean ± standard deviation.

	Control	Fragmented	Fragmented+Coffee
Peak Glucose (mmol·L^{-1})	8.20 ± 1.55	8.23 ± 1.46	8.96 ± 1.58*
Peak Insulin (pmol·L^{-1})	265 ± 116	235 ± 90	310 ± 145*
Time to Peak Glucose (min)	33 ± 15	28 ± 15	30 ± 10
Time to Peak Insulin (min)	36 ± 10	35 ± 10	38 ± 10
ISI Matsuda (au)	15.4 ± 4.5	15.3 ± 5.8	13.9 ± 5.0
HOMA2-IR (au)	0.40 ± 0.08	0.55 ± 0.60	0.43 ± 0.19

* Indicates difference from Fragmented ($p < 0.05$).

6.3.2 Order effects

Analysis of Sequence x Condition interactions revealed no effect of first trial on the observed effect of Fragmented+Coffee ($p = 0.101$). However, an order effect in plasma glucose iAUC was observed whereby values in participants first trial were higher than both the second and third trial (197.0 [173.4-220.6] *versus* 147.5 [125.7-169.3] *versus* 132.2 [106.5-158.0] mmol·L^{-1}·120 min).

6.3.3 Subjective Sleep Quality

Time to sleep (Median [Inter Quartile Range]; 23:30 [23:00-00:00] h *vs* 23:30 [23:00-00:01] h, *vs* 23:45 [23:05-24:10] h) and wake time (07:00 [06:52-07:17] h *vs* 07:00 [07:00-07:16] h *vs* 07:00 [06:55-07:20] h) did not differ between Control, Fragmented and Fragmented+Coffee conditions, respectively. Subjective ratings of sleep fragmentation (i.e. "How fragmented was your night's sleep?") were greater in the Fragmented, and Fragmented+Coffee conditions, relative to the Control condition (83 ± 18 *versus* 81 ± 14 *versus* 8 ± 12 mm/100, respectively).

6.3.4 Genotyping

Of the n=26 genotyped, 15 participants were homozygous for the A allele, with the remaining 11 carrying the C allele (n=2 CC; n=9 AC). The distribution of genotypes

was therefore as expected within the given population and therefore did not deviate from the Hardy – Weinberg equilibrium (Pearson's χ^2 test with 1 df, P >0·05).

6.3.5 Secondary Analysis: Glycaemia and insulinaemia by genotype.

Peak plasma glucose concentrations in the Fragmented+Coffee condition did not differ between the "fast" (AA: 8.86 ± 1.82 mmol·L^{-1}) and "slow metaboliser" genotypes (AC/CC*:* 9.13 ± 1.35 mmol·L^{-1}; *p* = 0.71) (**Figure 6.3A**). Likewise, plasma glucose iAUC was similar between "fast" (AA: 208 ± 163 mmol·L^{-1}·120min) and "slow" metabolisers (AC/CC*:* 189 ± 143 mmol·L^{-1}·120min; *p* = 0.54). Furthermore, ΔiAUC for glucose between the Fragmented and Fragmented+Coffee trials was similar between "fast" and "slow" genotypes respectively (81.8 ± 125.1 *versus* 55.7 ± 107.8 mmol·L^{-1}·120 min; *p* = 0.26).

Similarly, peak insulin concentration in the Fragmented+Coffee condition (AA: 310 ± 164 *versus* AC/CC: 346 ± 77 pmol·L^{-1}; *p* = 0.84), insulin iAUC (AA: 10.2 ± 6.7 *versus* AC/CC: 10.8 ± 5.3 nmol·L^{-1}·120min; *p* = 0.80), and ΔiAUC between the Fragmented and Fragmented+Coffee trials for insulin (2.68 ± 4.15 *versus* 2.05 ± 6.87 nmol·L^{-1}·120min; *p* = 0.75) did not differ between "fast metaboliser" or "slow metaboliser" genotype respectively.
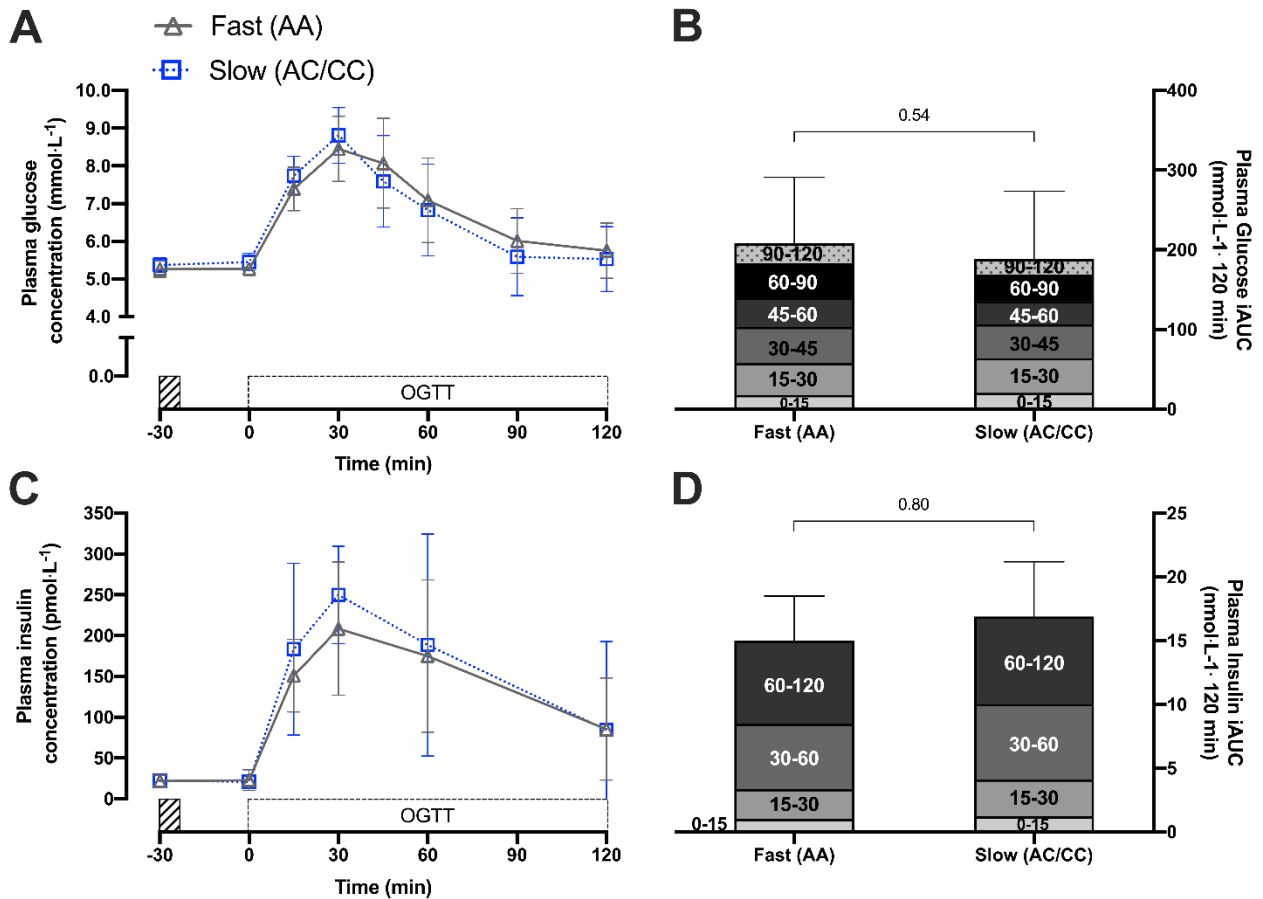
Figure 6.3: A) Time course plasma glucose B) Plasma glucose iAUC C) time course plasma insulin D) Plasma insulin iAUC for data split by "Fast" or "Slow" metaboliser CYP1A2 genotype. *$p < 0.05$. ▨ caffeinated coffee/boiled water. OGTT; Oral glucose tolerance test.

6.4 Discussion

The current study demonstrates that one night of hourly sleep fragmentation had no effect on next-day insulin sensitivity or glucose tolerance, relative to a habitual night of sleep, in young, healthy men and women. However, consumption of caffeinated coffee after sleep fragmentation increased glucose iAUC by ~50%.

A recent survey found that ~40% of people in the UK drink caffeinated coffee upon waking, and therefore the coffee condition in the current study provided an important ecological comparison to the sleep-fragmentation alone condition (Statista, 2017). Previous studies have demonstrated acute reductions in glucose tolerance when caffeinated coffee is ingested prior to assessment of glucose metabolism (Moisey *et al.*, 2010). In the present study, glucose iAUC was ~50% greater following caffeinated coffee relative to sleep fragmentation alone; this agrees with the one other study to have investigated the combination of caffeinated coffee and sleep disruption (Rasaei *et al.*, 2016), albeit in the reverse order (i.e. caffeine before *versus* after sleep) and in relation to overall sleep deprivation (i.e. lower total duration) rather than the fragmentation reported here. Indeed, in that previous study there was no habitual sleep control condition, and the caffeinated coffee was ingested during the sleep deprivation period; the present study therefore extends those findings by isolating the independent effects of sleep deprivation and caffeinated coffee, with the latter consumed following, rather than prior to sleep disruption.

Whilst not investigated in the current study, there are several potential mechanisms following caffeinated coffee ingestion that may explain the reduction in glucose tolerance. Primarily, within a circulating range of 25-40 $mmol \cdot L^{-1}$ caffeine acts as an antagonist for adenosine receptors, particularly in skeletal muscle where caffeine may inhibit glucose uptake via A1 adenosine receptor antagonism (Thong & Graham, 1985; Vergauwen *et al.*, 1994; Fredholm, 1995). Additionally, caffeine ingestion is a stimulant for the release of adrenaline, which suppresses the action of insulin through β-adrenergic receptor activation (Deibert & DeFronzo, 1980; Baron *et al.*, 1987; Avogaro *et al.*, 1996). It is also worth considering the lipolytic effects of caffeine ingestion on glucose uptake into skeletal muscle. The dose of caffeine provided in the current study

is likely sufficient to induce lipolysis *prior* to the OGTT, subsequently impairing glucose uptake into the muscle (Kovacs *et al.*, 1998; Gonzalez, 2014). Finally, the role of cortisol in disruption of glucose metabolism must also be considered. Specifically, cortisol is elevated following both caffeine ingestion (Lovallo *et al.*, 2005), and sleep disruption (Stamatakis & Punjabi, 2010), which elevates postprandial glucose responses (De Feo *et al.*, 1989).

The rate of caffeine metabolism is affected by the rs762551 single-nucleotide polymorphism in the CYP1A2 gene, with individuals classed as either fast (AA) or slow (AC/CC) metabolisers (Yang *et al.*, 2010a). Recent evidence suggests that postprandial glycaemic and insulinaemic responses to both chronic and acute caffeine ingestion are modulated by this polymorphism (Robertson *et al.*, 2018). Despite observing the expected distribution of the rs762551 SNP, no difference in the insulinaemic or glycaemic response to the glucose load was found between genotype following caffeinated-coffee ingestion. While this may suggest that the modulating effect of the rs762551 SNP was not present, this analysis was included on an exploratory basis and thus the study was not directly powered to detect differences between "fast" and "slow" metabolisers. Future work should therefore investigate the acute effect of this SNP on the response to coffee after a night of disrupted *versus* habitual sleep in a larger sample.

The current study observed no difference in insulin sensitivity or glucose tolerance following sleep fragmentation relative to a habitual night of sleep. This is interesting considering that previous work has shown even a single night of sleep restriction is sufficient to induce reductions in both peripheral and hepatic insulin sensitivity relative to a habitual night of sleep (Donga *et al.*, 2010). In similar fashion to Gonnissen *et al* (Gonnissen *et al.*, 2013), the current study observed one time-point at which postprandial plasma insulin concentration was lower following Fragmented *versus* Control, which in the former was speculated to be due to a difference in night-time glycogen use from waking. However, this cannot be directly concluded from one time point alone. Speculatively, lack of postprandial differences in the current and previous study (Gonnissen *et al.*, 2013) may be explained by the total magnitude of sleep

disruption achieved through hourly sleep fragmentation. In the current study, wake time was ~70-80 minutes total. Comparatively, Donga *et al* (Donga *et al.*, 2010) employed a broken sleep protocol (i.e. sleep time 2300-0100 h and 0500-0730 h) in healthy lean subjects, observing a ~22% increase in endogenous glucose production alongside a ~20% decrease in the rate of glucose disposal, indicative of reduced hepatic and peripheral insulin sensitivity, respectively. Similarly, *two* nights of sleep reduction (~50% of habitual sleep duration: 442 ± 78 min *versus* 235 ± 34 min) also reduced Matsuda insulin sensitivity index by ~19% relative to habitual sleep (Sweeney *et al.*, 2017). The total duration of sleep loss accumulated over one or more nights may therefore be proportionate to the effect on postprandial glycaemia. Speculatively, this could be explained by lesser disruption of slow-wave sleep (SWS - i.e., stage III of non-rapid eye movement sleep) with the current protocol compared to previous studies. As SWS is thought to be the most important mediator of metabolic, hormonal, and neurophysiological changes during sleep, studies observing greater reductions in insulin sensitivity are likely to have done so through employment of sleep disruption protocols that provide a greater degree of disruption to SWS (Tasali *et al.*, 2008; Donga *et al.*, 2010; Sweeney *et al.*, 2017). Conversely in the current study, participants were aroused from sleep every hour, which based on the average length of each stage of sleep (~5-15 mins) and one sleep cycle (~90-110 mins) would potentially not provide as much SWS disruption as previous literature (Roebuck *et al.*, 2014).

Whilst the dose of caffeine ingested in the current is above that typically consumed in one cup of coffee on a daily basis (~100-200 mg) (Desbrow *et al.*, 2007; Ludwig *et al.*, 2014), studies employing lower doses (~60 mg) have observed disrupted glucose control (Moisey *et al.*, 2010). Despite this, a strength of the current study is the perhaps more ecologically valid model of consumption following one night of sleep curtailment relative to previous research (Rasaei *et al.*, 2016). Furthermore, the effects shown in this study are present in a relatively large sample size for an acute study of this nature. One potential limiting factor in the interpretation of the present results is the apparent order effect whereby mean plasma iAUC was higher in participants first trial compared to both the second and third trials (197 ± 192 *versus* 148 ± 57 *versus* 132 ± 109 mmol·L^{-1}, respectively). However, repeated measures ANOVA revealed no interaction effect between which trial participants performed first and the effect of Coffee on

postprandial glycaemia relative to Fragmented and habitual sleep. Simply put, the higher responses in the Fragmented+Coffee condition were not due to the participants who performed the Fragmented+Coffee trial first. Furthermore, the addition of a 4th condition (i.e., habitual sleep+coffee) to the study would have provided an interesting comparison to fully assess the independent effects of caffeinated coffee relative to sleep disruption. The lack of assessment of hormones potentially linked to the mechanism of disruption (e.g., cortisol) also limit further discussion surrounding the effects reported. Finally, the current study also did not strictly control for the menstrual phase in female participants, and this may have influenced our primary outcomes (Valdes & Elkind-Hirsch, 1991). This is important to acknowledge given the apparent sex differences in the pharmacokinetics of caffeine metabolism, however the study was not sufficiently powered to detect Condition x Time x Sex interactions and therefore provides scope for future work (Rasmussen *et al.*, 2002; White *et al.*, 2016).

In summary, no effect of hourly fragmented sleep (totalling <80 mins) was found on postprandial glucose and insulin responses to breakfast the next morning. However, the common approach of consuming a strong caffeinated coffee following disrupted sleep resulted in a reduction in glucose tolerance. Following a night of disrupted sleep, individuals should balance the potential stimulating benefits of caffeinated coffee consumption with the potential to increase postprandial glucose excursions.

Chapter 7 – Acute metabolic and appetite responses of substituting carbohydrate for whey protein at breakfast relative to extended morning fasting

7.1 Introduction

Breakfast habits exhibit low day-to-day variability (i.e., we typically consume similar foods upon waking every day). Dietary surveys from various countries indicate that breakfast is consistently rich in carbohydrates but provides less than half the protein of other daily meals (Rousset *et al.*, 2003; Almoosawi *et al.*, 2012; Tieland *et al.*, 2015; NHANES, 2016). Nutritional interventions targeting breakfast therefore present an opportunity to influence nutrient intake recurrently and therefore to modify overall dietary profile, with the added potential for carry-over effects from that first meal on subsequent metabolic and behavioural responses later each day.

The acute effects of common breakfast foods relative to morning fasting are well established. Extending the overnight fast results in incomplete compensation of energy intake during an *ad libitum* lunch, such that total energy intake tends to be higher when breakfast is consumed (Astbury *et al.*, 2011; Gonzalez *et al.*, 2013; Chowdhury *et al.*, 2015, 2016b; Edinburgh *et al.*, 2019). However, whilst typical carbohydrate-rich breakfasts can result in acute hyperglycemia during the morning, this can be balanced against improved glucose control in the afternoon relative to when breakfast has been omitted (i.e., the 'second-meal effect'; (Chowdhury *et al.*, 2015, 2016b)). Considering that most humans in middle-high income countries spend the majority of waking hours in the post-prandial state (Ruge *et al.*, 2009b) and that the mean amplitude of post-prandial glycemic and insulinemic excursions are associated with inflammation and cardiometabolic disease risk, strategies to mitigate such perturbations are desirable (e.g., diet) (Fonseca, 2003; Quagliaro *et al.*, 2003; De Castro, 2004; Akasaka *et al.*, 2017).

It remains unclear whether a carbohydrate-rich breakfast and/or acute hyperglycemia in the morning are necessary to elicit the second-meal effect. In particular, there are several protein-mediated mechanisms leading to the hypothesis that substituting a portion of the carbohydrate typically consumed at breakfast for added whey protein

may elicit similar, greater and/or additional metabolic effects. Firstly, from a metabolic control perspective, co-ingestion of carbohydrate with added protein results in delayed gastric emptying along with a larger, more protracted insulin response (Swaminathan *et al.*, 1985; van Loon *et al.*, 2000; Stanstrup *et al.*, 2014), thus exerting lasting effects on both glucose appearance and disposal (Manders *et al.*, 2006b; Betts & Williams, 2010b) that may carry over to improve glucose tolerance at subsequent meals (Staub, 1921; Park *et al.*, 2015; Meng *et al.*, 2017; Chang *et al.*, 2019). Secondly, from an energy balance perspective, protein has a greater effect on diet-induced thermogenesis than other dietary macronutrients and so may increase metabolic requirements over the morning, potentially contributing towards weight-loss in the longer-term (Swaminathan *et al.*, 1985; Westerterp, 2004b; Bray *et al.*, 2015). Finally, from a behavioural perspective, the physical and chemical properties of protein directly exert a more satiating effect than other dietary macronutrients, so may help with appetite control at subsequent meals (Astbury *et al.*, 2010; Leidy *et al.*, 2013).

Previous studies have begun to examine the above hypotheses but with equivocal results. Allerton *et al* (2016) reported no effect of added protein at breakfast on post-lunch glycemia, although the breakfasts in that study varied not just in macronutrient composition but also energy content (Allerton *et al.*, 2016). Conversely, Meng *et al.* (2017) did demonstrate attenuated glycemic responses to a subsequent meal following earlier ingestion of protein, although the metabolic response to the initial meal was not characterized in that study. Similarly, there is also no consensus regarding the effects of added protein at breakfast on appetite regulation. Some studies have demonstrated greater appetite suppression and/or lower energy intake at lunch following a protein-rich breakfast (Leidy & Racki, 2010; Palacios *et al.*, 2018), whereas others have not (Veldhorst *et al.*, 2009; Sayer *et al.*, 2016).

The aim of this study was therefore to isolate the metabolic effects of protein at breakfast by comparing isoenergetic carbohydrate-rich and protein-enriched breakfasts relative to extended morning fasting as a control condition. Primary outcome measures will be the glucose and insulin responses to both breakfast and lunch; secondary outcomes included energy intake (appetite) and expenditure

(substrate oxidation). It was hypothesised that the reduced-carbohydrate protein-enriched breakfast would attenuate glycemic responses to breakfast and lunch, with greater satiation and thermogenesis than the other conditions.

7.2 Methods and Materials

7.2.1 Approach to the research question

The type of protein under investigation (whey protein isolate) was selected on the basis that our hypothesis is related to insulin-mediated mechanisms; whey (especially isolate) exhibits a relatively short gastric emptying time and thus rapid hyperaminoacidemia, with potent effects on both insulin secretion (Hall *et al.*, 2003; Smith *et al.*, 2021) and diet-induced thermogenesis (Acheson *et al.*, 2011). The dose of protein selected for investigation (15 g) was based on robust evidence from acute laboratory trials of dose-response effects of whey protein on energy intake later the same day. Specifically, the addition of 15 g of protein to an already protein-rich meal reduces energy intake at the next meal, such that the protein added at breakfast is more than fully compensated for by reduced energy/protein intake at the next meal alone (Astbury *et al.*, 2010). Furthermore, based on data from NHANES (NHANES, 2016), an additional 15 g of protein at breakfast would be appropriate to achieve a more even distribution of protein intake across the day (i.e. the protein content of daily meals is typically ~10 g at breakfast, ~20 g at lunch and ~35 g at dinner).

The above type and dose of protein was included within a typical breakfast food (porridge) that delivered carbohydrate in proportion to the individual resting metabolic requirements measured at each participant's first visit to the laboratory. Specifically, the target rate of carbohydrate delivery (7.3 mg·kJ RMR^{-1}) has been used in previous studies in our laboratory on the basis that it provides a sufficient metabolic challenge for the assessment of glucose tolerance. Within the context of the present study this was also appropriate as the unfortified porridge condition would not then deliver an excessive amount of protein. Furthermore, the chosen carbohydrate delivery rate also results in a total breakfast energy content that meets the commonly reported threshold definition that breakfast should constitute ≥20 % of daily energy intake (Timlin & Pereira, 2007). Importantly, standardisation of breakfast quantity to resting metabolic rate (RMR) reflects energy requirements during the trial (i.e., rested in bed) whilst also accounting for RMR as a primary driver of energy intake (Blundell *et al.*, 2012; Chowdhury *et al.*, 2015). The lunch meal was also based on previous work by Chowdhury *et al* (2015, 2016b), the composition and dose of which (pasta – see

below) again was selected on the basis that it would provide a sufficient challenge to glycemic control in order to meaningfully compare post-prandial responses.

7.2.2 Experimental Design

Participants underwent three trials in a randomised crossover design with a minimum washout period of 4 days between trials (12 ± 10 days; range 4-47 days). Trial sequence was stratified based on participants biological sex and habitual breakfast habit (>209 kJ [>50 kcal] intake within 2 h of waking on ≥ 4 d/week (de Castro, 1994; Betts *et al.*, 2016)) and was not counter-balanced. Diet was recorded for 48 h prior to participants' first visit to the laboratory and replicated before subsequent visits. Participants also standardised physical activity, whilst refraining from caffeine and alcohol for 24 h before each trial. Broadly, laboratory visits involved consumption of either a porridge breakfast (with or without substitution of oats for whey protein) or extended morning fasting, before an *ad libitum* pasta lunch.

7.2.3 Participants

Twelve healthy men and women participated in the study (**Table 1**). Exclusion criteria included body mass index outside of the range of 18·5-29·9 kg·m^{-2}, any diagnosed metabolic disease (e.g., Type 1 or Type 2 diabetes), any reported use of substances or behaviours (e.g., non-standard sleep–wake cycle, shift workers) that may pose undue personal risk or introduce bias into the experiment. All participants were informed of any potential risks and discomfort involved in the study prior to providing written and oral informed consent. The study was given a favourable ethical opinion by the Research Ethics Approval Committee for Health (REACH) at the University of Bath (EP 17/18 260) and was registered on clinicaltrials.gov (NCT03866720). All procedures were performed in accordance with the Declaration of Helsinki.

Table 7.1. Participant characteristics (mean ± SD, *n* = 12)

Characteristics	Combined	Male (*n*=8)	Female (*n*=4)
Age (years)	22 ± 2	23 ± 3	21 ± 2
Body mass (kg)	75.7 ± 11.2	80.2 ± 11.0	66.7 ± 7.2
Height (m)	1.77 ± 0.08	1.81 ± 0.05	1.69 ± 0.01
Body mass index (kg·m^{-2})	24.1 ± 3.6	24.5 ± 3.3	23.3 ± 2.6
Waist:Hip	0.78 ± 0.05	0.80 ± 0.05	0.74 ± 0.04
RMR (kcal·d^{-1})	1851 ± 241	1960 ± 253	1634 ± 208
Habitual breakfast consumers[†] (*n*)	11	8	3

[†]Habitual breakfast consumption defined as >209 kJ (>50 kcal) intake within 2 h of waking on ≥ 4 d/week (de Castro, 1994; Betts *et al.*, 2016).

7.2.4 Experimental protocol

Participants arrived at the laboratory at 0800 ± 1 h following an overnight fast of ≥10 h and voided prior to assessment of body mass (Tanita-BC543, Japan) and waist:hip circumference ratio (SECA). In accordance with best practice for measuring resting metabolic rate, participants rested in a semi-supine position (~60⁰) prior to collection of expired breath samples via the Douglas bag method (Compher *et al.*, 2006). An intravenous cannula was placed in an antecubital vein and a baseline 10 mL sample of blood was drawn (BD Venflon Pro; BD, Switzerland). Cannulae were kept patent by flushing with 0.9% sodium chloride infusion (B. Braun; UK). Participants then either ingested one of the two porridge breakfasts or remained in an overnight fasted state, initiating the beginning of the 180 min morning period with their first mouthful. Further blood samples (10 mL) were drawn every 15, 30, and 60 min for the first-, second- and third hours respectively, alongside hourly expired gas samples and visual analogue scales. Upon completion of the post-breakfast period participants then were then provided with an *ad libitum* lunch over a 30-min duration. Blood samples were collected every 15 and 30 mins for the first- and second-hours post-lunch respectively, alongside hourly expired gas and visual analogue scales.
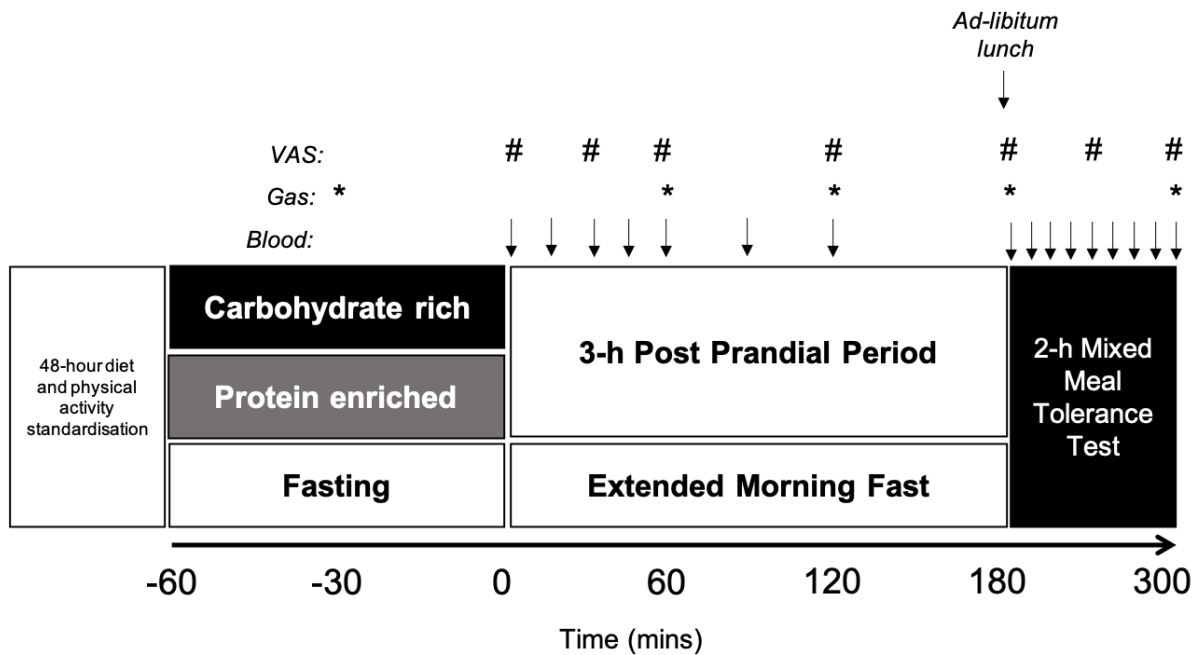
Figure 7.1 – Schematic representation of the study protocol.

7.2.5 Breakfast

Breakfast meals were isoenergetic and consisted of boiling water added to instant porridge oats (Quaker, UK), either unfortified/standard (*carbohydrate-rich*) or fortified with whey protein isolate (*protein-enriched;* MyProtein, Northwich, UK). The carbohydrate-rich breakfast provided 7.3 mg of carbohydrate per kJ of each individual's resting metabolic rate. In the protein-enriched breakfast, substitution of 18.3 g of whey protein isolate powder (i.e., 15 g whey protein) into the porridge resulted in a carbohydrate delivery of 5.73 ± 0.13 mg CHO·kJ^{-1} RMR. The difference in macronutrient compositions of the breakfasts is reported in **Table 7.2**. During the *extended morning fast* only plain water was permitted across the morning (standardized across trials) until the *ad libitum* lunch in the *extended morning fast* condition. The comparative overnight fasting period between the breakfast and fasting trials was therefore ≥ 10 h and ≥ 14 h, respectively.

Table 7.2 – Macronutrient composition of the provided breakfast meals

	Carbohydrate rich breakfast	Protein enriched breakfast
Energy (kJ)	1334 ± 209	1334 ± 209
Carbohydrate (g)	56.0 ± 8.7	45.0 ± 7.9
Sugar (g)	24.6 ± 3.8	19.8 ± 3.4
Fibre (g)	4.7 ± 0.7	3.6 ± 0.7
Fat (g)	4.6 ± 1.0	4.6 ± 0.4
Porridge protein (g)	11.9 ± 1.9	9.4 ± 1.7
Whey protein (g)	-	15
Total Protein (g)	11.9 ± 1.9	24.4 ±1.7

7.2.6 Ad libitum lunch

The lunch meal consisted of penne pasta (Sainsbury's, UK; Per 100 g: Carbohydrate 32.3 g, Fat 0.7 g, Protein 5.2 g) including tomato and herb bolognese sauce (Sainsbury's, UK; Per 100 g: Carbohydrate 6.2 g, Fat < 0.5 g, Protein 1.3 g); prepared at a ratio of 1:1 uncooked mass. Each pasta meal was prepared according to standardised cooking times (20 mins), with the energy density of the cooked pasta calculated (to account for any differences in water absorption) and the final masses of pasta and sauce combined noted, to calculate the energy density for the homogeneous mixture of pasta and sauce. The nutritional information for the lunch meal is reported in **Table 7.3**. Pasta meals were prepared the night before each trial and were heated in a microwave at 700 Watts for 5 mins prior to serving to participants. The pasta meal was provided in a large bowl that was replenished every 10 min or when participants were close to finishing to minimize visual feedback relating to consumed volume and to prevent any tendency to finish the portion provided. Participants were asked to eat until "comfortably full" (Chowdhury *et al.*, 2015, 2016b; Hengist *et al.*, 2020) and were otherwise left alone during the lunch. *Ad libitum* water intake was permitted during participants first trial, and this volume was replicated upon subsequent visits.

Table 7.3 – Macronutrient composition of the *ad libitum* lunch meal.

	Extended morning fast	Carbohydrate rich breakfast	Protein enriched breakfast
Mass (g)	697 ± 219	667 ± 211	654 ± 226
Carbohydrate (g)	89.2 ± 25.7	84.5 ± 25.2	86.8 ± 31.2
Sugar (g)	18.7 ± 5.5	17.5 ± 5.2	18.1 ± 6.7
Starch (g)	68.9 ± 19.8	66.4 ± 19.7	67.1 ± 24.1
Fat (g)	4.7 ± 1.4	4.4 ± 1.4	4.5 ± 1.7
Protein (g)	15.1 ± 4.4	14.5 ± 4.3	14.8 ± 5.5

7.2.7 Blood analysis

Blood samples were immediately transferred into tubes treated with EDTA or clotting activators (plasma and serum respectively; Sarstedt, UK) and were left to clot at room temperature for 15 mins prior to centrifugation (10 min, 4000 x *g*, 4°C), before plasma/serum supernatant was aliquoted into Eppendorf tubes and stored at -80°C for subsequent analysis. Plasma samples were analysed for glucose, non-esterified fatty acids (NEFA), urea, and insulin as described in Chapter 3.6.1 and 3.6.2. Incremental area under the curve (iAUC), peak concentration ($mmol \cdot L^{-1}$) and time to peak (mins) were calculated using the Time Series Response Analyser (TSRA) tool (Narang *et al.*, 2020).

7.2.8 Expired gas analysis

Expired gases were collected using 150 L Douglas bags, with concentrations of O_2 and CO_2 analyzed in a known volume of sample, using paramagnetic and infrared analyzers, respectively (Mini HF 5200; Servomex Group Ltd., Crowborough, UK). These analysers were also used to determine the composition of inspired air to adjust for variation in atmospheric O_2 and CO_2 concentrations (Betts & Thompson, 2012). Total volumes of expired gas were determined using a dry gas meter (Harvard Apparatus, Holliston, MA) and temperature measured using a digital thermometer (Edale Instruments, Longstanton, UK). Substrate utilization was determined using the equations of Frayn (Frayn, 1983). Diet-induced thermogenesis was calculated as the difference in increment in energy expenditure ($kcal \cdot min^{-1}$) between the extended morning fasting trial and each of the fed trials (i.e., carbohydrate-rich and protein-enriched), thus controlling for the influence of circadian variation in resting metabolic

rate on the thermic effect of feeding (Ruddick-Collins *et al.*, 2021). Incremental area under the curve was then calculated from these values using the Time Series Response Analyser (Narang *et al.*, 2020).

7.2.9 Appetite ratings

Paper-based visual analogue scales with 100-mm horizontal axes were employed to assess subjective appetite as described in Chapter 3.7.

7.2.10 Statistical analysis

Statistical analyses were performed in GraphPad Prism (Prism 9, GraphPad Software). The distribution of paired differences was assessed using the Shapiro-Wilk test with a paired *t*-test or Wilcoxon test employed for normally and non-normally distributed data, respectively. The separate research questions concerning the post-breakfast and post-lunch periods were addressed by applying a separate two-way condition x time mixed model analysis of variance (ANOVA) to each period. Wherever there were multiple contrasts for such time series data, the Greenhouse-Geisser correction was adopted for epsilon <0.75 and the Huynh-Feldt correction adopted for less severe asphericity. Interaction effects were then followed up with Ryan-Holm-Bonferroni stepwise correction to adjust the resulting *p*-values for multiple comparisons and thus avoid inflation of type I error rate (Ludbrook, 2000; Atkinson, 2002). Simple contrasts between conditions (i.e., without time-series data) such as the energy intake data and incremental area under the curve data were analysed using one-way ANOVA, with *post-hoc t*-tests employed to identify the location of variance between the 3 conditions.

In text values are reported as mean ± standard deviation unless otherwise stated. Error values on figures are normalized confidence intervals corrected for between-participant variation (SPSS, IBM v28 & Microsoft Excel v16.0.13801.21072). This correction is such that the magnitude of these confidence intervals infer the contrast between paired means at each time point rather than variance of individual values around each mean (Loftus & Masson, 1994). Using this approach, non-overlapping

intervals between contrasted values would typically be deemed significantly different according to conventional null hypothesis testing (i.e., $p \leq 0.05$).

As an exploratory analysis into the relationship between pre-lunch circulating NEFA concentrations and post-lunch glycemic/insulinemic responses, simple linear regressions were performed between post-breakfast plasma NEFA concentrations with iAUC values for plasma glucose and insulin. Finally, given the cross-over design, the potential for trial order effects was examined by analysing the iAUC/tAUC, peak, and time-to-peak data of participants' first, second and third laboratory visits using a one-way ANOVA; if a significant effect of trial sequence was identified then a follow up two-way condition x sequence ANOVA was performed (Wellek & Blettner, 2012; Betts *et al.*, 2020) – none were present.

Based on a post lunch difference in insulin incremental area under the curve of 23.4 nmol·L^{-1}·180 min in previous work contrasting the metabolic responses to a subsequent meal following breakfast or an extended overnight fast, 15 participants were deemed sufficient to achieve >80% power with an alpha level of 0.05 between groups (G*Power 3.1) (Chowdhury *et al.*, 2015).

7.3 Results

7.3.1 Morning postprandial metabolism

Ingestion of breakfast increased plasma glucose concentrations such that morning peak glucose was higher in both the *carbohydrate-rich* (7.71 ± 0.68 mmol·L^{-1}; *p* < 0.001) and *protein-enriched* (7.35 ± 0.76 mmol·L^{-1}; *p* < 0.001) conditions relative to *extended morning fasting* (5.38 ± 0.22 mmol·L^{-1}; **Figure 7.2A**). Peak glucose was similar between *carbohydrate-rich* and *protein-enriched* breakfasts (*p* = 0.27). As such, morning incremental area under the curve (iAUC) for glucose was higher following both *carbohydrate-rich* (119 ± 36 mmol·L^{-1}·180 min; *p* < 0.0001), and *protein-enriched* (87.7 ± 36.0 mmol·L^{-1}·180 min; *p* = 0.001) breakfasts compared to *extended morning fasting* (22.9 ± 24.4 mmol·L^{-1}·180 min) (**Figure 7.3A**). Notably, plasma glucose iAUC was also higher following ingestion of the carbohydrate-rich *versus* protein-enriched breakfast (*p* = 0.03).

Likewise, regardless of composition, breakfast ingestion elicited a similar increase in insulin concentrations across the morning such that peak insulin was higher following *carbohydrate-rich* (326 ± 180 pmol·L^{-1}; *p* = 0.0002) and *protein-enriched* (362 ± 135 pmol·L^{-1}; *p* < 0.001) breakfasts relative to *extended morning fasting* (32 ± 11 pmol·L^{-1}). Plasma insulin iAUC was also then higher across the morning in both the *carbohydrate-rich* (14.2 ± 7.9 nmol·L^{-1}·180 min; *p* = 0.0002) and *protein-enriched* (16.3 ± 5.9 nmol·L^{-1}·180 min; *p* < 0.001) conditions compared to *extended morning fasting* (0.5 ± 0.7 nmol·L^{-1}·180 min) with no difference between the two breakfasts (*p* = 0.06) (**Figure 7.3C**).

Ingestion of breakfast suppressed plasma NEFA across the morning to a similar extent between *carbohydrate-rich* and *protein-enriched breakfasts* in contrast to the gradual increase observed with extended morning fasting (condition x time *p* = 0.006; **Figure 7.2C**). Consequently, total area under the curve (tAUC, **Figure 7.3E**) for NEFA across the morning was lower following both the *carbohydrate-rich* (28.8 ± 16.6 mmol·L^{-1}·180 min; *p* = 0.02) and *protein-enriched* (24.6 ± 10.6 mmol·L^{-1}·180 min; *p* = 0.003) breakfasts relative to the extended morning fasting (55.9 ± 26.9 mmol·L^{-1}·180 min). tAUC was similar between breakfasts (*p* = 0.38).

Breakfast composition altered the late morning plasma urea response (condition x time *p* = 0.05). Whereas concentrations remained relatively stable following the *protein-enriched breakfast* and during the *extended morning fast*, plasma urea concentration gradually decreased in the second hour of the morning period following the *carbohydrate-rich breakfast* such that plasma concentration was lower than the *protein-enriched breakfast* at 120 mins (Figure 3D). Despite this, tAUC for plasma urea was similar between conditions (*p* = 0.10).
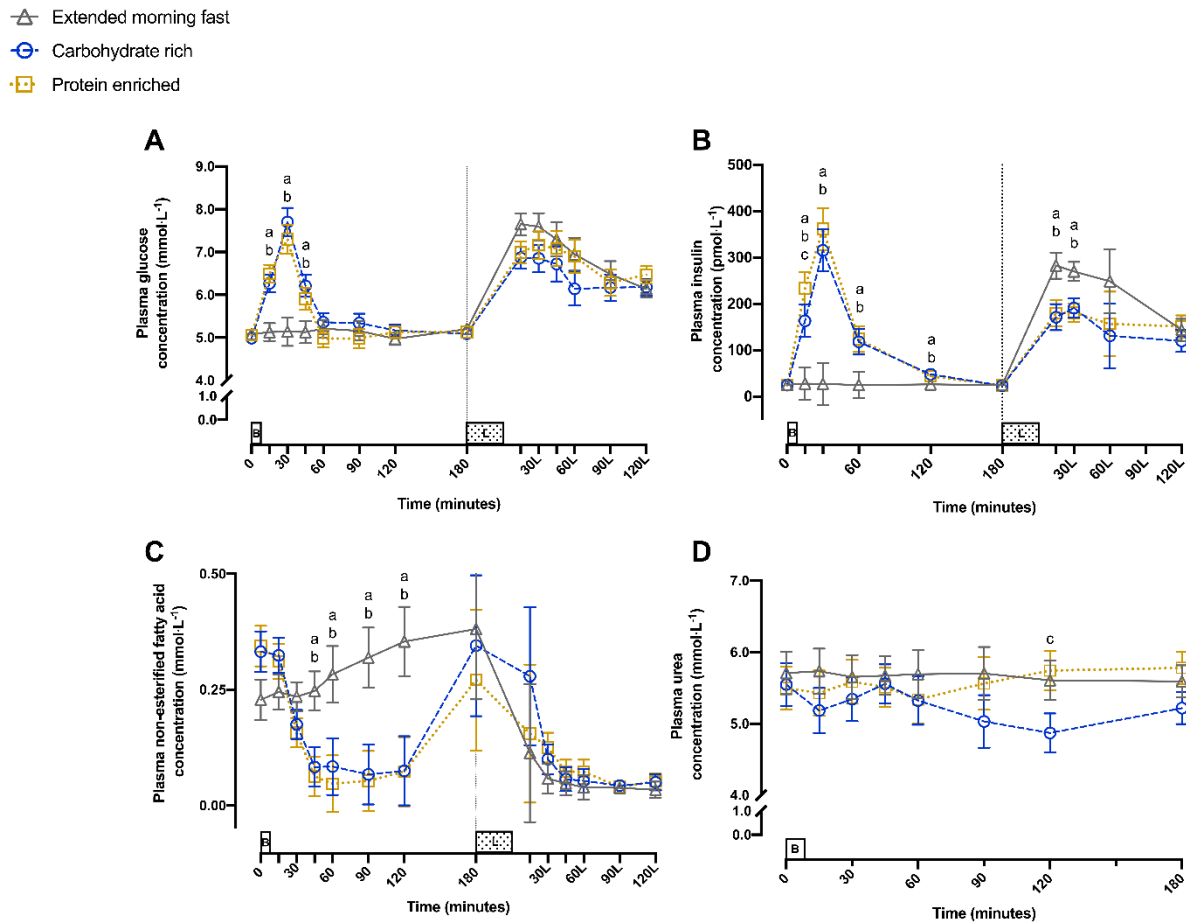
Figure 7.2 - Metabolic responses for the trial duration (A) plasma glucose (B) plasma insulin (C) plasma non-esterified fatty acid (NEFA) (D) plasma urea. Means ± normalised CI. a denotes difference ($p < 0.05$) between carbohydrate rich breakfast and extended morning fasting. b denotes difference ($p < 0.05$) between protein enriched breakfast and extended morning fasting. c denotes difference ($p < 0.05$) between carbohydrate rich and protein enriched breakfasts. The dashed vertical line denotes the end of the breakfast (B) period and start of the lunch (L) period.
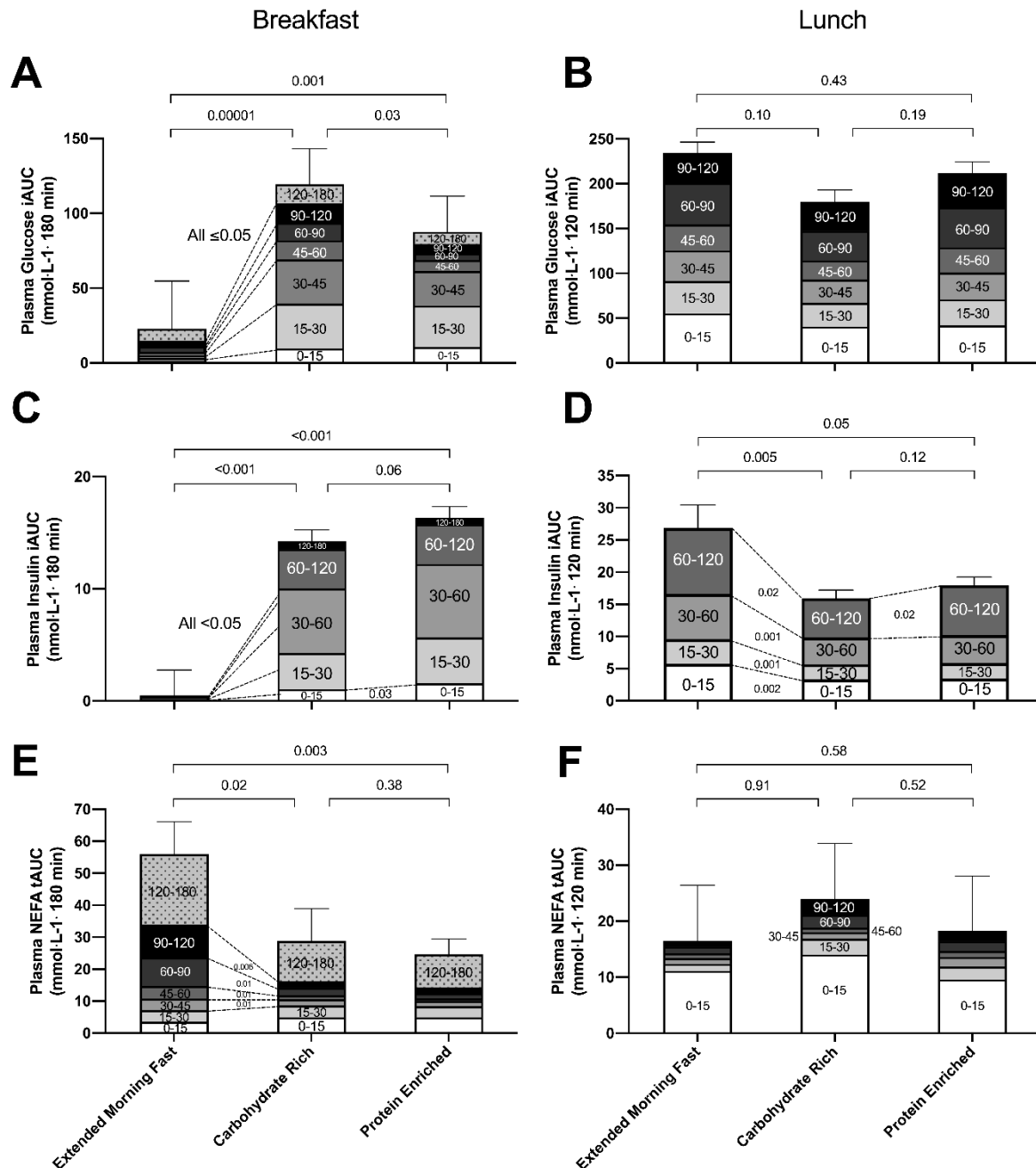
Figure 7.3 - Incremental area under the curve (iAUC) data for (A) plasma glucose, (B) plasma insulin. Total area under the curve data for (C) plasma NEFA. Means ± normalised CI. Each bar is comprised of incremental or total AUC for each time increment.

7.3.2 Morning respiratory data

Breakfast consumption resulted in an increase in energy expenditure across the morning relative to extended morning fasting (condition x time $p < 0.001$; **Figure 7.4A**). However, dietary induced thermogenesis across the post-breakfast period was similar between the *carbohydrate-rich* and *protein-enriched* breakfasts (19.0 ± 11.5 *versus* 23.5 ± 13.7 kcal·180 min; $p = 0.25$) (**Figure 7.4B**).

At the group level, peak respiratory exchange ratio (RER) was greater following both the *carbohydrate-rich* (0.94 + 0.06; $p = 0.01$) and *protein-enriched* (0.90 ± 0.04; $p = 0.05$) breakfasts relative to *extended morning fasting* (0.86 ± 0.04) (**Figure 7.4C**). However, individual peak values across the morning were similar between *extended morning fasting* (0.92 ± 0.06), *carbohydrate-rich breakfast* (0.92 ± 0.06), and *protein-enriched breakfast* (0.91 ± 0.05) ($p = 0.43$).
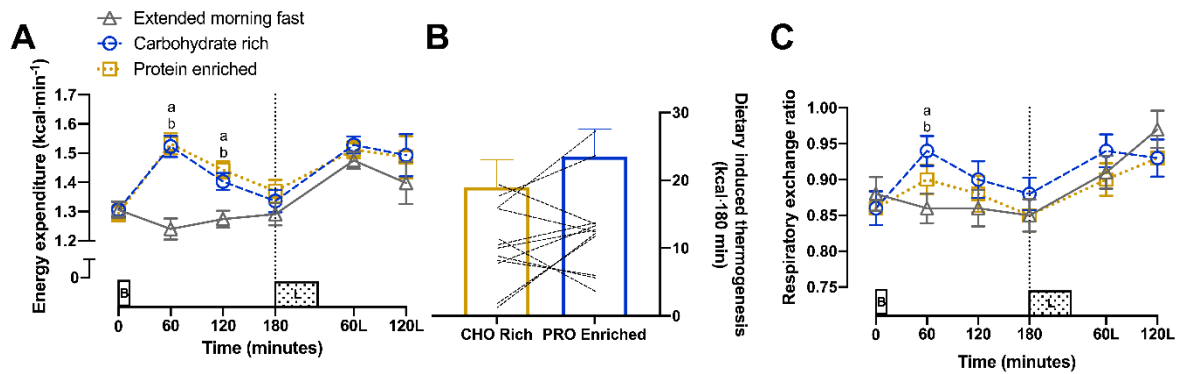
Figure 7.4 - Respiratory data collected from Douglas bags for the trial duration. (A) energy expenditure (B) Measured thermic effect of feeding (C) respiratory exchange ratio. a denotes difference ($p < 0.05$) between carbohydrate rich breakfast and extended morning fasting. b denotes difference ($p < 0.05$) between protein enriched breakfast and extended morning fasting. c denotes difference ($p < 0.05$) between carbohydrate rich and protein enriched breakfasts. The dashed vertical line denotes the end of the breakfast (B) period and start of the lunch (L) period.

7.3.3 Morning subjective appetite

Composite appetite fell comparably following consumption of the *carbohydrate-rich* and *protein-enriched* breakfasts (**Figure 7.5A**). Conversely, subjective appetite gradually increased across the morning during the extended morning fast (condition x time $p < 0.001$). Total area under the curve (tAUC) for composite appetite across the morning was therefore higher following extended morning fasting (44534 ± 9680 mm·180 min) compared to both the carbohydrate-rich (28242 ± 9867 mm·180 min; $p < 0.001$) and protein-enriched (28416 ± 9204 mm·180 min; $p < 0.001$) breakfasts.

7.3.4 Energy intake

During the two breakfast conditions, participants consumed a prescribed breakfast of 1337 ± 211 kJ (320 ± 50 kcal) (i.e., variance proportionate to inter-individual differences in RMR). Energy intake during the *ad-libitum* lunch was not different between conditions, with participants consuming 3305 ± 948 kJ (790 ± 227 kcal) following the extended morning fast *versus* 3149 ± 931 kJ (753 ± 223 kcal) following the carbohydrate-rich breakfast ($p = 0.50$) and 3217 ± 1157 kJ (769 ± 278 kcal) following the protein-enriched breakfast ($p = 0.75$, **Figure 7.5B**). As such, total energy intake across the trial was lower in extended morning fasting condition (3306 ± 948 kJ [790 ± 227 kcal]) relative to both the carbohydrate-rich (4486 ± 1003 kJ [1072 ± 240 kcal]; $p = 0.001$) and protein-enriched (4555 ± 1217 kJ [1088 ± 291 kcal]; $p = 0.002$) breakfasts respectively.
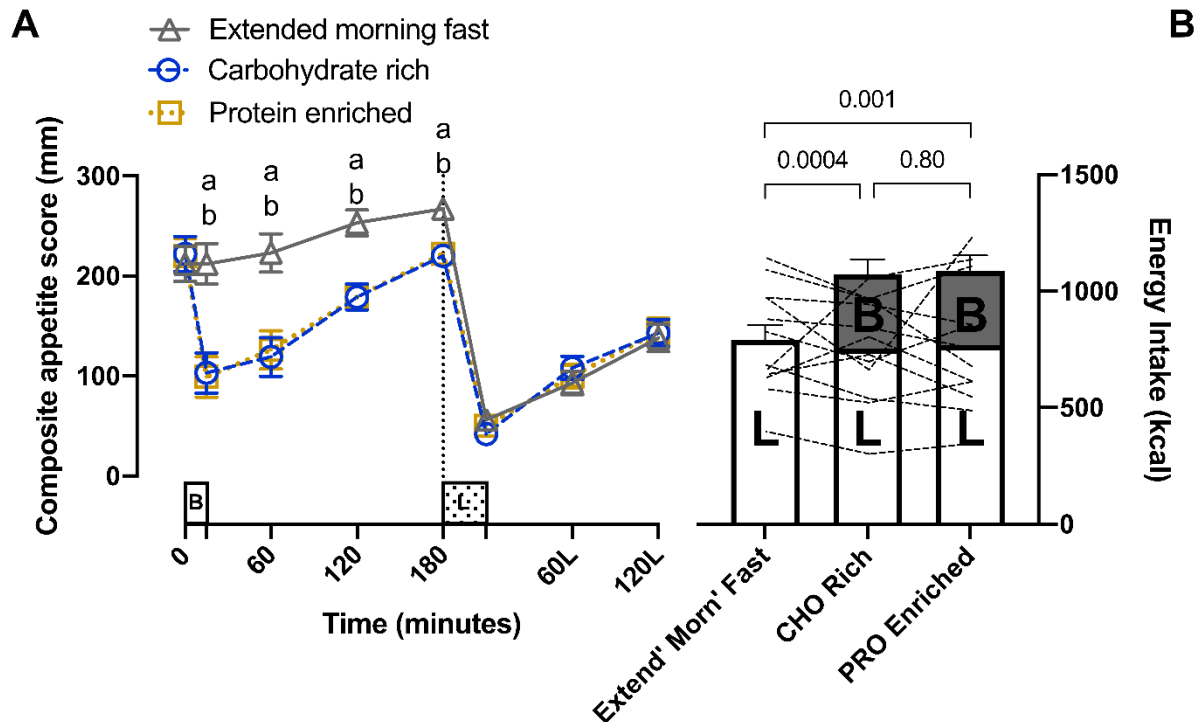
Figure 7.5 - (A) Composite appetite score and (B) energy intake for the trial duration. Means ± normalised CI. a denotes difference (*p* < 0.05) between carbohydrate rich breakfast and extended morning fasting. b denotes difference (*p* < 0.05) between protein enriched breakfast and extended morning fasting. c denotes difference (*p* < 0.05) between carbohydrate rich and protein enriched breakfasts. The dashed vertical line denotes the end of the breakfast (B) period and start of the lunch (L) period.

7.3.5 Post-lunch post-prandial metabolism

Neither breakfast consumption nor composition influenced plasma glucose across the afternoon (condition x time p = 0.16). However, peak plasma glucose concentration was lower following ingestion of the *carbohydrate-rich* breakfast (7.76 ± 0.97 mmol·L^{-1}; p = 0.01) relative to the *extended morning fast* condition (8.76 ± 1.35 mmol·L^{-1}), which was similar to peak glucose following the *protein-enriched* breakfast (8.08 ± 0.93 mmol·L^{-1}; p = 0.09). Time to post-lunch peak glucose was similar between conditions (p = 0.44). Post-lunch plasma glucose iAUC was similar between *carbohydrate-rich* (179 ± 92 mmol·L^{-1}·120 min; p = 0.10), and *protein-enriched* (211 ± 84 mmol·L^{-1}·120 min; p = 0.43) breakfasts relative to *extended morning fasting* (234 ± 112 mmol·L^{-1}·120 min) (**Figure 7.2B**). Post-lunch plasma glucose iAUC was also similar following *carbohydrate-rich* and *protein-enriched breakfasts* (p = 0.19).

Post-lunch peak insulin was lower following both the *carbohydrate-rich* (225 ± 82 pmol·L^{-1}; p = 0.01) and *protein-enriched* (228 ± 75 pmol·L^{-1}; p = 0.01) breakfast conditions relative to the extended morning fast condition (403 ± 215 pmol·L^{-1}) but was similar between each breakfast condition (p = 0.81). Time to post-lunch peak insulin was similar between conditions (p = 0.19). Post-lunch plasma insulin iAUC was lower following both *carbohydrate-rich* (16.0 ± 7.7 nmol·L^{-1}·120 min; p = 0.005) and *protein-enriched* (18.0 ± 8.0 nmol·L^{-1}·120 min; p = 0.05) breakfasts compared to *extended morning fasting* (26.9 ± 13.5 nmol·L^{-1}·120 min) (**Figure 7.3D**). Post-lunch plasma insulin iAUC was similar between the *carbohydrate-rich* and *protein-enriched* breakfasts (p = 0.12).

While post-lunch plasma NEFA concentrations fell to a similar extent in all conditions (**Figure 7.2C**), the most rapid post-prandial suppression in the afternoon was following extended morning fasting. As such, total area under the curve (tAUC) for NEFA following lunch was similar following extended morning fasting (16.5 ± 7.6 mmol·L^{-1}·120 min) compared to both the carbohydrate-rich (23.9 ± 29.2 mmol·L^{-1}·180 min; P = 0.45) and protein-enriched (18.3 ± 11.8 mmol·L^{-1}·180 min; P = 0.58) breakfasts. Likewise, post-lunch tAUC was similar between *carbohydrate-rich* and *protein-*

enriched breakfasts (p = 0.56). Pre-meal plasma NEFA concentration was not associated with post-lunch glucose or insulin iAUC (**Figure 7.6**).

Repeated measures one-way ANOVA revealed an effect of trial sequence on post-lunch glucose iAUC (p = 0.04) whereby, at the group level, iAUC was higher in the first trial (248.3 ± 107.1 mmol·L^{-1}·120 min) relative to the second (192.0 ± 93.6 mmol·L^{-1}·120 min; p = 0.05) and third (185.3 ± 84.2 mmol·L^{-1}·120 min mmol; p = 0.04) trials respectively. However, no Sequence x Condition interactions in post-lunch plasma glucose iAUC responses were evident (p = 0.16).
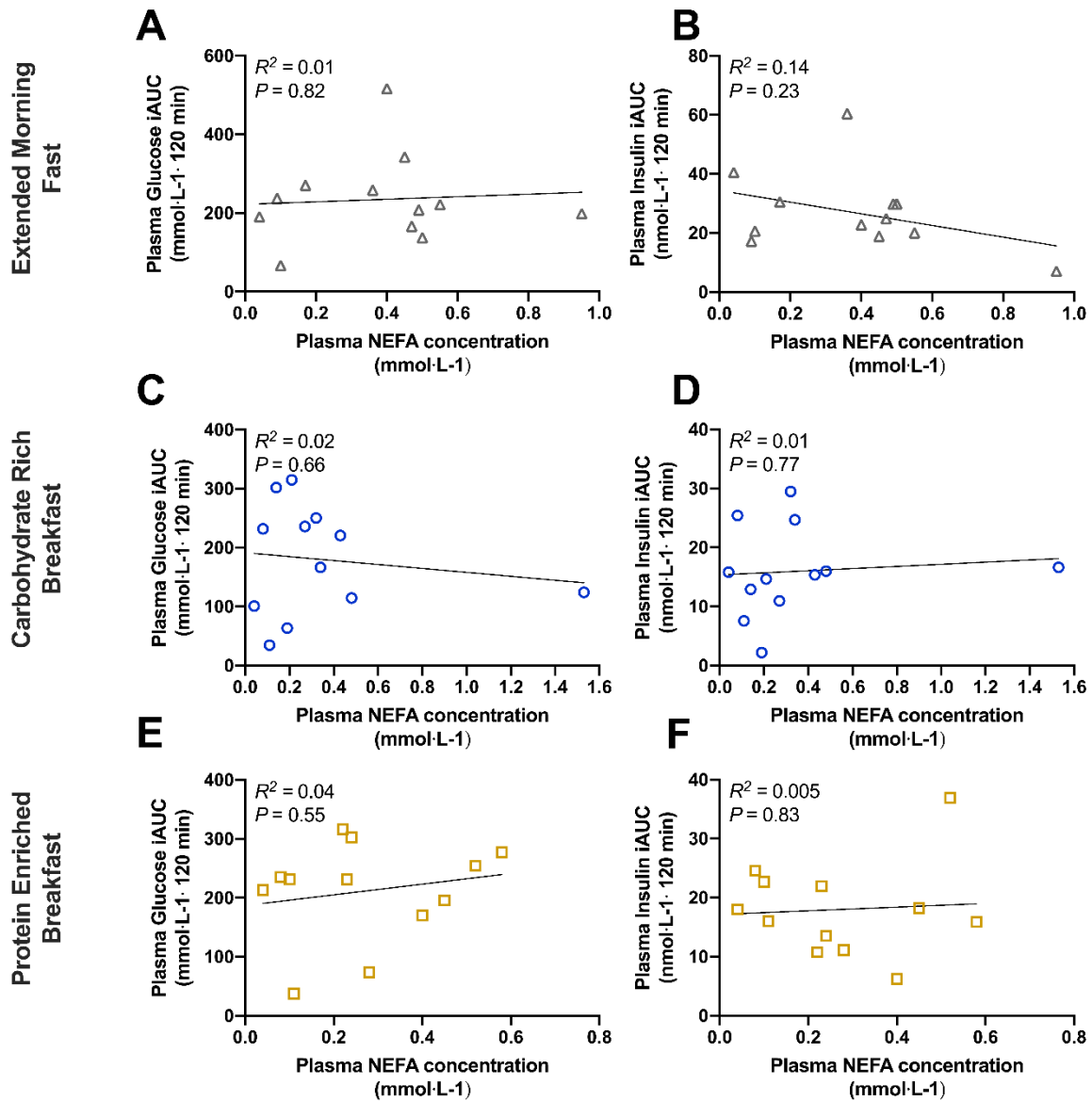
Figure 7.6 – Simple linear regression between 180 min post breakfast plasma NEFA concentration and (A) Extended morning fast Breakfast Glucose iAUC (B) Extended morning fast Breakfast Insulin iAUC (C) Carbohydrate Rich Breakfast Glucose iAUC (D) Carbohydrate Rich Breakfast Insulin iAUC (E) Protein Enriched Breakfast Glucose iAUC (F) Protein Enriched Breakfast Insulin iAUC.

7.3.6 Post-lunch appetite

Following ingestion of the lunch meal, composite appetite was suppressed in all conditions before gradually increasing throughout the post-lunch period. Post-lunch appetite values were similar at each time point (all $p > 0.05$) such that tAUC was also similar between conditions ($p = 0.72$).

7.3.7 Post-lunch respiratory data

Energy expenditure was similar at the pre-lunch baseline between all three conditions. Following the lunch meal, energy expenditure increased to a similar extent in all conditions and remained similar across all post-lunch time points (**Figure 4A**). RER was similar at the pre-lunch baseline between all three conditions. Following the lunch meal, RER increased to a similar extent in all conditions and remained similar across all post-lunch time points (**Figure 4C**).

7.2.8 Combined morning and post-lunch glycemic, insulinemic, and NEFA responses

Plasma glucose iAUC across the laboratory visit (i.e., morning and post-lunch) was similar ($p = 0.27$) for extended morning fasting (257 ± 110 mmol·L^{-1}·300 min), carbohydrate-rich breakfast (299 ± 99 mmol·L^{-1}·300 min), and protein-enriched breakfast (299 ± 110 mmol·L^{-1}·300 min; **Figure 7A**). Likewise, combined morning and afternoon plasma insulin iAUC was similar ($p = 0.14$) between extended morning fasting (27.4 ± 13.4 nmol·L^{-1}·300 min), carbohydrate-rich (30.2 ± 14159 nmol·L^{-1}·300 min), and protein-enriched breakfasts (34.3 ± 13.3 nmol·L^{-1}·300 min) (**Figure 7B**). Finally, combined NEFA tAUC was also similar ($p = 0.15$) regardless of extended morning fasting (72 ± 33 mmol·L^{-1}·300 min), carbohydrate-rich breakfast (53 ± 44 mmol·L^{-1}·300 min), or protein-enriched breakfast (43 ± 21 mmol·L^{-1}·300 min; **Figure 7C**).
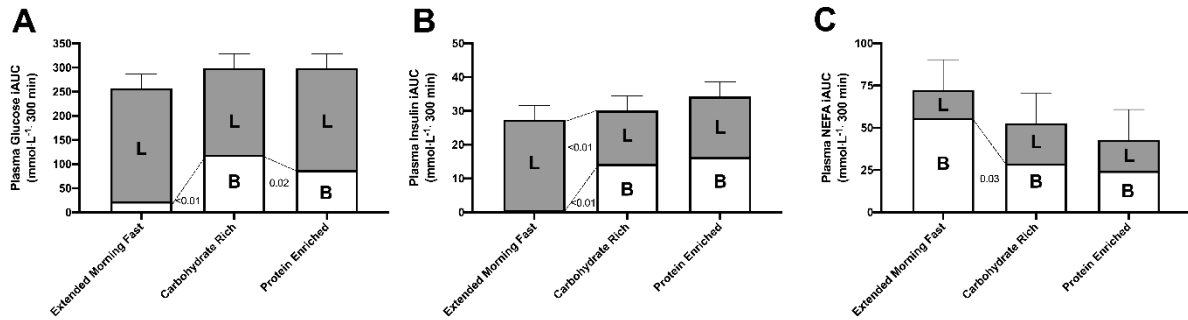
Figure 7.7 - Combined morning and afternoon Incremental area under the curve (iAUC) data for (A) plasma glucose, (B) plasma insulin. Combined morning and afternoon Total area under the curve data for (C) plasma NEFA. Means ± normalised CI. **B** = breakfast; **L** = Lunch.

7.4 Discussion

The present study revealed that ingestion of breakfast, as opposed to extended morning fasting, can attenuate the post-prandial insulin response to lunch. This observation was consistent irrespective of whether a typical carbohydrate-rich breakfast was ingested or a reduced-carbohydrate, protein-enriched breakfast was ingested.

Despite a lower glycemic response to the protein-enriched breakfast, the second-meal effect was nonetheless effectively stimulated. Whilst glycemic responses to the *ad libitum* lunch meal were similar between all conditions, consumption of either breakfast attenuated the insulinaemic responses to lunch. Increases in circulating insulin (and subsequent suppression of plasma NEFA concentrations) following an initial meal primes the pancreas and metabolic tissues in preparation for a subsequent meal (Lee *et al.*, 2011; Lopez *et al.*, 2011; Deshmukh, 2016; Moore *et al.*, 2017) and therefore could represent an important metabolic signal in broad alignment with waking to maintain glucose homeostasis throughout the day (Jakubowicz *et al.*, 2017). This is especially pertinent considering the robust effects of insulin upon the circadian clock in the peripheral tissues that deal with ingested nutrients (Crosby *et al.*, 2019; Tuvia *et al.*, 2021). Interestingly, this second-meal response following either breakfast occurred despite differing post-prandial metabolic responses between the two isoenergetic breakfasts; ingestion of the protein-enriched breakfast reduced post-prandial glycaemia and accentuated the early phase insulin response relative to the carbohydrate-rich breakfast. This is consistent with previous research demonstrating acutely attenuated post-prandial glycaemia with higher protein meals (Claessens *et al.*, 2008; Power *et al.*, 2009; Acheson *et al.*, 2011) and is likely explained by dietary protein-induced stimulation of glucagon and insulin to maintain euglycemia (Ang *et al.*, 2019), as well as the direct reduction in carbohydrate dose (Samkani *et al.*, 2018).

Although previous work investigating the effects of breakfast *per se* on sequential meal metabolism demonstrated a distinct second-meal effect for glucose that was not apparent in the current study (Chowdhury *et al.*, 2015, 2016b), this could be explained by differences in population age between our study and Chowdhury et al (Chowdhury

et al., 2015, 2016b), whereby the regulatory effects of insulin are more apparent in younger populations (Chowdhury *et al.*, 2015, 2016b; Hengist *et al.*, 2020). Finally, total glycemic and insulinemic responses across the day were similar, which is remarkable considering not only the divergent responses between conditions during the morning but especially the fact that the fasting condition involved substantially less total food ingestion, thus highlighting the extent of poorer metabolic control when lunch was consumed in the fasted state.

In a somewhat more experimental paradigm, Smith *et al* (2021) investigated the effects of a large dose of nocturnal (0400 h) protein on next meal (i.e., breakfast) glycemic control. Nocturnal feeding of protein resulted in worsened glycemic control at breakfast, which is most likely explained by the large dose of protein ingested (~75 g) (Smith *et al.*, 2015) but may be due to feeding at a time misaligned from typical rhythms (Garlick *et al.*, 1980). In the current study, the initial feed was broadly aligned with the commencement of the typical active/light phase for humans. Consequently, breakfast was consumed at a time during which the body is typically more insulin sensitive and anticipating energy intake (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Qian & Scheer, 2016). Aside from the smaller dose of protein used in the current study (~25 g) in contrast to Smith *et al.* (2021), these data could suggest that the time of day during which the initial meal is fed may also influence the second-meal response.

Omission of breakfast resulted in a gradual increase in circulating NEFA concentrations across the morning, whereas ingestion of either breakfast resulted in a similar degree of NEFA suppression (**Figure 7.3C**). This is consistent with the possibility that NEFA exposure is a key mechanism of the second-meal effect, and/or that insulin priming reaches a threshold whereby further insulin has no further priming effect. This was expected given the established effects of fasting and insulin upon secretion of NEFA from adipose tissue to fuel metabolism (Frayn, 2010b). Conversely, ingestion of breakfast resulted in a similar suppression of NEFA, regardless of the whey protein content of the breakfast meal. Considering the acute detrimental influence of NEFA upon insulin action in healthy populations (Randle *et al.*, 1963;

Balent *et al.*, 2002; Kruszynska *et al.*, 2002; Kehlenbrink *et al.*, 2012), it is plausible that the elevation in NEFA during an extended morning fast contributes to the exaggerated insulin and/or glucose response following the lunchtime meal. However, our exploratory analysis revealed no association between pre-lunch NEFA concentrations and plasma glucose or insulin iAUC (**Figure 7.6**).

Appetite ratings following ingestion of breakfast were similar regardless of protein content. This is perhaps surprising given the established satiating effects of isoenergetic protein relative to carbohydrate but is consistent with previous breakfast literature (Allerton *et al.*, 2016). Conversely, subjective appetite rose across the morning during the extended morning fast – consistent with previous literature (Chowdhury *et al.*, 2015; Allerton *et al.*, 2016; Chowdhury *et al.*, 2016b). Despite differences in appetite between breakfast omission and consumption (regardless of composition), energy intake during the *ad libitum* lunch was almost identical between all conditions such that total morning energy intake (i.e., breakfast + lunch) was higher. Lack of difference in energy intake between carbohydrate-rich and whey protein-enriched breakfasts may be explained by the time between breakfast and lunch ingestion. Whilst the 3-hour post-prandial period following breakfast ingestion is more reflective of free-living conditions, this duration may have been too long for the previously established effects of protein content of a preload on *ad libitum* energy intake to carry-over. Astbury *et al* (2010) observed a clear dose-response effect of a protein preload on energy intake during a subsequent meal 90-minutes later, which is almost half the post-prandial duration of the current study. Chungchunlam *et al* (2012) also reported an effect of whey protein on energy intake relative to other preloads regardless of whether this was 30, 90, or 120 mins before assessing energy intake, however this is still 1 hour shorter than the post-breakfast period in the current study during which time the effects of added protein on appetite may have deteriorated.

Despite differing in protein content, dietary induced thermogenesis did not differ between breakfasts. Energy expenditure increased to a similar extent following ingestion of breakfast whilst remaining stable across the morning in the extended morning fasting condition. Dietary induced thermogenesis was similar between trials

(~19 *vs* ~24 kcal·180 min in the carbohydrate-rich and protein-enriched conditions, respectively). The value for the carbohydrate breakfast is at the lower end of the ranges of values reported by Westerterp (2004b) (Carbohydrate: 5-10 %, Fat 0-3 %, Protein 20-30 %). The measured increase in thermogenesis following the protein-enriched breakfast is lower than even the bottom end of this range (Westerterp, 2004b). This is surprising given that whey protein appears to have a relatively higher thermic effect relative to casein and soy (Acheson *et al.*, 2011).

Ingestion of breakfast meals differing in protein and carbohydrate content following an overnight fast resulted in similar metabolic substrate oxidation. Specifically, RER increased in response to both breakfasts, reflective of an increase in carbohydrate oxidation, before gradually decreasing across the morning. In contrast, substrate selection did not vary across the morning during the extended morning fasting, reflecting a greater contribution of lipids to fuel metabolism during this period (Frayn, 1983). No difference was observed in RER post-lunch between trials; however, the pattern of response is interesting to note between the three conditions. Whilst RER peaked 60 mins post-lunch at the group level, RER continued to increase through to 120 mins post-lunch in both the extended morning fast and protein-enriched conditions. Whilst the lack of difference in post-breakfast plasma urea is initially surprising given the difference in protein content between the 2 breakfasts, data from Witard *et al.* (2014) suggests that protein oxidation (and therefore plasma urea concentrations) is unlikely to differ between breakfasts containing 10 *vs* 20 g and may only increase when protein is fed in doses above 40 g. However, the lack of difference in plasma urea may therefore suggest that protein oxidation is not contributing towards any differences in RER across the post-breakfast period, although urine was not collected in the current study so could not be analysed for urea to conclusively state this.

The current data must be considered in light of the recruitment of both male and female participants. Whilst there are apparent sex differences in glucose metabolism and appetite regulation (Varlamov *et al.*, 2014; Bédard *et al.*, 2015), it is unclear whether or not there are sex differences in protein metabolism (Tipton, 2001; Markofski & Volpi,

2011). However, with 8 male and 4 female participants, the current study is clearly not suitably powered to detect any sex differences in the effects of enriching the breakfast meal with protein.

In summary, consumption of breakfast reliably elicits the second-meal effect, which can be achieved equally with consumption of either a typical carbohydrate-rich porridge breakfast, or an energy matched porridge breakfast enriched with whey protein isolate. Interestingly, this occurred despite a lower immediate glycemic response to the whey protein-enriched breakfast. However, despite the established metabolic effects no additional effect of whey protein upon appetite was observed.

Chapter 8 – Effect of regular high-protein breakfast on free-living energy balance and associated health outcomes

8.1 Introduction

The majority of adults in the UK report eating breakfast on a daily basis (Reeves *et al.*, 2013). However, relative to other eating occasions across the day, the breakfast meal tends to be relatively low in protein and rich in carbohydrates (NHANES, 2016). The data reported in Chapter 4 contrasted the acute (i.e., minutes; hours) metabolic and behavioural responses to enrichment of a typically carbohydrate-rich breakfast with whey protein. It is now necessary to establish how these effects translate to a longer-term free-living context.

Relative to the complete omission of breakfast (i.e., zero energy intake until midday), ingestion of a carbohydrate-rich breakfast can result in higher physical activity thermogenesis (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Higher glycaemic responses to breakfast relative to extended fasting were parallel with elevated physical activity thermogenesis in previous studies, consistent with the possibility that greater exposure to exogenous carbohydrates might drive spontaneous increase in energy expenditure (Betts *et al.*, 2014; Smith *et al.*, 2017). However, the breakfast consumed in previous studies was both carbohydrate-rich and very large, which has implications for both the effect on glucose and net energy balance. Therefore, it would be valuable examine the effects of breakfasts that are less energy and carbohydrate dense to achieve a better balance of outcomes (i.e., higher physical activity, with attenuated glycaemia and lower energy intake). This can be achieved through manipulation of the protein and carbohydrate content of this meal. Protein-enrichment of a carbohydrate-rich meal may delay gastric emptying, thereby protracting the insulin response and potentially exerting lasting effects on both glucose appearance and disposal (Swaminathan *et al.*, 1985; van Loon *et al.*, 2000; Manders *et al.*, 2006a; Betts & Williams, 2010a; Stanstrup *et al.*, 2014). Furthermore, supplementation of the diet with protein stimulates resting thermogenesis as measured over 24 h in a metabolic chamber therefore further accentuating elevated energy expenditure (Bray *et al.*, 2012). Finally, the established satiating effects of protein relative to other dietary macronutrients, may help with appetite control at subsequent meals (Astbury *et al.*, 2010; Leidy *et al.*, 2013). Collectively, enrichment of the breakfast meal with whey

protein may favour negative energy balance alongside reduced glycaemia. However, it is remarkable that so few studies have studied the chronic effects of high-protein breakfasts on energy balance and associated health outcomes.

The majority of protein distribution research focusses on tissue turnover especially in response to exercise stimuli. The current evidence around the metabolic and behavioural effects of daily consumption of a high protein breakfast, however, remains unclear. Two weeks of higher protein intake at breakfast had no apparent effect on non-breakfast energy intake, fasting glucose or insulin, nor did it affect postprandial responses of these biomarkers in overweight adults (Amankwaah et al., 2017). Supplementing both breakfast and lunch with a milk-based protein matrix for 24 weeks resulted in a more balanced distribution of protein intake across the day, accompanied by an increase in lean tissue mass in older individuals (Norton *et al.*, 2016). However, it is difficult to isolate the effects of protein at breakfast when the lunch meal was also supplemented. There is an apparent need therefore, to establish the effects of breakfast protein content on the major components of energy balance, and associated metabolic health outcomes.

To this end the aim of this study was to establish the short/mid-term (i.e., days; weeks) free-living behavioural and metabolic responses when introducing of daily protein-enriched breakfasts relative to isocaloric carbohydrate-rich breakfasts or to complete omission of morning meals.

8.2 Methods and Materials

8.2.1 Approach to the research question

As described in Chapter 7, whey protein isolate was selected on the basis that whey (especially isolate) exhibits a relatively short gastric emptying time and thus rapid hyperaminoacidemia, with potent effects on both insulin secretion (Hall *et al.*, 2003; Ma *et al.*, 2009; Smith *et al.*, 2021) and diet-induced thermogenesis (Acheson *et al.*, 2011; Bray *et al.*, 2015). The dose of protein enrichment selected (15 g) was based on acute laboratory trials showing addition of 15 g of protein to an already protein-rich meal reduces energy intake at the next meal (Astbury *et al.*, 2010). Furthermore, an additional 15 g of protein at breakfast would result in a more even distribution of protein intake across the day (NHANES, 2016).

On this basis, both the *Carbohydrate-Rich* (7.3 mg CHO·kJ RMR^{-1}), and *Protein-enriched* breakfasts were identical to those fed in Chapter 7.0. As such, carbohydrate delivery, and therefore total energy content of this meal was fed relative to resting metabolic rate, therefore accounting for this variable as a driver of energy intake (Blundell *et al.*, 2012; Blundell *et al.*, 2015). Furthermore, feeding at this prescribed rate of carbohydrate delivery results in a total breakfast energy content that meets the commonly reported threshold definition that breakfast should constitute ≥20 % of daily energy intake (Timlin & Pereira, 2007).

Pre- and post-intervention visits were standardised such that all participants were fed the *Carbohydrate-rich* breakfast (7.3 mg CHO·kJ RMR^{-1}) on the basis that it provides a sufficient metabolic challenge for the assessment of post-prandial metabolic control. The lunch meal was identical to that described in Chapter 7.0, based on previous work to provide a sufficient challenge to glycaemic control in order to meaningfully compare post-prandial responses (Chowdhury *et al.*, 2015, 2016b).

8.2.2 Research Design

A parallel-arm independent measures randomised controlled trial was employed to contrast the effects of daily enrichment of breakfast with whey protein with a carbohydrate-rich breakfast and the total omission of breakfast. Specifically, all participants completed a 1-week energy balance monitoring phase prior to their randomisation in a 1:1:1 allocation stratified according to biological sex (male/female)

and habitual breakfast habits (consumer: ≥50 kcal within 2 hours of waking most days of the week; Skipper: <50 kcal within 2 hours of waking most days of the week) thus ensuring equal distribution of sex and breakfast habit (Betts *et al.*, 2011; Betts *et al.*, 2016). The randomisation plan was generated and managed by an individual who was not involved in participant recruitment, data collection or analysis. Participants completed a pre-intervention laboratory visit, after which they received their group allocation and adhered to the prescribed breakfast intervention for 28-d (with monitoring of free-living diet and energy expenditure during), before returning for the post-intervention visit. The three experimental conditions were as follows: daily consumption of a whey protein enriched breakfast (*Protein-enriched*), daily consumption of a Carbohydrate rich breakfast (*Carbohydrate-rich*), or the daily complete omission of breakfast (*Extended morning fast*) for 28 days.

All participants were informed of any potential risks and discomfort involved in the study prior to providing written and oral informed consent. The study was given a favourable ethical opinion by the Yorkshire & The Humber NHS research ethics committee (20/YH/0310), as well as the Research Ethics Approval Committee for Health (REACH) at the University of Bath (EP 17/18 260) and was registered on clinicaltrials.gov (NCT04417205).

8.2.3 Experimental Protocol

Following the provision of written informed consent, participant screening was undertaken through completion of a general health and validated chronotype questionnaires to assess habitual sleep patterns and diurnal preferences (Horne & Ostberg, 1976; Buysse *et al.*, 1989; Roenneberg *et al.*, 2003). Eligible participants then undertook the 5-week protocol depicted in **Figure 8.1**. All laboratory sessions took part at the University of Bath, before which participants were asked to refrain from caffeine, alcohol, smoking, and strenuous exercise for 24 hours. Participants reported to the laboratory at 0730 ± 0100 h in an overnight-fasted state (≥ 10 h) having consumed ~500 mL of water upon waking. Menstrual phase was standardised within female participants (i.e., tested at the same phase, regardless of phase) to obtain a heterogenous sample of female responses to the intervention.
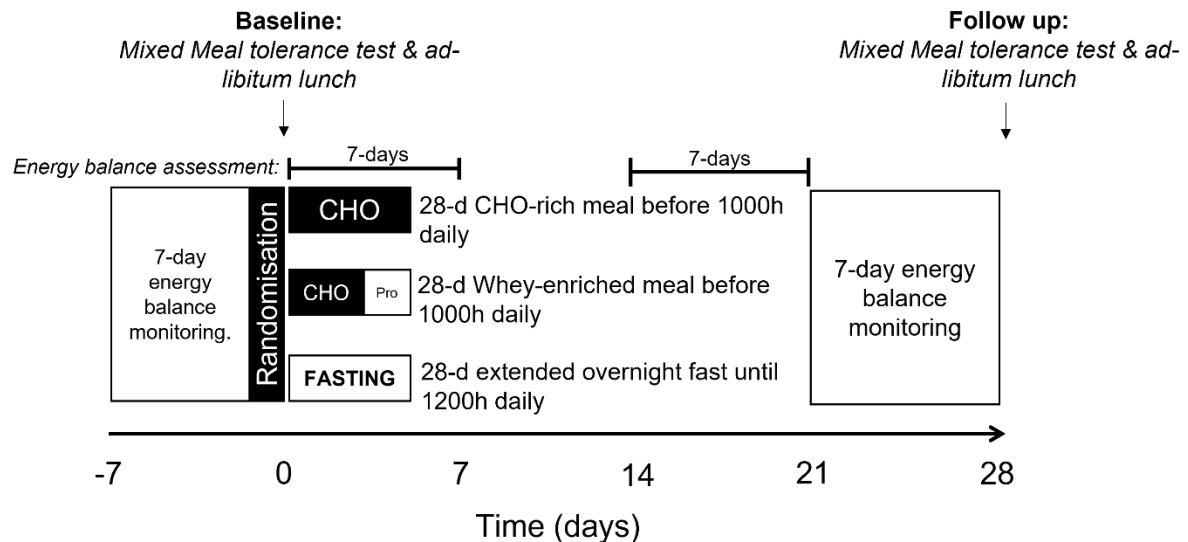
Figure 8.1 – Schematic of the 5-week randomised control trial study design.

8.2.3.1 Preliminary Laboratory Protocol

Prior to the one week of energy balance monitoring, participants visited the laboratory to undergo a short exercise test to calibrate the Actiheart™ monitor (as described in full detail in Chapter 3.8.2). Briefly, following an overnight fast, participants' resting metabolic rate was assessed; in accordance with best practice for measuring resting metabolic rate, participants rested in a semi-supine position (upper body at ~60°) prior to collection of 3 x 5 min expired breath samples via the Douglas bag method (Compher *et al.*, 2006). Participants then completed the 12-minute sub-maximal treadmill protocol described in Chapter 3.8.2.

8.2.3.2 Pre- and Post-Intervention Laboratory Protocol

Upon entering the laboratory for each visit, participant's body mass, height, waist:hip circumference, and sagittal-abdominal diameter was measured prior to assessment of resting metabolic rate and blood pressure. Following this, an intravenous cannula was placed, and a 10 mL blood sample was drawn before ingestion of a carbohydrate-rich breakfast (7.3 mg carbohydrate·kJ RMR^{-1}; 1127 ± 202 kJ, 73/12/15 % CHO/FAT/PRO).

A 3-hour postprandial period followed the ingestion of breakfast, with blood samples collected at 15-, 30- and 60-minute intervals during the first, second and third hour,

respectively, alongside hourly expired gas samples and assessment of subjective appetite and mood. Upon completion of the 3-hour post-breakfast period, participants were provided with a mixed-meal pasta lunch (penne pasta with a tomato and herb sauce as described in Chapter 7.2.6), with post-lunch blood samples being collected at 15- and 30-minute intervals during the first and second hour, respectively, alongside hourly subjective appetite and mood assessment.

Upon completion of the 28-day intervention, participants visited the laboratory at the University of Bath to repeat this visit.

8.2.3.3 Free-living Breakfast Intervention

Participants were randomised to one of three breakfast conditions for 28 days. These included:

1.   Carbohydrate-rich breakfast: Consumption of a carbohydrate-rich breakfast before 1200 h daily for 28-days. This provided 7.3 mg·kJ RMR^{-1} of carbohydrate per breakfast meal and was provided in pre-weighed Quaker porridge pots (**Table 8.1**).

2.   Protein-enriched breakfast: Participants were provided with and asked to consume a protein-enriched breakfast before 1200 h daily for 28-days. This provided ~6 mg·kJ^{-1} of carbohydrate per breakfast meal with an additional 15 g of whey protein to match the carbohydrate-rich breakfast for energy content. This was provided to participants in the form of pre-weighed golden syrup instant porridge pots (Quaker, UK) with 18.3 g of MyProtein whey isolate (**Table 8.1**) added (to achieve 15 g of total whey protein).

3.   Extended morning fast: Participants were asked to remain fasted (aside from water, and non-caloric decaffeinated drinks) until 1200h daily for 28-days.

During week 1 and week 4 of the free-living intervention participants were provided with equipment to monitor physical activity (Actiheart, CamnTech, UK; **Chapter 3.8.2**), as well as food scales (SmartWeigh), and were set up with a digital food diary application (Libro, Nutritics, Ireland) for assessment of free-living energy balance.

Table 8.1 – Macronutrient composition of the provided daily breakfast meals for each group

	Carbohydrate-rich	Protein-enriched
Energy (kJ)	1208 ± 227	1098 ± 181
Carbohydrate (kJ)	866 ± 154	577 ± 130
Sugars (kJ)	260 ± 52	205 ± 59
Fat (kJ)	165 ± 47	150 ± 23
Porridge Protein (kJ)	188 ± 45	126 ± 29
Whey Protein (kJ)	-	250
Total Protein (kJ)	188 ± 45	371 ± 28

8.2.4 Blood analysis

Blood samples were immediately transferred into tubes treated with EDTA or clotting activator beads (plasma and serum respectively; Sarstedt, UK) and were left to clot at room temperature for 15 mins prior to centrifugation (10 min, 4000 x *g*, 4°C), before plasma/serum supernatant was aliquoted into Eppendorf tubes and stored at -80°C for subsequent analysis. Plasma samples were analysed for glucose, non-esterified fatty acids (NEFA), urea, and insulin as described in Chapter 3.6.1 and 3.6.2.

8.2.5 Expired gas analysis

Expired gases were collected using 150 L Douglas bags, with concentrations of O_2 and CO_2 analysed in a known volume of sample, using paramagnetic and infrared analysers, respectively (Mini HF 5200; Servomex Group Ltd., Crowborough, UK). These analysers were also used to determine the composition of inspired air to adjust for variation in atmospheric O_2 and CO_2 concentrations (Betts & Thompson, 2012). Total volumes of expired gas were determined using a dry gas meter (Harvard Apparatus, Holliston, MA) and temperature measured using a digital thermometer (Edale Instruments, Longstanton, UK). Substrate utilisation was determined using the equations of Frayn (Frayn, 1983).

8.2.6 Appetite ratings

Paper-based visual analogue scales with 100-mm horizontal axes were employed to assess subjective appetite as described in **Chapter 3.7**.

8.2.7 Statistical Analysis

All analyses were performed using SPSS 25.0 (IBM) and Microsoft Excel. As described in Chapter 3.9, primary contrasts were examined using a two-way group x time mixed-model of variance (ANOVA), with experimental group (Group) as a between subjects factor and baseline/follow-up or habitual/intervention (Time) as a within subjects factor. For time series data (i.e., postprandial responses), a three-way ANOVA was employed to include timepoint as an additional within subjects factor (Group x Time x Timepoint). The Greenhouse-Geisser correction was adopted for Greenhouse-Geisser epsilon <0.75 and the Huynh-Feldt correction adopted for >0.75 (Greenhouse & Geisser, 1959). Significant interaction effects were followed up with multiple unpaired t-tests to identify the location of variance. Holm-Bonferroni stepwise corrections were then used to prevent inflation of the type 1 error rate by multiple comparisons (Ludbrook, 1998; Atkinson, 2002). Based on a previously observed difference in mean energy expenditure of 442 kilocalories between participants either consuming or not consuming breakfast, 18 participants per group (3 groups) would be sufficient to achieve >85% power with an alpha level of 0.05 between groups consuming energy for breakfast (e.g., carbohydrate/protein) and those not consuming breakfast (i.e., water) (Betts et al., 2014).

8.3 Results

8.3.1 Participants

Baseline characteristics of the participants in each intervention group are reported in **Table 8.1**. A total of 121 potential participants expressed interest in the study. Of those to express interest, 52 were screened for eligibility, 46 were deemed eligible to participate and 42 provided informed consent. Of these participants, 39 completed the baseline laboratory session. After randomisation, 5 participants withdrew from the study (citing discomfort with the activity monitor, illness x 2, no reason, unwilling to go ahead with the intervention). Thirty-four men and women participated in the study (**Table 8.1**). Exclusion criteria included body mass index outside of the range of 18.5-29.9 kg·m^{-2}, any diagnosed metabolic disease (e.g., type 1 or type 2 diabetes), any reported use of substances or behaviours (e.g., non-standard sleep–wake cycle, shift workers) that may pose undue personal risk or introduce bias into the experiment. Of the 21 participants to be allocated to receive breakfast for 28 d, 8 reported that they felt able to identify which breakfast they received. Of those 8, only 3 correctly identified which group they had been allocated to.

Table 8.2 – Participant characteristics of the study cohort. Data are presented as mean ± SD.

Characteristic	Extended morning fast	Carbohydrate-rich	Protein-enriched	Combined
Sex (M/F)	4/9	4/7	3/7	34 (11/23)
Age (y)	36 ± 06	35 ± 14	33 ± 18	35 ± 16
Height (m)	1.72 ± 0.08	1.68 ± 0.10	1.67 ± 0.10	1.70 ± 0.09
Body Mass (kg)	65.9 ± 8.0	70.6 ± 8.3	67.0 ± 12.8	67.7 ± 9.6
Body Mass Index (kg·m^{-2})	22.2 ± 2.2	24.9 ± 2.6	23.8 ± 3.5	23.6 ± 2.9
Resting Metabolic Rate (kcal·day^{-1})	1554 ± 233	1702 ± 345	1546 ± 275	1599 ± 286
Resting Metabolic Rate (kcal·kg^{-1}·day^{-1})	24 ± 2	22 ± 8	23 ± 3	23 ± 5
Average sleep duration (hh:mm)*	08:22 ± 00:25	08:00 ± 00:52	08:24 ± 00:34	08:13 ± 00:42
Midsleep time (hh:mm)*	03:55 ± 00:36	03:48 ± 00:24	03:51 ± 00:26	03:50 ± 00:24
Horne-Östberg Score	60 ± 10	57 ± 10	56 ± 8	58 ± 9
Pittsburgh Sleep Quality Index	4 ± 2	6 ± 3	6 ± 1	6 ± 2
Epworth Sleepiness Scale	3 ± 3	6 ± 4	2 ± 3	4 ± 4

*Determined from the Munich Chronotype Questionnaire (Roenneberg *et al.*, 2003)

8.3.2 Anthropometry

Four weeks of extended morning fasting (-0.5 ± 1.3 kg), daily consumption of carbohydrate-rich breakfast (-0.1 ± 1.4 kg), or protein-enriched breakfast (-0.2 ± 1.4 kg) did not result in a significant change in body mass (Group x Time $p = 0.68$) (**Table 8.3**). Waist:Hip ratio was also similar between groups at baseline and follow-up (**Table 8.3**)

Table 8.3 – Anthropometric responses to breakfast-based interventions. Data are Mean ± SD.

	Extended morning fast		Carbohydrate rich breakfast		Protein enriched breakfast		*P* value		
	Pre	ΔPost	Pre	ΔPost	Pre	ΔPost	Time	Group	Interaction
Body mass (kg)	65.9 ± 8.0	-0.5 ± 1.3	70.6 ± 8.3	-0.1 ± 1.4	67.0 ± 12.8	-0.2 ± 1.4	0.27	0.46	0.68
Body mass index (kg·m^{-2})	22.2 ± 2.2	-0.2 ± 0.5	24.9 ± 2.6	-0.02 ± 0.5	23.8 ± 3.5	-0.1 ± 0.5	0.41	0.06	0.69
Waist circumference (cm)	76.3 ± 11.2	-1.1 ± 3.8	82.2 ± 9.7	-1.1 ± 4.8	79.4 ± 11.2	-0.1 ± 3.0	0.28	0.34	0.78
Hip circumference (cm)	97.1 ± 5.0	-0.5 ± 1.9	99.8 ± 5.5	-1.1 ± 3.8	98.9 ± 6.9	-0.8 ± 3.0	0.11	0.60	0.88
Waist:Hip (cm:cm)	0.79 ± 0.06	-0.01 ± 0.04	0.82 ± 0.06	0.00 ± 0.05	0.80 ± 0.07	0.00 ± 0.04	0.89	0.35	0.83
RMR (kcal·d^{-1})	1554 ± 233	28 ± 172	1702 ± 345	51± 252	1546 ± 275	13 ± 134	0.99	0.54	0.50
RMR (kcal·kg^{-1}·day^{-1})	24 ± 2	0.7 ± 2.6	22 ± 8	0.3 ± 1.8	23 ± 3	-0.6 ± 3.3	0.73	0.87	0.33

RMR = Resting metabolic rate.

8.3.3 Energy Balance

8.3.3.1 Energy Intake

Allocation to consume breakfast daily resulted in a reduction in energy intake from habitual levels in contrast to extended morning fasting (Time *p* = 0.02). Reported average daily energy intake when extending the morning fast to 1200 h each day was 2130 ± 546 kcal·d^{-1} in contrast to daily intakes of 1980 ± 545 kcal·d^{-1} and 1809 ± 238 kcal·d^{-1}, with daily consumption of a carbohydrate rich and protein enriched breakfasts, respectively (**Figure 8.2A**).

A
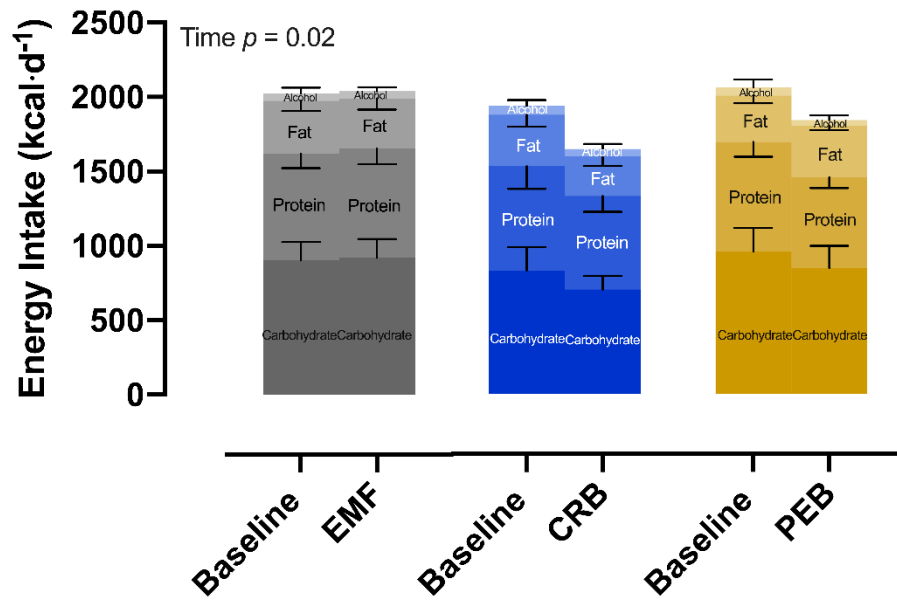
B
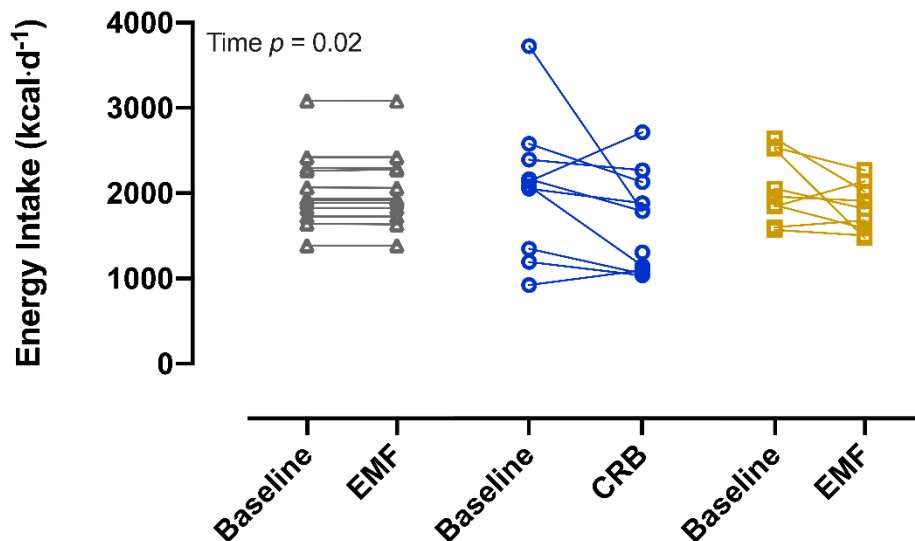
Figure 8.2 – A) Energy and macronutrient intake at baseline and during the intervention phases in the extended morning fast, carbohydrate rich, and protein enriched breakfasts. B) Individual energy intake responses. Energy was derived from the respective macronutrients calculated by multiplying the reported intake in grams by the accompanying Atwater general factor. Ns denotes $p > 0.05$ for Condition x Time effects.

8.3.3.2 Energy Expenditure

8.3.3.2a Physical activity thermogenesis

Objectively assessed daily physical activity thermogenesis during extended morning fasting condition was (1261 ± 822 kcal·d^{-1}), compared to carbohydrate-rich (1264 ± 341 kcal·d^{-1}) and protein-enriched (1061 ± 470 kcal·d^{-1}) breakfasts (Group p = 0.51) and was similar to baseline (Time p = 0.06). As such, group allocation did not alter physical activity thermogenesis from baseline (**Figure 8.3B**) (Group x Time p = 0.95).

8.3.3.2b Dietary induced thermogenesis

Using established constants for the thermogenic effect of reported macronutrient intake as assessed by food diaries, there was no difference in daily DIT between *extended morning fasting* (174 ± 25 kcal·d^{-1}), *carbohydrate rich breakfast* (167 ± 31 kcal·d^{-1}) or *protein enriched breakfast* (167 ± 13 kcal·d^{-1}) (Condition p = 0.57). DIT was therefore not altered by allocation to any breakfast intervention (Condition x Time p = 0.14).

8.3.3.2c Resting metabolic rate

Resting metabolic rate assessed at follow up revealed no difference in response to extended morning fasting (1604 ± 238 kcal·d^{-1}), carbohydrate-rich (1656 ± 336 kcal·d^{-1}) and protein-enriched breakfasts (1559 ± 311 kcal·d^{-1}) (Condition x Time p = 0.50) (**Figure 8.3A**). This can also be expressed relative to body mass, to provide an indication of adaptive thermogenesis, however this equally shows no change in response to the breakfast-based interventions (**Table 8.3**).
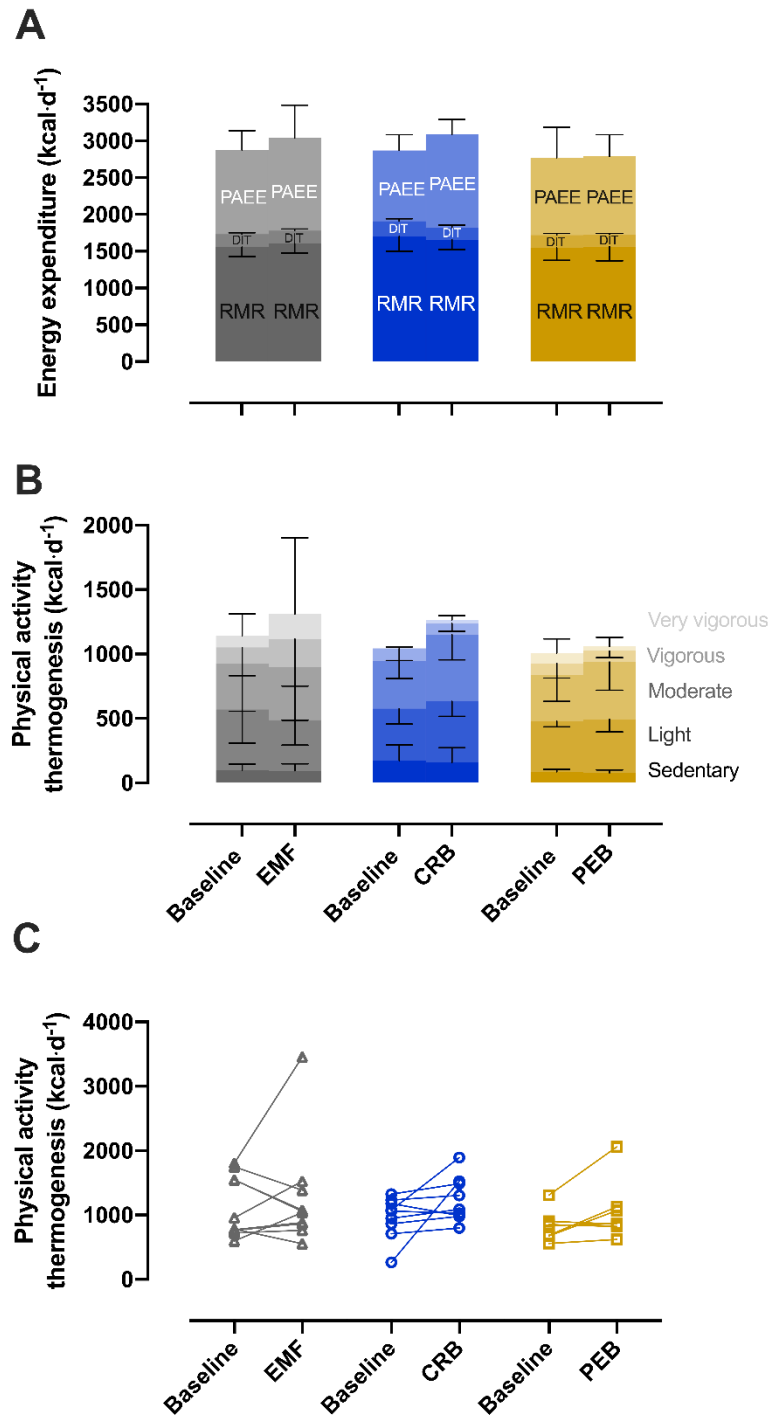
Figure 8.3 – A) Major components of total daily energy expenditure at baseline and in response to each of the three intervention arms. B) Physical activity thermogenesis and component intensity thresholds at baseline and in response to each of the three intervention arms. C) Morning-afternoon physical activity thermogenesis between conditions. Ns denotes $p > 0.05$ for Condition x Time effects.

8.3.4 Postprandial Metabolism

None of the breakfast-based dietary interventions differentially affected fasting or postprandial responses of glucose, non-esterified fatty acids, or insulin (Condition x Time x Timepoint $p > 0.05$) (**Figure 8.4**). Fasting triglycerides were higher at follow up in the protein enriched breakfast group (0.96 ± 0.30 mmol·L^{-1}) relative the extended morning fasting group (0.51 ± 0.27 mmol·L^{-1}) and remained higher during the first 90 minutes of the breakfast period (Condition x Time x Timepoint $p = 0.004$). Energy expended over the 3-h within laboratory post-breakfast period was similar between baseline and follow up in all groups (Group x Time $p = 0.30$) (**Figure 8.5**). Likewise, the rate of carbohydrate oxidation (Group x Time $p = 0.41$), lipid oxidation (Group x Time $p = 0.28$), and protein oxidation (Group x Time $p = 0.63$) that contribute to energy expenditure over this period, were all similar at follow up to baseline and did not differ between groups.
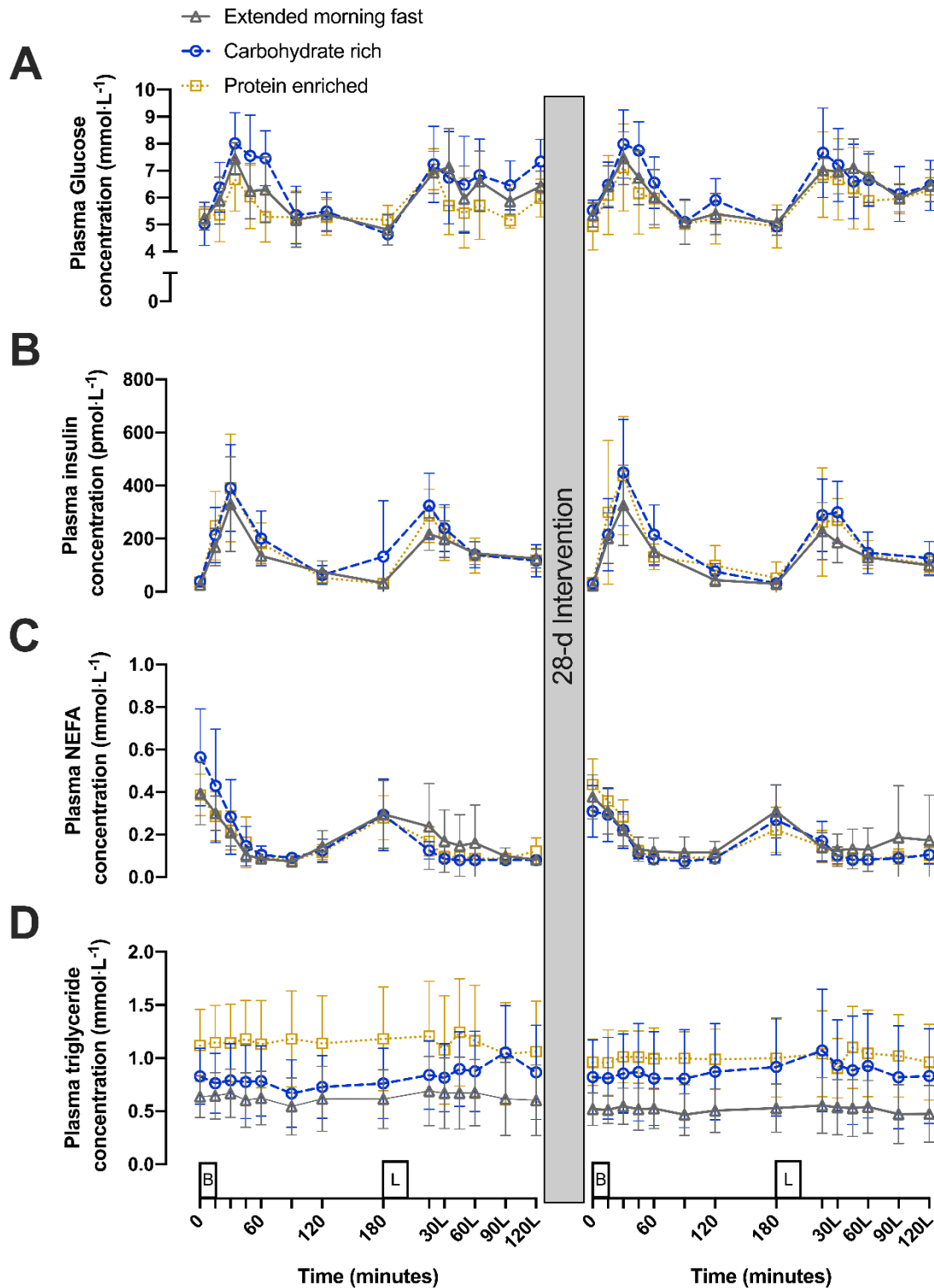
Figure 8.4 - Postprandial A) glucose B) insulin C) non-esterified fatty acids D) triglyceride responses to standardised breakfast and lunch at baseline (left side of panel) and at follow up (right side of panel). "a" denotes difference between protein enriched and extended morning fasting group concentrations ($p \le 0.05$).
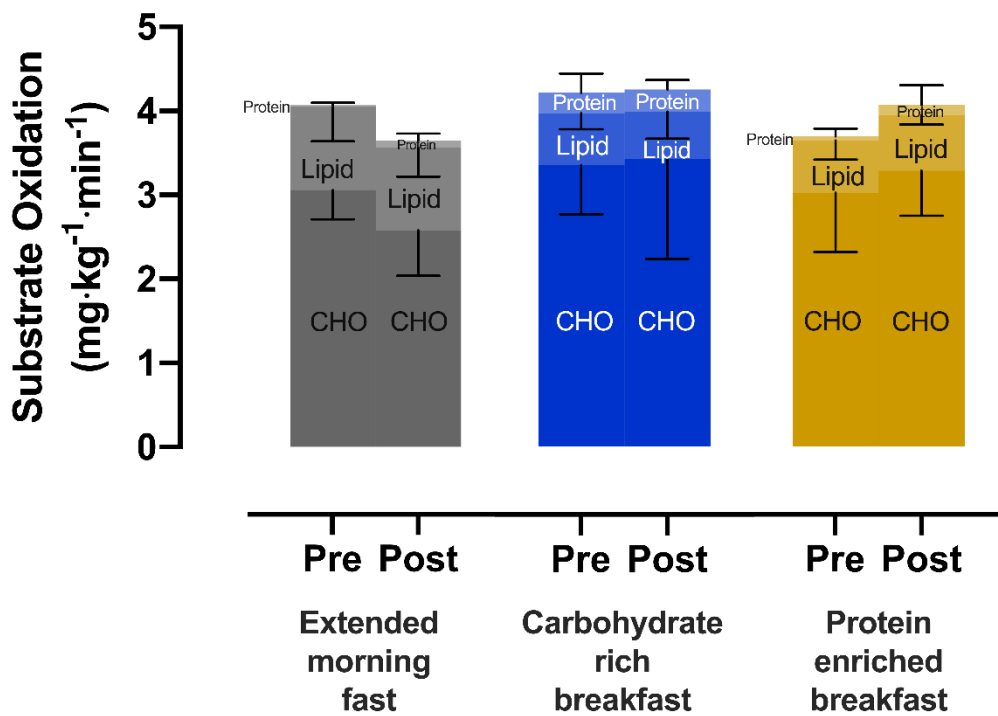
Figure 8.5 – Average substrate oxidation responses to the within laboratory standardised breakfast meal before and after 4-weeks of extended morning fasting, carbohydrate rich breakfast, or protein enriched breakfast.
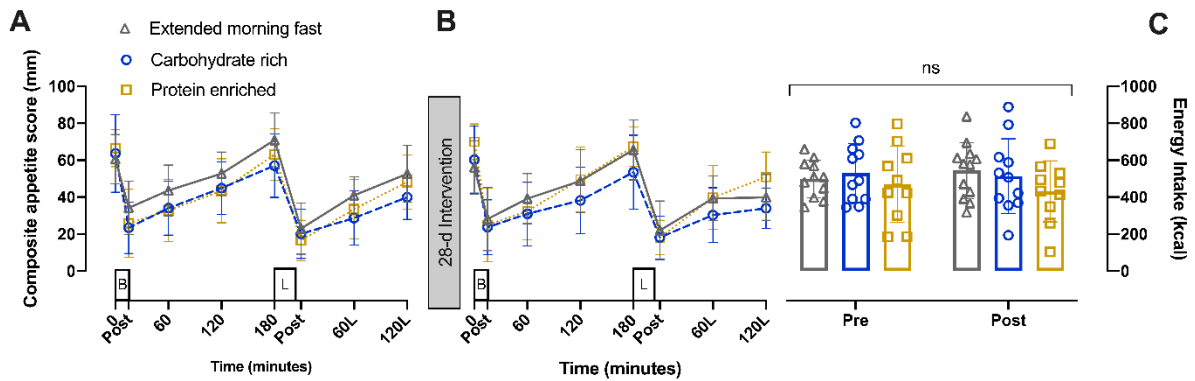
Figure 8.6 – A) Within laboratory composite appetite responses to the standardised breakfast and lunch meals at baseline and B) at follow up. C) Energy intake at the *ad libitum* test lunch. Ns denotes *p* > 0.05 for Condition x Time effects.

8.3.4 Appetite

Neither daily consumption of a carbohydrate rich breakfast or protein enriched breakfast altered postprandial appetite to sequential standardised meals relative to daily omission of breakfast (Condition x Time x Timepoint *p* = 0.89) (**Figure 8.6A**). Equally, energy intake during the *ad libitum* lunch meal did not change in response to daily extended morning fasting (41 ± 108 kcal), daily carbohydrate rich breakfast (-17 ± 128 kcal), or daily protein enriched breakfast (-22 ± 100 kcal) (Condition x Time *p* = 0.26) (**Figure 8.6B**).

8.4 Discussion

This is the first study to report all major components of energy balance with daily consumption of a protein enriched breakfast relative to carbohydrate rich breakfast and daily extended morning fasting, measured under free-living conditions. Paradoxically and contrary to previously published research, the prescription of both breakfasts resulted in a reduction in total energy intake, such that less energy was consumed in those conditions relative to when no breakfast at all was consumed. However, neither breakfast consumption nor the composition of the breakfast affected physical activity thermogenesis, body mass changes, or postprandial metabolic responses.

This was surprising considering that a relative wealth of literature has consistently shown no or incomplete compensation of energy intake in response to skipping breakfast, therefore resulting in lower energy intake across the day (Levitsky & Pacanowski, 2013a; Betts *et al.*, 2016; Yoshimura *et al.*, 2017; Edinburgh *et al.*, 2019; Sievert *et al.*, 2019). This is an intuitive effect, as if breakfast is omitted with incomplete compensation later in the day, then any given individual skipping breakfast will be consuming less than if they had consumed breakfast. The use of self-reported energy intake does lend itself to bias, with previous reports of participants under-reporting total energy intake by ~34 % (Whitton *et al.*, 2011). However, even if this was the case in the current study, this bias could be assumed to be consistent across groups. Furthermore, previous studies using the same tools to assess energy intake have demonstrated a clear effect of skipping breakfast suggest that the reduction in energy intake with daily consumption of breakfast is likely not to be driven by under-reporting (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Looking at the individual responses in Figure 8.2 it appears the reduction in energy intake resulting from carbohydrate breakfast is fairly consistent for the majority of participants, although perhaps primarily driven by a couple of individuals. In contrast the effect of protein enriched breakfast on energy intake, while numerically smaller than that in the carbohydrate rich breakfast, appears to be more consistent between individuals.

Therefore, assuming no type I (i.e., false positive) or type II (i.e., false negative) error, there could be several plausible explanations for the effects of breakfast on energy intake observed presently. Whey protein was chosen to enrich the daily breakfast for its established satiating effects, however consistency in energy intake responses

between the carbohydrate rich and protein enriched breakfasts suggest that this is not the case (Westerterp-Plantenga *et al.*, 1999a; Astbury *et al.*, 2010). Speculatively, the daily consumption of a warm porridge breakfast at a time normally associated with a cold meal may have altered appetite across the afternoon as cold foods are expected to be less satiating than hot foods (Baskentli *et al.*, 2021). What is perhaps more likely is that the size of the breakfast meal was sufficient to reduce energy intake across the remainder of the day (from 1200 h onwards) with little or no compensation for the relatively smaller breakfast prescribed compared to previous studies. This is conceivable as there seems to be a relationship between calorie distribution and daily appetite whereby greater energy intake in the morning suppresses appetite across the day (Ruddick-Collins *et al.*, 2022). Whilst this has been demonstrated with breakfasts of different sizes, the principle very much could apply to the current scenario whereby the comparison is no energy intake relative to consumption of a breakfast containing ~250-300 kcal.

The lack of effect of daily extended morning fasting on physical activity thermogenesis directly contradicts the established relationship between fasting and physical activity (Betts *et al.*, 2016; Templeman *et al.*, 2021a). Specifically, breakfast consumption appears to result in higher reported and objectively assessed physical activity in comparison to breakfast skipping (Wyatt *et al.*, 2002; Corder *et al.*, 2011; Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). A proposed mechanism for this effect is the glycaemic excursion in response to consumption of breakfast (relative to a stable concentration of plasma glucose with fasting). However, introduction of protein to the breakfast meal reduces postprandial glycaemia (Chapter 7) compared to the carbohydrate rich breakfast yet no difference in the physical activity responses between these groups was apparent. When looking at the spread of the data presented in Figure 8.3C it is apparent that some individuals physical activity decreased in response to fasting, balanced by no change or slight increase in physical activity. One participant however displayed an incredibly large change in physical activity levels in response to fasting however the group level physical activity response to extended morning fasting persists even when their data are excluded. It must also be considered whether it is the absence of food (i.e., fasting) that reduces physical activity thermogenesis and/or the prescription of food (i.e., breakfast) that increases it. Previous studies provide proof

of concept that a matched morning fasting duration over 6 weeks results in a reduction in energy expenditure from baseline (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Furthermore, a longer fasting period appears to result in bigger reduction in physical activity, especially when fasting occurs during the afternoon (Templeman *et al.*, 2021a). This could suggest that in order to observe an effect on physical activity fasting periods may have to be for a longer duration, or longer period each day and/or in the afternoon too to see clear effects. Finally, it is interesting to consider the effects of baseline physical activity on the response to fasting. Habitual physical activity levels in the extended morning fasting group were 1144 ± 485 kcal·d^{-1} which was relatively low in comparison to Betts *et al.* (2014) in which the clear effect of fasting on physical activity thermogenesis was observed. Interestingly this same effect was apparent but less clear in individuals with lower habitual physical activity, hinting at a possible floor effect of fasting on physical activity responses (Chowdhury *et al.*, 2016b; Templeman *et al.*, 2021a).

The time effect observed in DIT is most likely a reflection of the reduction in energy intake observed within both breakfast group, especially in the carbohydrate-rich breakfast condition. Whilst it was hypothesised that enrichment of a typically carbohydrate rich meal would increase morning DIT, it appears that even if this was true, this did not amount to a change in total energy expenditure. Furthermore, Lack of change in resting metabolic rate reflects the stability in body mass from baseline to follow up but is also consistent with the effects of fasting/breakfast reported previously (Betts *et al.*, 2014). Expression of RMR relative to body mass showed a similar response, which in the context of the current study, suggests that neither protein content of the breakfast meal, or fasting, resulted in adaptive thermogenesis. Collectively, the measurement of the major components of energy balance during 4-weeks of daily extended morning fasting, carbohydrate-rich breakfast, or protein-enriched breakfast reveal no large or consistent changes in energy balance, as reflected in the similarity in body mass responses between groups.

If there is any effect of fasting, breakfast, or protein at breakfast on postprandial metabolism it was not present in this study. Our data therefore extends from previous

studies showing that there is no metabolic adaptation in response to skipping or consuming breakfast by showing that increased protein and decreased carbohydrate intake at breakfast does not alter this relationship (Neumann *et al.*, 2016; Amankwaah *et al.*, 2017; Chowdhury *et al.*, 2018; Chowdhury *et al.*, 2019). The observed effect in follow up postprandial triglycerides should be interpreted with caution due to the high degree of variability apparent in measured values. The remarkably similar time course responses to standardised breakfast and lunch meals before and after 28 days of extended morning fasting necessarily reflects the lack of change in body mass as a result of an absence of large or consistent effects in the major components of energy balance, especially physical activity (Balkau *et al.*, 2008; Clamp *et al.*, 2017).

Recruitment of both male and female participants in the current study necessarily requires acknowledgement of potential sex differences in responses to breakfast-based interventions. Previous research has identified sex differences in glucose metabolism and appetite regulation (Varlamov et al., 2014; Bédard et al., 2015). However, it is unclear the extent to which sex differences in protein metabolism exist (if at all) (Tipton, 2001; Markofski & Volpi, 2011). Aside from these metabolic differences, females tend to be more inactive and participate in less moderate-to-vigorous physical activity compared to males (Khuwaja & Kadir, 2010; Silva *et al.*, 2014). However, intervention groups were stratified on biological sex such that distribution of males and females was equal across groups, to avoid sex differences driving any effects between groups. Furthermore, as sex differences were not a primary experimental focus, this study was not appropriately powered to detect sex differences (if any existed).

The absence of previously established effects of breakfast and fasting on the component energy balance is a common theme in the data reported here. The most consistent query arising from these data relate to the effects of the energy content of the breakfast meal consumed daily. Whilst providing a less stark contrast between fasted and fed states in terms of total energy, this is perhaps reflective of a more subtle yet realistic change (i.e., people could feasibly expect to consume a slightly smaller breakfast instead of skipping breakfast all together). Further consideration should be

paid to the duration and/or timing of the fasting period in driving compensatory changes in physical activity thermogenesis, providing focus for future work.

In summary, our study shows that daily consumption of either a protein enriched, or carbohydrate rich breakfast did not result in large or consistent changes in any component of energy balance compared to extended morning fasting. The absence of detectable effects may be explained by the relatively modest energy content of the breakfast, which was more reflective of typical breakfasts compared with prior research, therefore providing greater external validity.

Chapter 9 – General Discussion

9.1 Recap of thesis aims and objectives

The aim of the work described in this thesis was to adopt a multi-faceted approach to studying the metabolic and behavioural effects of meal timing, with focus on both absolute timing (i.e. when nutrients arrive in relation to clock time) and relative timing (i.e. when nutrients arrive in relation to other inter-related events across the day). With this in mind, the following objectives were outlined:

1. Characterise the diurnal hormonal and metabolite rhythms in the context of time-series skeletal muscle gene expression.
2. Contrast the metabolic response to two distinct temporal nutrient delivery patterns over 24-h.
3. Determine whether hourly sleep disruption influences next morning metabolic control.
4. To establish the acute (hours; day) metabolic response to sequential feedings when enriching a typical breakfast with whey protein.
5. To establish the short/mid-term (days; weeks) free-living behavioural and molecular responses when introducing daily protein-enriched breakfasts.

This series of studies has revealed underlying concurrent rhythmicity in skeletal muscle metabolic regulation and systemic metabolic/endocrine markers and established the influence of contrasting enteral feeding patterns on these rhythms. Furthermore, this work shows no effect of hourly sleep fragmentation upon next day glycaemic control. Finally, exploration of the effects of enriching the breakfast meal with protein reveals that protein-enriched breakfasts are just as effective as a typical carbohydrate-rich breakfast at eliciting the second meal effect. However, this does not confer any beneficial metabolic adaptations in metabolic control, nor does it meaningfully alter energy balance when this is prescribed over a 28-d period (**Figure 9.1**)
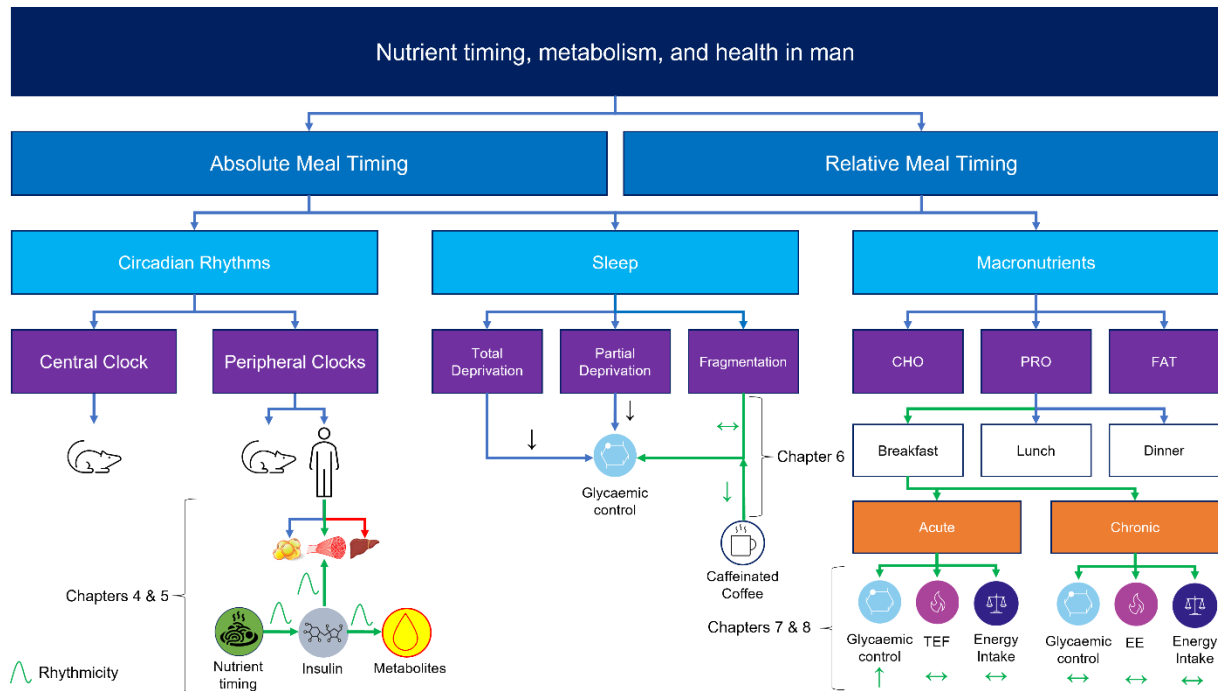
Figure 9.1 – Updated overview of the main themes and objectives of the thesis along with graphical depictions of knowledge gained from the work described in this thesis. Chapters 4 & 5 focused on peripheral tissue and metabolite responses to changes in absolute meal timing. Chapter 6 combined absolute and relative meal timing. Chapters 7 and 8 focused more specifically on relative meal timing and macronutrient composition. Green arrows denote contributions of the research described in this thesis. Horizontal green arrows denote no effect of a given intervention. Downward green arrows represent a negative effect and upward green arrows denote a positive effect. Red arrows represent areas that are unknown in humans. Blue arrows denote knowledge from previous literature.

9.2 Collective findings in context and novelty

The data in Chapter 4.0 were the first round the clock measures of skeletal muscle metabolism in humans. Simultaneous collection of blood samples therefore provided further novelty and has improved our knowledge of 24 h tissue and systemic metabolic regulation. Most interestingly rhythmicity in genes relating to carbohydrate, and lipid metabolism in the muscle logically aligned with respective circulating markers of nutrient metabolism (e.g. insulin, glucose, NEFA). However, whilst a semi-constant routine allows for study of the diurnal influences of cycles in sleep-wake and fasting-feeding, an inherent limitation of the protocol design using oral nutrition is that nutrients cannot then be provided during the night whilst participants were sleeping. This in turn limited the ability to conclude whether observed rhythms were driven endogenously or exogenously. The use of enteral feeding in Chapter 5 therefore built upon the design of the study in Chapter 4 by allowing for provision of nutrition over 24 h, including through the night during sleep. An additional contrast (i.e., Bolus feeding) was also included in Chapter 5 to examine the metabolic responses between the minimum frequency of feeding whilst ensuring evenly balanced delivery of nutrients across 24 h (i.e. two feeds at 12-h intervals), relative to the maximal possible frequency of nutrient delivery (i.e. continuous delivery of nutrients).

Daily profiles of plasma glucose were consistent between Chapters 4 & 5, regardless of feeding patterns (**Figure 9.2A**). Briefly, plasma glucose was stable throughout the day, with rhythmic peaks occurring overnight, consistent with previous research previous studies employing circadian misalignment, constant routine, and forced desynchrony - therefore providing further evidence for robust control of plasma glucose by the endogenous circadian system (Van Cauter *et al.*, 1989; Van Cauter *et al.*, 1991; Van Cauter *et al.*, 1992; Qian & Scheer, 2016). Interestingly, whilst the broad rhythm was similar between feeding regimes, variation in the timing of peak glucose was apparent. Bolus enteral feeding advanced the acrophase of the rhythm in plasma glucose relative to both continuous feeding and when nutrition was limited to waking hours. Collectively this adds to evidence that modifying the pattern and/or timing of nutrient intake alters the temporal profile of plasma glucose (**Figure 9.2A**) (Wehrens *et al.*, 2017a; Gu *et al.*, 2020).

Consistency between the rhythmicity in expression of genes relating to glucose metabolism with the observed rhythm in systemic insulin adds to our knowledge of the

role of circadian rhythms in facilitating daily fluctuations in glucose control (Perrin *et al.*, 2018b). Peak *GLUT4, PPARGC1A* and *HK2* expression in skeletal muscle coincided with rising/peak insulin concentration and were lowest when insulin also reached its nadir. Collectively, alignment of rhythmic profiles of these skeletal muscle genes with rhythmic plasma insulin reflects their involvement in glucose uptake and their potential to influence daily patterns of glucose metabolism (Schalin-Jäntti *et al.*, 1994; Mandarino *et al.*, 1995; Watkins *et al.*, 1997; Michael *et al.*, 2001; Dimitriadis *et al.*, 2011).

Insulin was found to be rhythmic across 24 h under a "typical" feeding-fasting cycle, and when nutrition was provided continuously by nasogastric feeding. However, despite a clear acute "meal response" to both morning and evening boluses in Chapter 5, plasma insulin was not deemed to be rhythmic by way of cosinor analysis. This raised important questions about the appropriateness of using cosinor analysis for assessing 24-h rhythmicity in physiological outcomes that are episodic in nature. For example, both insulin and GLP-1 displayed a "typical meal response" to bolus feeding in the morning and evening. On inspection of the data it would appear that these outcomes are at least visually rhythmic (i.e., regular repeated pattern). However, cosinor analysis is not able to detect rhythmicity as the frequency and/or similarity of events (i.e., equal spikes in insulin at 0800 and 2000 h) results in the 24-h mean value being determined the best fit for the data (i.e. a flat line; Figure 9.2G). In this regard, whereas cosinor is a valuable tool for detecting 24-hour rhythms, ANOVA is the more effective analysis to examine acute/episodic changes in plasma metabolites in response to altered patterns of nutrient availability.

Twenty-four-hour rhythms in NEFA and glycerol were apparent across the various prescribed feeding patterns in Chapters 4 & 5 (**Figure 9.2B**). Under the influence of diurnal factors, plasma NEFA and glycerol were suppressed during the day and peaked overnight, coinciding with when the rhythm in insulin was at its highest and lowest, respectively. Conversely plasma NEFA and glycerol rhythms in Chapter 5 opposed those reported in Chapter 4 peaked during waking hours, with lowest values occurring during the night. Intriguingly, as in Chapter 4, the rhythm in NEFA and glycerol mirrored the rhythmicity of insulin, suggesting that any observed rhythms in

circulating levels of these adipose-derived lipids may reflect the function of insulin in regulating adipose tissue lipolysis (Jelic *et al.*, 2009). Given the episodic profile of plasma insulin in response to an influx of insulinogenic nutrients through bolus or meal feeding (Chapters 5, 6 and 7), it is apparent that rhythmicity in plasma NEFA and glycerol is determined by nutrient availability and therefore can be affected by meal-timing.

As well as regulating the mobilisation of lipids from adipose tissue, it is also apparent that the observed rhythm in systemic insulin, underpins rhythmic expression of genes in skeletal muscle that are involved in lipid utilisation. The rhythmic expression of *PDK4* mirrored the 24 h profile of insulin such that highest expression occurred at ~0530 h when insulin was low and systemic NEFA was highest, consistent with the role of this gene in stimulating fatty acid utilisation (Lee *et al.*, 2004; Zhang *et al.*, 2014b; Pettersen *et al.*, 2019). The profile of genes in skeletal muscle involved in regulating of fatty acid solubility, mobility, and transport (e.g., *CPT1B* and *FABP3*) are also consistent with the profile of insulin 24 h. As insulin rose to peak in the early evening, *CPT1B* and *FABP3* expression gradually declined, although to a less dramatic magnitude than PDK4 expression. The alignment of these genes with 24 h insulin could reflect fluctuating lipid utilisation as well as the role of these genes in facilitating this response (Bonnefont *et al.*, 2004; Furuhashi & Hotamisligil, 2008). This is supported by broad alignment between *UCP3* expression (involved in mitochondrial fatty acid oxidation) with the profile of systemic NEFA, which is an important predictor of lipid utilisation in the muscle (Sacchetti *et al.*, 2002; Frayn, 2010b).

Semi-constant routine also revealed rhythmicity in plasma triglycerides, with lowest circulating concentrations in the morning before rising to a peak at ~2330 h, approximate to the reported DLMO (**Figure 9.2D**). This was consistent with studies of constant routine and forced desynchrony, suggesting that rhythms in plasma triglycerides occur independent of feeding patterns (Dallmann *et al.*, 2012; Kasukawa *et al.*, 2012; Chua *et al.*, 2013; Brøns *et al.*, 2016; Wehrens *et al.*, 2017a; Yuan *et al.*, 2020). However, rhythmicity was not evident from cosinor analysis in either continuous or bolus feeding. With the aforementioned limitations of cosinor analysis in mind, it is more informative to look at the temporal traces of mean plasma concentration across time points. When fed continuously across 24 h, plasma triglyceride concentration steadily increased across the period. In comparison, plasma triglyceride concentration

increased across the day (with hourly oral nutrition), before declining overnight (i.e., coinciding with the withdrawal of oral nutrition overnight). The steady increase in plasma triglycerides with more continual patterns of nutrient provision may reflect a state of sustained postprandial hypertriglcyeridaemia, which are extended when nutrition is provided through the night. On the other hand, the response to the bolus feeds more closely reflected the typical postprandial triglyceride response to a fat containing meal (Lopez-Miranda *et al.*, 2007).

Daily responses of plasma urea displayed remarkable consistency across feeding patterns. Generally, circulating urea tended to be lowest between mid-morning (~1000 h) and mid-afternoon (~1600 h) with rhythmic peaks occurring overnight during sleep (**Figure 9.2E**). Enteral feeding pattern only resulted in small alterations in the pattern of rhythmic urea, with an ~30 min difference in peak between continuous (~0245 h) and bolus feeding (~0215 h), although this rhythmic peak in systemic urea occurred ~2 h earlier under the diurnal influence of feeding-fasting (~0045 h). Notably the pattern of plasma urea responses report in Chapter 5 has implications for assessment of protein oxidation through measurement of urea in plasma and urine. Current practice for this method is to assess urea concentration in plasma samples at the beginning and end of a given period, alongside measurement of nitrogen excreted as urea in total urine output throughout the same period (described in Chapter 3.3.3). Whilst urea concentration at the end of the total period was similar between conditions, the pattern of response in plasma urea (and thus total systemic uraemia) was vastly different when fed in a bolus pattern compared to continuously. Consequently, total exposure to plasma urea was greater across any given period, suggesting a greater rate of amino acid oxidation and ureagenesis (Witard *et al.*, 2014). Assessment of plasma urea at the beginning and end of a given period may not therefore give an accurate reflection of protein oxidation in response to any given nutritional intervention.

As well as studying the influence of feeding patterns on the 24-h profiles of nutrient metabolism, it was also important to establish how feeding time affected markers of tissue turnover. The most striking of these responses was in plasma profiles of C-terminal telopeptide (CTX), which is a circulating marker of bone resorption (Rosen *et al.*, 2000). Plasma CTX tended to be lower during waking hours and elevated overnight, yet rhythmic parameters were clearly modified by feeding pattern (**Figure 9.2H**). Most notably, bolus feeding resulted in a blunting of the daily rhythm in plasma

CTX that was seen with both feeding-fasting and continuous feeding. Previous research suggests this is at least in part facilitated by postprandial increases in plasma total GLP-1, which certainly aligns with the responses of both GLP-1 and CTX seen in Chapter 5 (Iepsen *et al.*, 2015; Jensen *et al.*, 2021).

The lack of rhythmicity in serum testosterone across Chapters 4 and 5 was a surprising outcome considering that numerous studies report a clear rhythm, whereby highest values are apparent in the morning (~0800 h) and corresponding nadir in the evening (~2000 h) (Resko & Eik-nes, 1966; Marrama *et al.*, 1982; Bremner *et al.*, 1983; Plymate *et al.*, 1989). Feeding-related responses of this anabolic marker may exist but these effects may have been masked by rises in cortisol across the day in response to muscle biopsies, or the noise associated with the use of commercial enzyme-based immuno-assays rather than gold standard measurement by liquid chromatography mass spectrometry (Cumming *et al.*, 1983; Handelsman & Wartofsky, 2013; Handelsman *et al.*, 2018; Gagliano-Jucá *et al.*, 2019).

Autophagy is a vital component of the adaptive response of skeletal muscle to variable nutrient supply, and the data presented in this thesis provides novel insights in the response of this process to feeding patterns (Kim & Lee, 2014; Yin *et al.*, 2016; Martinez-Lopez *et al.*, 2017; Ryzhikov *et al.*, 2019). The use of a semi-constant routine revealed peak expression of skeletal muscle *FBXO32,* and *FOXO3* during the morning period while cortisol was rising; consistent with the related action of these genes in promoting the expression of downstream autophagy-related proteins (Bodine *et al.*, 2001; Gomes *et al.*, 2001). This could suggest that autophagy in skeletal muscle is upregulated in the morning, however this would be surprising given that at this time participants were consuming an hourly iso-caloric feed (i.e., we would expect autophagy to be low) (Kim & Lee, 2014). However, autophagy was not directly measured in this study, so the above statements remain speculative.

Through direct measurement of autophagic flux (albeit in isolated neutrophils from whole blood rather than skeletal muscle), a potential effect of feeding patterns on autophagic flux was revealed. In particular, it appears that absolute levels of autophagic flux may be higher under continuous feeding in contrast to bolus feeding. This was an entirely plausible outcome given that a sufficient meal stimulus triggers the mTOR pathway, which itself downregulates autophagy (Ganley *et al.*, 2009; Kim

et al., 2011; Khapre *et al.*, 2014; Kim & Guan, 2015; Tao *et al.*, 2022). Equally, this also suggests that continuous feeding did not provide a sufficient metabolic stimulus to inhibit autophagy (as reflected in respective glucose and insulin responses). A further interesting outcome from the assessment of autophagic flux in neutrophils was that autophagic flux was seemingly suppressed compared to continuous feeding at 2000 h, 12 h after receiving the morning bolus. This supports previous work suggesting that ≥16-19 h are needed to reach a "true" post-absorptive state (Cahill, 1970; Soeters *et al.*, 2012; Templeman *et al.*, 2020). The apparent patterns between conditions in Chapter 5 lend support to the notion that autophagy aligns with intermittent rather than continuous feeding, to best respond to fluctuating nutrient availability (Tao *et al.*, 2022). However, further work is necessary to provide a clearer picture of how different feeding patterns affect autophagic responses in different tissues and cell types as well as further research in critically ill patients.

Collectively, the studies outlined in Chapters 4.0 and 5.0 provide novel context of daily temporal systemic and tissue metabolic responses to contrasting feeding patterns. Whilst endogenous clocks are likely to underpin the majority of rhythms reported, it is certainly evident from the data reported that feeding patterns can distinctly alter the profile of these rhythms, primarily due to the acute responsiveness of feeding related hormones such as insulin. Considering that the effect of physical activity was effectively removed by having participants in bed for 24 h future work should now aim to establish the effects of physical activity and/or exercise on core clock machinery within skeletal muscle. Moreover, future work should also seek to explore interactions between nutrient timing and exercise timing; investigate the molecular mechanisms underpinning tissue and systemic responses to feeding patterns across the day including the use of stable isotope tracer methodologies to explore metabolite kinetics.

Aside from improving our basic physiological knowledge of the metabolic response to of divergent feeding patterns, study of feeding patterns has important implications for clinical practice. Based on the diverse metabolic responses to altering nutrient delivery patterns clinicians should seek to ensure appropriate controls are employed prior to sampling, such as time of day of sampling, and time since last meal, allowing for more appropriate interpretation of clinical results. Furthermore, direct contrast of enteral

feeding patterns described in Chapter 5 can be used to formulate evidence-based approaches to enteral feeding in critically ill patients. Whereas bolus feeding resulted in what would be seen as the "typical meal response" (i.e., increases in circulating glucose, insulin, and GLP-1, with reciprocating profiles of adipose tissue derived lipids NEFA and glycerol), the metabolic and endocrine response to continuous feeding appears to present in a similar manner to fasting. This was initially evident from the autophagic flux data reported in Chapter 8, showing that continuous feeding did not provide a sufficient metabolic stimulus to inhibit autophagy, which has implications for cellular homeostasis and is vital in both the physiological and pathophysiological state (Ravikumar *et al.*, 2010). However, this is also apparent when comparing the morning glucose, NEFA and insulin responses to extended morning fasting reported in Chapter 7 to the pattern of these metabolic and endocrine markers when healthy participants are provided with continuous 24-h enteral nutrition.

Continuous administration of nutrition is the norm amongst critically ill patients in intensive care, with practice primarily dictated by convenience. However, continuous delivery of enteral nutrition may in fact be contributing to the prevalence of insulin resistance by increasing insulin requirements to maintain euglycemia during bed rest (Woolfson *et al.*, 1976; Dirks *et al.*, 2016; Luttikhold *et al.*, 2016; Gonzalez *et al.*, 2020). Extension of the overnight fast until midday in Chapter 7 resulted in a greater insulin response at lunch relative to if the same lunch meal was preceded by breakfast, providing further support to the idea that continuous feeding can be likened to a fasted state, therefore increasing insulin requirements to maintain glycaemia. Interestingly, interstitial glucose responses to a standardised breakfast were greater the day after bolus feeding. However, this only further supported the narrative that continuous feeding did not provide a sufficient meal stimulus and it may be the case that the peripheral tissues remained insulin sensitive to replenish glycogen stores (Iwayama *et al.*, 2020). However, this interpretation is made with the limitation that enteral feeding patterns were only studied for one day and does not preclude the possibility that longer term continuous feeding results in poorer metabolic health outcomes (Gonzalez *et al.*, 2020). Further interesting findings in response to differing enteral feeding patterns include the use of bolus feeding to attenuate rises in triglycerides in critically ill patients, as well as the apparent feeding related effects on bone resorption as seen in the CTX

data reported. Clearly there is a need to continue this line of research, especially in an applied context, studying clinical outcomes in critically ill patients.

In order to study 24-h plasma metabolite and muscle responses in chapters 4 and 5, nocturnal sampling was required while participants slept. Participants were instructed to sleep on their back for this period, which provided easy access to the intravenous cannula and researchers aimed not to wake participants for these samples. To minimise disruption to sleep, incisions for the 0000 and 0400 h biopsies were made prior to the 2000-0700 h sleep opportunity. Participants were woken briefly for these samples but continued to wear the eye mask while samples were taken by torch light. Whilst every precaution was exercised to minimise disruption to sleep, it is unavoidable that a least some disruption to typical sleep patterns would occur as a result of a) nocturnal sampling procedures and b) an unfamiliar environment (Tamaki *et al.*, 2016). The prevailing question based on this information was how this would impact next-day metabolic control, especially in light of the interstitial glucose measures on days 1 and 3. As described in Chapter 6, hourly sleep fragmentation *per se* did not impair next morning glucose control. Therefore, even if participants did wake even briefly for every single sample during the night, it was not expected that this would greatly influence next morning results.

The metabolic effects of sleep fragmentation remain relatively understudied compared to total and partial sleep deprivation. Taken alongside previous literature the data reported in Chapter 6 suggest total duration of sleep loss accumulated over one or more nights may therefore be proportionate to the effect on postprandial glycaemia. Further novel context was added to the study of sleep fragmentation with the addition of a strong caffeinated coffee to examine the metabolic effects of common stimulating remedies following a poor night of sleep. Consumption of caffeinated coffee after hourly sleep fragmentation increased glucose iAUC by ~50%. A recent survey found that ~40% of people in the UK drink caffeinated coffee upon waking, this finding has therefore added important novel context on metabolic responses to typical daily behaviours, and suggests that morning coffee should perhaps be consumed after breakfast rather than before (Statista, 2017).
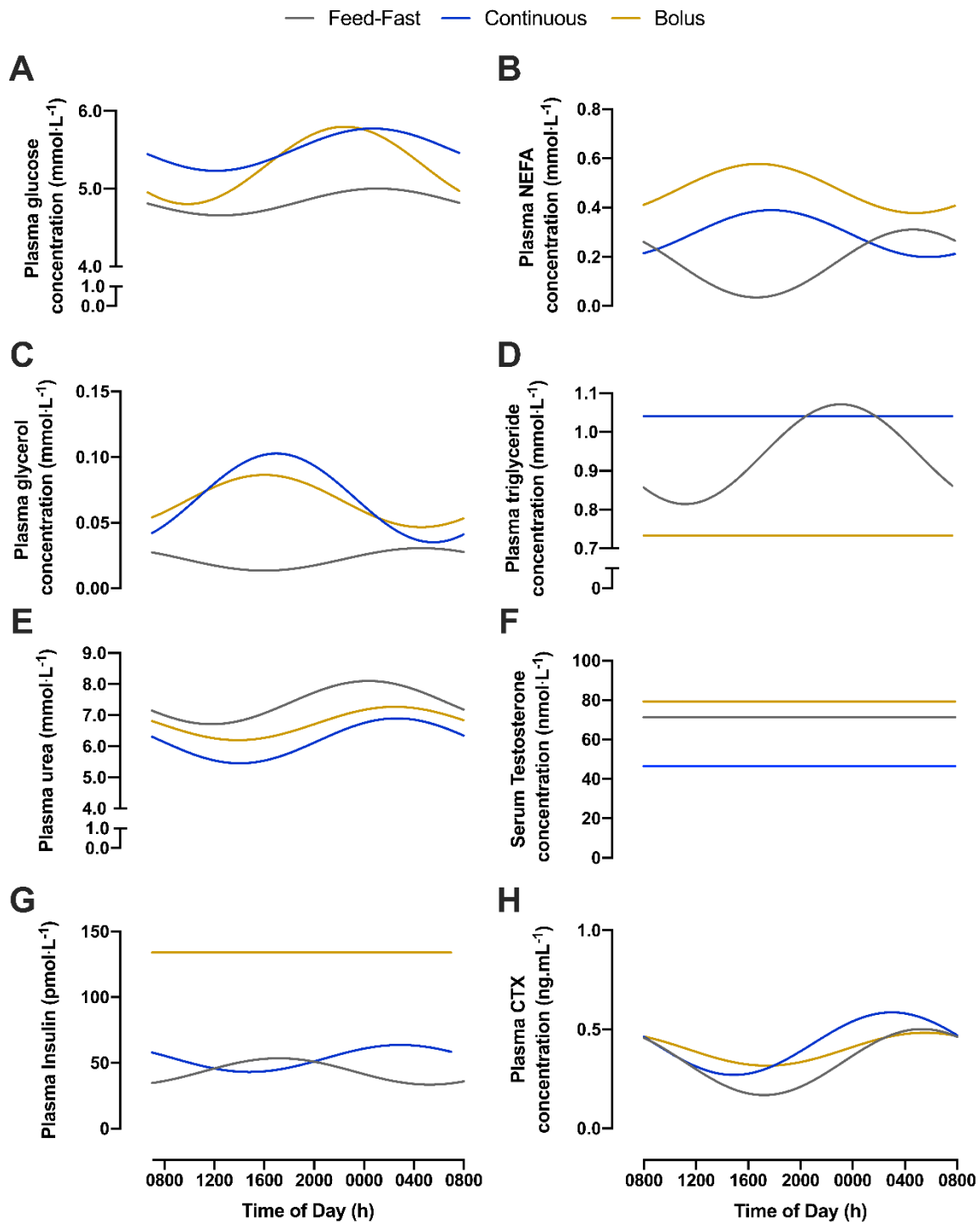
Figure 9.2 – Contrast of rhythmic outcomes of cosinor analysis between feeding patterns employed in Chapters 4.0 and 5.0 for A) plasma glucose B) plasma NEFA C) plasma glycerol D) plasma triglyceride E) plasma urea F) serum testosterone G) plasma insulin H) Plasma CTX.

Chapters 7 and 8 aim to establish the acute (hours; day) and chronic (days; weeks) metabolic and behavioural responses to enrichment of a typical carbohydrate-rich breakfast with whey protein relative to extended morning fasting. Characterisation of the acute metabolic responses to the designed breakfasts was also necessary to determine how and/or whether the acute effects of breakfasts differing in protein content translate to chronic free-living conditions. Despite a lower glycaemic response to the protein-enriched breakfast, the second-meal effect in insulin was effectively elicited to an equal degree as consumption of an energy-matched typical carbohydrate-rich porridge breakfast. Conversely, extended morning fasting resulted in a higher insulin response to the lunch meal such that total insulin exposure was equal across the laboratory visit, regardless of the pattern or amount of energy consumed. However, daily consumption of the same protein-enriched, or carbohydrate-rich breakfast did not confer any apparent metabolic or behavioural adaptations when contrasting the baseline and follow up metabolic responses to a standardised breakfast meal and lunch. Along with previous work demonstrating the ability of breakfast to elicit the second-meal effect, the data reported in Chapters 7 and 8 collectively suggest chronic elicitation of the second-meal effect does not confer any postprandial metabolic adaptation. However, it must be noted that acute study of the second-meal effect took place in controlled conditions (i.e., bed rest) and that free-living physical activity and/or exercise would no doubt influence the metabolic responses to these meals and therefore potentially erode the effect of breakfast composition *per se* (Gonzalez *et al.*, 2013; Edinburgh *et al.*, 2018; Edinburgh *et al.*, 2020).

Aside from glycaemic control, addition of protein to the breakfast meal was also chosen to target the satiating effect of higher protein meals. In a controlled acute setting, subjective appetite was suppressed to a similar extent in response to either whey protein-enriched, or carbohydrate-rich breakfast meals. Conversely, extended morning fasting was characterised by a gradual increase in subjective appetite across the morning. Despite this, energy intake during an *ad libitum* lunch test meal was similar between conditions, such that total energy intake across the laboratory visit was lower when breakfast was omitted. However, daily consumption of protein-enriched breakfast or carbohydrate-rich breakfast, or extended morning fasting resulted in adaptations in subjective appetite (Chapter 8), with no differences in *ad*

libitum energy intake between conditions at the 4-week follow up. The acute data reported here therefore support previous studies of breakfast omission *versus* consumption hinting at the *potential* for breakfast to acutely induce negative energy balance through reduction in energy intake and extends upon this by showing no effect of 15 g whey protein enrichment compared to a typical breakfast.

When looking at free living energy balance during the chronic breakfast intervention, consumption of a carbohydrate-rich or protein-enriched breakfast each day for 4-weeks resulted in a small reduction in energy intake from baseline, which contradicts the acute effects of the same breakfasts report in Chapter 7. Whilst under-reporting of energy intake is common and meaningful (i.e., ~34% under reporting in kcal) in free-living studies, it is safe to assume this would apply equally across all conditions *and* previous studies employing the same tools to assess dietary intake (i.e., that have not seen the same effect as reported currently) (Whitton *et al.*, 2011). Assuming no type I or type II error the size of the breakfast meal may have simply been sufficient to suppress appetite and therefore reduce energy intake across the remainder of the day (from 1200 h onwards). This is entirely conceivable based on the recently reported effects of greater morning caloric intake on appetite across the day (Ruddick-Collins *et al.*, 2022). Equally, no clear effects on physical activity (nor the other major components of energy expenditure) were present. One of the proposed mechanisms potentially explaining higher PAEE in breakfast consumers is parallel elevations of glycaemia across the morning compared to fasting (Betts *et al.*, 2014). A lower glycaemic response to the protein-enriched breakfast was observed to the protein-enriched breakfast in Chapter 7, so it is therefore interesting that physical activity responses were similar between breakfast consuming groups in Chapter 8. This suggests that glycaemia across the morning is not a meaningful driver of spontaneous changes in physical activity. A more likely explanation could be that the duration of the fast is a better predictor for adaptive physical activity responses to fasting. Specifically, longer durations of fast appear to result in a greater reduction in physical activity, with the absence of effect in Chapter 8 potentially being explained by a floor effect relating to low habitual physical activity levels (Templeman *et al.*, 2021a).

As outlined in Chapter 6, caffeinated coffee resulted in an exaggeration of postprandial glycaemia. On the basis of the proposed mechanism of glycaemia in driving spontaneous changes in PAEE it was decided that allowing caffeine across the morning in Chapter 8 could mask any possible effect of the breakfast *per se*. With that said caffeine (especially in the form of coffee) is one of the most widely consumed substances in the world and its potent stimulatory effects predispose earlier consumption in the day, either with breakfast or at some point across the morning. This is particularly pertinent given that caffeine can impact energy balance (Harpaz *et al.*, 2017). Future work should consider the specific interactions between breakfast-fasting and caffeine consumption and its effects on metabolism, energy balance, and health. In particular characterising time of day effects of caffeine intake would be incredibly informative for understanding these potential interactions.

Collective measurement of the major components of energy balance during 4-weeks of daily extended morning fasting, carbohydrate-rich breakfast, or protein-enriched breakfast reveal no large or consistent changes in the major components of energy balance. The collective information gained from the studies in Chapters 7 and 8 extend upon previous research examining all major components of energy balance in response to breakfast consumption or omission by providing a more subtle yet realistic intervention (i.e., people could feasibly expect to consume a slightly smaller breakfast instead of skipping breakfast all together). The current research complements and extends upon previous work showing that whilst macronutrient composition of breakfast acutely leads to alterations in metabolic control compared to fasting, daily consumption of the same breakfasts does not confer adaptation in postprandial metabolism across sequential meals.

9.3 Translation of findings to public health

Chapter 4 characterised rhythmicity under aligned conditions in which feeding is restricted to waking/light hours only. This provides greater understanding of nutrient metabolism across the day under conditions which are more reflective of free living (i.e., individuals tend to spend the majority of waking hours in a fed state) (Ruge *et al.*, 2009b). This knowledge may provide practical relevance for clinicians for diagnostic blood sampling, allowing for greater context and more accurate interpretation of results, especially relating to metabolic control (e.g., glucose, lipids). Furthermore, characterisation of such rhythms may also inform the basis of future research into optimal eating patterns (e.g., Time restricted eating based on glucose rhythmicity to avoid eating at times when glucose tends to be highest).

Chapter 5 improves our understanding of the basic physiological responses to divergent feeding patterns across 24 h. This holds clinical relevance for enteral feeding practice in critical care. Current practices are based mainly on convenience, rather than replicating the physiological norm of intermittent feeding. Insulin resistance is particularly common in enterally fed patients, which could be explained by muscle disuse, injury/illness associated inflammation, and/or endocrine disruption resulting from an inappropriate enteral nutrition delivery pattern (Woolfson *et al.*, 1976; Dirks *et al.*, 2016; Luttikhold *et al.*, 2016). Replicating physiologically normal feeding patterns therefore could be at least one method of maintaining peripheral insulin sensitivity during bed rest/illness (Gonzalez *et al.*, 2020). Characterisation and comparison of the metabolic, endocrine, and tissue responses to these feeding patterns in healthy individuals, can form the basis of a stronger evidence base on which clinical enteral feeding practice can be informed. It is now important to follow this work up in a clinical setting.

Hourly sleep fragmentation did not appear to negatively affect next morning glycaemic control in Chapter 6. This was surprising given the consistency of which sleep disruption appears to dysregulate glucose metabolism in previous research (Donga *et al.*, 2010; Sweeney *et al.*, 2017). However, what this study does reveal is the potential dose dependent nature in which sleep disruption disturbs next day metabolic control,

suggesting that perhaps one night of fragmented sleep is not detrimental, but any form of sleep deprivation or sequentially fragmented nights could increase cardiometabolic disease risk at the population level. Furthermore, this study revealed the negative affect of a strong caffeinated coffee on glycaemic control (i.e., ~50% increase in exposure to circulating glucose). If repeated chronically, this could further increase the risk of developing cardiometabolic disease (Cavalot *et al.*, 2011). To counter this, a lower dose of caffeine could be consumed, or caffeinated coffee could be ingested after, rather than before a breakfast meal.

Considering the breakfast meal, consuming a higher protein, lower carbohydrate meal to start the day may also be an effective method of avoiding higher morning postprandial glycaemic responses, whilst also improving glucose control across the day (Chapter 7). Consumption of a relatively small breakfast (regardless of composition) does not appear to also alter appetite beneficially such that energy intake at lunch is the same as when breakfast is skipped. Consequently, omission of breakfast acutely reduces energy intake. However, when consumed in the extended morning fasted state, approximately equal energy intake at lunch results in an insulinaemic response that equates to the insulinaemic response to 2 meals within the same time frame. Individuals may want to consider balancing these outcomes when deciding whether to skip or consume breakfast (regardless of macronutrient composition).

Despite acute differences in postprandial metabolism between breakfasts of differing composition daily consumption of the same breakfasts 28-d did not confer any metabolic benefits, nor did it consistently or meaningfully alter the major components of energy balance (Chapter 8). It is likely that the lack of consistent changes between groups allocated to skip breakfast, consume a carbohydrate rich or whey protein enriched breakfast may be due to the size of the breakfast provided, as this tended to be smaller than individuals typical breakfast intake (if consumed regularly). Whilst the smaller breakfast provided perhaps represents a more achievable or realistic change in daily breakfast habit than skipping breakfast the data in Chapter 8 could be taken alongside previous research to advocate consumption of a larger breakfast meal in

order to alter the major components of energy balance (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a; Chowdhury *et al.*, 2019; Ruddick-Collins *et al.*, 2022)

9.4 Conclusion

In summary, the collective body of research outlined in this thesis highlights that alteration nutrient feeding patterns affects episodic and rhythmic responses of tissue and circulating markers of nutrient metabolism. Amongst typical feeding responsive markers (e.g., insulin), direct measurement of autophagy under different feeding conditions highlights the inability of continuous feeding to elicit a sufficient "meal response". This has important implications for current practices in enteral feeding in critically ill patients, but more work is required in this regard. Interestingly, more subtle changes in response to altered nutrient timing (whilst difficult to assess in a free-living context) have furthered our understanding of the relationship between morning fasting and energy balance. The results gathered in this thesis therefore support emerging evidence demonstrating that nutrient/meal timing is an important metabolic regulator and should equally be considered alongside dietary quality (i.e., what we eat) and quantity (i.e., how much we eat).

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Appendix A

University of Bath

Department for Health

Entry Criteria Questionnaire

The purpose of this questionnaire is to ensure we minimise the risk of anyone being adversely affected by participating in this study.

Please read each of the questions below carefully, and if you are unsure about why a question is being asked or whether your answer is relevant, please discuss this with the researcher. If you would prefer not to answer any of the questions below, the researchers will respect your wish however you must answer ALL the below questions in order to participate in the study.

Participant Code: _____

Date of Birth: _____

Weight: _____kg

Height: _____m

BMI: _____kg.m^{-2}

1. What is your sex (i.e. at birth)? (please circle):

Male   Female   Prefer not to say

Has this changed since birth? (please circle):   Yes   No

Prefer not to say

1b. *If yes, please provide some additional information (i.e. hormone supplementation):*

2a. What is your current cigarette smoking status?

NON-SMOKER   SMOKER   QUITTING

2b. *If you circled QUITTING, when was your most recent cigarette?*

3a. Have you been diagnosed with any physical disorders/diseases?

Arthritis   ☐

                             ☐

Cardiac-arrhythmia

Coronary Heart Disease ☐

Type 1 or 2 Diabetes ☐

Cancer ☐

Liver disease (alcohol or non-alcohol related) ☐

Other ☐

3b. If you answered OTHER please provide details.

4a. Are you currently/were you recently on any medication (e.g. lipid-lowering or undergoing medical treatment?

Metformin ☐

Statins ☐

Verapamil ☐

Disulfiram ☐

☐

Other

4b. *If you answered OTHER, please provide details.*

5. How would you best describe your breakfast eating habits?

Regular consumer (≥50 kcal within 2 hours of waking most days of the week)

Regular Skipper (<50 kcal within 2 hours of waking most days of the week)

6. Do you follow any dietary patterns or have any special dietary requirements (e.g. vegetarian, vegan, coeliac, etc.)? (please circle):

        No   Yes

6b. *If 'yes', please state information on type, duration, and any other relevant information below:*

7. Do you take any dietary supplements (i.e. vitamin tablets)? (please circle):

No   Yes

7b. *If circled 'yes', please provide information on type, duration, or any other relevant information below:*

8. Do you work night-shifts or regularly skip sleep at night? (please circle):

No   Yes

8b. *If circled 'yes', please provide any other relevant information below:*

9. Females only, which of the below statements most appropriately describes your menstrual cycle? (please write A-E):

Regular menstrual cycle; 28 ± 7 days

Irregular menstrual cycle; >35 days

Absence of menstrual cycle; >90 days but <365 days

Postmenopausal (absence of menstrual cycle for ≥365 days)

Using Contraception (of any kind)

Most applicable……….

9b. *Based on your choice above, please answer the respective question(s) overleaf:*

If you have a regular menstrual cycle (28 ± 7 days), please state number of days since onset of last menstruation: ……. Day(s). And average menstrual cycle length: …... Days.

If you have an irregular menstrual cycle (>35 days), please state number of days since onset of last menstruation: ……. Day(s). And average menstrual cycle length: …... Days.

If you experience absence of the menstrual cycle (>90 days but <365 days), please state approximately number of days since onset of last menstruation: ….. Day(s)

If Postmenopausal, approximately how long since your last menstruation:

..... Year(s)   ….. Month(s)   ….. Day(s)

If using contraception, please describe method / type below (e.g. combined pill, IUD, dose):

Appendix B

GENERAL HEALTH QUESTIONNAIRE

UNIVERSITY OF
BATH

Identification Code: Date:

GP's Name: …………………………….

GP's Address and telephone number: ……………………………………………

……………………………………………

Date of Birth:

Age:

Are you a Smoker/Non Smoker?

If yes, how many cigarettes do you smoke per day?: ……….

How much alcohol do you drink on average per week (units per week)?

(1 pint = 2 units; 1 measure of alcohol = 1 unit)

None   1-4 units   5-14   15 or more

<u>How many cups of tea/coffee do you drink a day?</u>

<u>Any known eye conditions</u> (glaucoma, cataract)?

<u>Eye Colour:</u>

<u>Are you colour blind?</u>

<u>Have you given more than 400 ml of blood in the past 3 months?</u>

<u>Have you travelled across more than 2 time zones in the last 2 weeks?</u>

<u>Do you regularly work night shifts?</u>

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continued well-being and (ii) to avoid the possibility of introducing bias into the study outcomes. Your responses to these questions may therefore show us you are not eligible to participate in the study.

Please complete this brief questionnaire to confirm your eligibility to participate:

1.   **At present,** do you have any health problem for which you are:

(a)   on medication, prescribed or otherwise   Yes ☐   No ☐

(b)   attending your general practitioner   Yes ☐   No ☐

(c)   on a hospital waiting list   Yes ☐   No ☐

2.   **In the past two years,** have you had any illness which required you to:

(a)   consult your GP   Yes ☐   No ☐

(b)   attend a hospital outpatient department   Yes ☐   No ☐

(c)   be admitted to hospital   Yes ☐   No ☐

3.   **Have you ever** had any of the following:

(a)   Convulsions/epilepsy Yes ☐   No ☐

(b)   Asthma   Yes ☐   No ☐

(c)   Eczema   Yes ☐   No ☐

(d)   Diabetes   Yes ☐   No ☐

(e)   A blood disorder   Yes ☐   No ☐

(f)   Head injury   Yes ☐   No ☐

(g)   Digestive problems   Yes ☐   No ☐

(h)   Heart problems   Yes ☐   No ☐

(i)   Problems with bones or joints   Yes ☐   No ☐

(j)   Disturbance of balance/coordination Yes ☐   No ☐

(k)   Numbness in hands or feet   Yes ☐   No ☐

(l)   Disturbance of vision Yes ☐   No ☐

(m)   Ear / hearing problems   Yes ☐   No ☐

(n)   Thyroid problems   Yes ☐   No ☐

(o)   Kidney or liver problems   Yes ☐   No ☐

(p)   Allergy to nuts Yes ☐   No ☐

4.   **Has any,** otherwise healthy, member of your family under the

age of 35 died suddenly during or soon after exercise?   Yes ☐   No ☐

Continued over…

If YES to any question, please describe briefly if you wish (e.g. to confirm problem was/is short-lived, insignificant or well controlled.)

....

....
.....………

…………………………………………………………………………………………………..

....
.....………

…………………………………………………………………………………………………..

....
.....………

…………………………………………………………………………………………………..

....
.....………

…………………………………………………………………………………………………..

Additional questions relevant to provision of muscle samples:

Do you have any form of bleeding disorder or are taking any

medication which can affect the ability of your blood to clot

(e.g. Warfarin, aspirin-like drugs, etc.)? …......….….…..... Yes ☐   No ☐

Are you aware of having any tendency towards keloid scarring

(i.e. scars which expand outside the area of the wound)?   Yes ☐   No ☐

Are you currently taking any medication which impacts upon

your immune system (e.g. steroids)?   Yes ☐   No ☐

Thank you for your cooperation!

Appendix C

<u>GENERAL SLEEP QUESTIONNAIRE</u>

Identification Code: ……………………….   **Date:……………..**

On a normal day how many times do you go outside?

< 1/day   1/day   > 1/day

Would you regard your mobility as:

good   fair   poor

What time do you normally go to bed (h:min)?

How many hours per night do you sleep on average?

How long does it take you to fall asleep?

< 10 min   10-30 min   > 30 min

How often do you wake up during the night?

How often do you get up during the night?

Why do you get up during the night?

What time do you normally wake up (h:min)?

Do you go through phases of getting to sleep earlier and earlier or later and later each night?

Do you have any difficulty getting up in the morning? Y/N

If yes, what kind of difficulty?

Do you fall asleep during the day? Y/N

If yes, how often and for how long?

Do you feel you have any problems sleeping? Y/N

If yes - What kind of problems?

Does it occur regularly?

Do you do anything to try and overcome this problem?

(i.e. sleeping pills, relaxation techniques, herbal remedies, routine)

Are these strategies effective?

How long have you had this sleep problem?

< 6 months   6-12 months   >12 months

Do you ever skip a night's sleep?

Is your sleep pattern different during work free periods (e.g. holidays, weekends)?

If yes, how is it different?

Does your sleep pattern affect your social or occupational life? Y/N If yes, how?

If yes, in what way?

Appendix D

UNIVERSITY OF
BATH

Subject Code: ……………… Date:………………

Munich ChronoType Questionnaire

All aspects of this study have received a favourable ethical opinion from the University of Surrey Ethics Committee.

Important: *in the following sections, please give your answers as they hold true in your current, present-time situation. For changing conditions (for example, beginning of work), please choose the most frequent or appropriate value.*

On workdays *(dont' fill out if you are retired)*

I have to get up at	……………o'clock
I need	……………min to wake up
I regularly wake up	……………min before the alarm / with the alarm
	(please delete one)
From	……………o'clock, I am fully awake
At around	……………o'clock, I have an energy dip
On nights before workdays, I go to bed at	……………o'clock…
… and it then takes me	……………min to fall asleep

If I get the chance, I like to take a siesta/nap…

correct	I then sleep for min
not correct	I would feel terrible afterwards

On free days (please only judge normal free days, i.e., without parties etc.)…

My dream would be to sleep until   ……………… o'clock

I normally wake up at   ……………… o'clock

If I wake up at around the normal (workday) alarm time, I try to get back to sleep

Correct /   Not correct

If I get back to sleep, I sleep for another   .... min

I need   .... min to wake up

From   .... o'clock, I am fully awake

At around   .... o'clock, I have an energy dip

On nights before free days, I go to bed at   .... o'clock

… and it then takes me   .... min to fall asleep

If I get the chance, I like to take a siesta/nap…

correct   I then sleep for min

not correct   I would feel terrible afterwards

Once I am in bed, I would like to read for   .... min,…

… but generally fall asleep after no more than .... min

I prefer to sleep in a completely dark room	Correct /	Not correct
I wake up more easily when morning light shines into my room	Correct /	Not correct

How long per day do you spend on average outside (really outside) exposed to day light?On work days: .... hrs   min

On free days: .... hrs   min

Appendix E

Self-assessment

After you have answered the preceding questions, you should have a feeling of the chronotype you belong to. If, for example, you like (and manage) to sleep quite a bit longer on free days than on workdays, or if you cannot get out of bed on Monday mornings, even without a Sunday-night party, then you are more likely a late type. If, however, you regularly wake up and feel fresh right from the moment you jump out of bed, and if you would go to bed early rather than to an evening concert, then you are more an early type. In the following questions, please characterize yourself and your family members in terms of early/late types.

Extreme early type		**Moderate early type**	**Slight early type**	**Normal Type**	**Slight late type**	**Moderate late type**	**Extreme late type**
I am…	0	1	2	3	4	5	6
As a child, I was…	0	1	2	3	4	5	6
As a teenager, I was…	0	1	2	3	4	5	6
In case you are older than 65: in the middle of my life, I was…							
	0	1	2	3	4	5	6
My parents are/were…							
Mother…	0	1	2	3	4	5	6
Father…	0	1	2	3	4	5	6
My siblings are/were… (please underline <u>Brother</u> or <u>Sister</u>)							
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6
Brother/Sister	0	1	2	3	4	5	6

Brother/Sister	0	1	2	3	4	5	6

My partner (girl/boyfriend, spouse, significant other) is/was…

	0	1	2	3	4	5	6

Appendix F

Subject Code: ……………… Date:…………………

Horne-Östberg questionnaire

All aspects of this study have received a favourable ethical opinion from the University of Surrey Ethics Committee.

This questionnaire will used to assess whether you are a "morning" type (i.e. a lark) or an "evening" type (i.e. an owl) person.

Please read each question very carefully before answering.

Answer all questions

Answer questions in numerical order

Each question should be answered independently of others. Do **NOT** go back and checkyour answers.

For some questions you are required to respond by placing a cross alongside your answer. Insuch cases, select **ONE** answer only.

Please answer each question as honestly as possible. Both your answers and results will bekept in strict confidence.

QUESTION 1

Considering only your own "feeling best" rhythm, at what time would you get up if you were entirely freeto plan your day?

```
AM    5      6      7      8      9      10     11     12
```
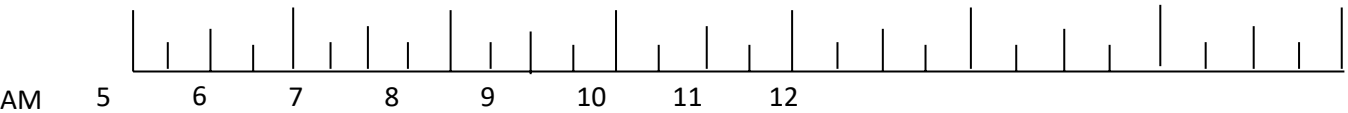
QUESTION 2

Considering only your own "feeling best" rhythm, at what time would you go to bed if you were entirelyfree to plan your evening?

```
PM    8      9      10     11     12  AM 1      2      3
```
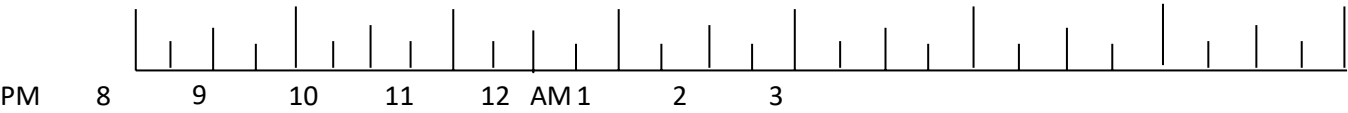
QUESTION 3

If there is a specific time you have to get up in the morning, to what extent are you dependent on beingwoken up by an alarm clock?

a.	Not at all dependent	[	]
b.	Slightly dependent	[	]
c.	Fairly dependent	[	]
d.	Very dependent	[	]

QUESTION 4

Assuming adequate environmental conditions, how easy do you find getting up in the morning?

a.	Not at all easy	[	]
b.	Not very easy	[	]
c.	Fairly easy	[	]
d.	Very easy	[	]

QUESTION 5

How alert do you feel during the first half hour after having woken in the morning?

    a.   Not at all alerts   [   ]

    b.   Slightly alert   [   ]

    c.   Fairly alert   [   ]

    d.   Very alert   [   ]

QUESTION 6

How is your appetite during the first half hour after having woken in the morning?

a.   Very poor   [   ]

b.   Fairly poor   [   ]

c.   Fairly good   [   ]

d.   Very good   [   ]

QUESTION 7

During the first half hour after having woken in the morning, how tired do you feel?

a.   Very tired   [   ]

b.   Fairly tired   [   ]

c.   Fairly refreshed   [   ]

d.   Very refreshed   [   ]

QUESTION 8

When you have no commitments the next day, at what time do you go to bed compared to your usualbedtime?

a.   Seldom or never later   [   ]

b.   Less than one hour later   [   ]

c.   1-2 hours later   [   ]

d.   More than 2 hours later   [   ]

QUESTION 9

You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice aweek and the best time for him is between 0700 and 0800h. Bearing in mind nothing else but your own inclinations, how do you think you would perform?

a.   Would be on good form   [   ]

b.   Would be on reasonable form   [   ]

c.   Would find it difficult   [   ]

d.   Would find it very difficult   [   ]

QUESTION 10

At what time in the evening do you feel tired and as a result in need of sleep?

PM   8   9   10   11   12   AM 1   2   3
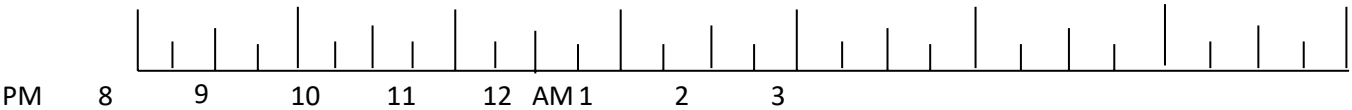
QUESTION 11

You wish to be at your peak for a test which you know is going to be mentally exhausting and lasting fortwo hours. You are entirely free to plan your day and considering only your own "feeling best" rhythm, which ONE of the four testing times would you choose?

a.   0800 – 1000   [   ]

b.   1100 – 1300

                                             [   ]

c.   1500 – 1700

                                             [   ]

d.   1900 – 2100

                                             [   ]

QUESTION 12

If you went to bed at 2300h at what level of tiredness would you be?

a.   Not at all tired   [   ]

b.   A little tired   [   ]

c.   Fairly tired   [   ]

d.   Very tired   [   ]

QUESTION 13

For some reason you have gone to bed several hours later than usual, but there is no need to get up at anyparticular time the next morning. Which ONE of the following events are you most likely to experience:

a.   Wake up at the usual time and not go back to sleep   [   ]

b.   Wake up at the usual time and doze   [   ]

c.   Wake up at the usual time and go back to sleep   [   ]

d.   Wake up later than usual   [   ]

QUESTION 14

One night you have to remain awake between 0400 and 0600h. You have no commitments the next day.Which ONE of the following suits you best:

Not to go to bed until 0600h   [   ]

Nap before 0400h and sleep after 0600h   [   ]

Sleep before 0400h and nap after 0600h   [   ]

Sleep before 0400h and remain awake after 0600h   [   ]

QUESTION 15

You have to do two hours of hard physical work. You are entirely free to plan your day and consideringonly your own "feeling best" rhythm which ONE of the following times would you choose?

a.	0800 – 1000	[	]
b.	1100 – 1300		
		[	]
c.	1500 – 1700		
		[	]
d.	1900 – 2100		
		[	]

QUESTION 16

You have decided to engage in some physical exercise. A friend suggests that you do this between 2200 and2300h twice a week. Bearing in mind nothing else but your own "feeling best" rhythm how well do you think you would perform?

a.   Would be on good form   [   ]

b.   Would be on reasonable form   [   ]

c.   Would find it difficult   [   ]

d.   Would find it very difficult   [   ]

QUESTION 17

Suppose that you can choose your own work hours. Assume that you worked a FIVE-hour day (includingbreaks)) and that your job was interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you select:

12 1 2 3 4 5 6 7 8 9 10 11 12 1   2 3 4 5 6 7 8 9 10 11 12

MIDNIGHT   NOON   MIDNIGHT

QUESTION 18

At what time of day do you think that you reach your "feeling best" peak?

12 1 2   3 4 5 6 7 8 9 10 11 12 1   2 3 4   5   6 7 8   9
10   11 12

MIDNIGHT   NOON   MIDNIGHT

QUESTION 19

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

Morning type   [   ]

More morning than evening   [   ]

More evening than morning   [   ]

Evening type   [   ]

Appendix G

Subject Code: ………………………. Date:……………..

Pittsburgh Sleep Quality Index

All aspects of this study have received a favourable ethical opinion from the University of Surrey Ethics Committee.

The following questions relate to your usual sleep habits **during the past month *only***. Your answers should indicate the most accurate reply for the *majority* of days and nights in the past month. Please answerall the questions.

During the past month, when have you usually gone to bed at night?

Usual bed time

During the past month, how long (in minutes) has it usually taken you to fall asleep each night?

Number of minutes

During the past month, when have you usually got up in the morning?

Usual getting up time

During the past month, how many hours of actual sleep did you get at night? (This may bedifferent from the number of hours spent in bed.)

Hours of sleep per night

For each of the remaining questions, check the one best response. Please answer all questions.

During the past month, how often have you had trouble sleeping because you…..

Cannot get to sleep within 30 minutes

Not during the Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Wake up in the middle of the night or early morning

Not during the Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Have to get up to use the bathroom

Not during the Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Cannot breathe comfortably

Not during the Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Cough or snore loudly

Not during the Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Feel too cold

Not during the   Less than   Once or twice   Three or more

past month_____   once a week____   a week_   times a week___

Feel too hot

Not during the   Less than   Once or twice   Three or more

past month_____   once a week____   a week_   times a week___

Had bad dreams

Not during the   Less than   Once or twice   Three or more

past month_____   once a week____   a week_   times a week___

Have pain

Not during the   Less than   Once or twice   Three or more

past month_____   once a week____   a week_   times a week___

Other reason(s), please describe

_____

_____

_____

How often during the past month have you had trouble sleeping because of this?

Not during the   Less than   Once or twice   Three or more

past month_____   once a week____   a week_   times a week___

During the past month, how would you rate your sleep quality overall?

Very good_____

Fairly good_____

Fairly bad_____

Very bad_____

During the past month, how often have you taken medicine (prescribed or "over the counter") tohelp you sleep?

Not during the   Less than   Once or twice   Three or more

past month_____   once a week___   a week_   times a week__

During the past month, how often have you had trouble staying awake while driving, eating meals,or engaging in social activity?

Not during the   Less than   Once or twice   Three or more

past month_____   once a week___   a week_   times a week__

During the past month, how much of a problem has it been for you to show enthusiasm to getthings done?

No problem at all_____

Only a very slight problem_____

Somewhat of a problem_____

A very big problem_____

Do you have a bed partner or roommate?

No bed partner or roommate?__

Partner/roommate in other room_____

Partner in same room, but not same bed_____

Partner in same bed____

If you have a roommate or bed partner, ask him/her how often in the past month you have had)…

Loud snoring

Not during the   Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Long pauses between breaths while asleep

Not during the   Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Legs twitching or jerking while you sleep

Not during the   Less than   Once or twice   Three or more

past month____   once a week___   a week_   times a week___

Episodes of disorientation or confusion during sleep?

Not during the   Less than   Once or twice   Three or more

 past month____   once a week___   a week_   times a week__

Other restlessness while you sleep; please describe

_____

_____

_____

Not during the   Less than   Once or twice   Three or more

 past month____   once a week___   a week_   times a week__

Appendix H

Subject Code: ………………………. Date:……………..

Beck Depression Inventory

All aspects of this study have received a favourable ethical opinion from the University of Surrey Ethics Committee.

Choose one statement from among the group of four statements in each question that best describes how you have been feeling during the **past few days**. Circle the number beside your choice.

1	I do not feel sad.	8	I don't feel I am any worse than anybodyelse.
	I feel sad.		I am critical of myself for my weaknesses or mistakes.
	I am sad all the time and I can't snapout of it.		I blame myself all the time for my faults.
	I am so sad or unhappy that I can'tstand it.		I blame myself for everything bad thathappens.
2	I am not particularly discouragedabout the future.	9	I don't have any thoughts of killing myself.
	I feel discouraged about the future. **2** I feel I have nothing to look forwardto.		I have thoughts of killing myself, but I wouldnot carry them out.
			I would like to kill myself.
			I would kill myself if I had the chance.

	3 I feel that the future is hopeless and that things cannot improve.		
3	I do not feel like a failure. I feel I have failed more than the average person. As I look back on my life, all I cansee is a lot of failure. I feel I am a complete failure as a person.	10	I don't cry any more than usual. I cry more now than I used to. I cry all the time now. I used to be able to cry, but now I can't cryeven though I want to.
4	I get as much satisfaction out ofthings as I used to. I don't enjoy things the way I usedto. I don't get any real satisfaction outof anything anymore. I am dissatisfied or bored with everything.	11	I am no more irritated by things than I everam. I am slightly more irritated now than usual. I am quite annoyed or irritated a good deal ofthe time. I feel irritated all the time now.
5	I don't feel particularly guilty. I feel guilty a good part of the time.**2** I feel quite guilty most of the time. **3** I feel guilty all of the time.	12	I have not lost interest in other people. I am less interested in other people than Iused to be. I have lost most of my interest in otherpeople. I have lost all of my interest in other people.

6	I don't feel I am being punished. I feel I may be punished. I expect to be punished. I feel I am being punished.	13	I make decisions about as well as I evercould. I put off making decisions more than I usedto. I have greater difficulty in making decisionsthan before. I can't make decisions at all anymore.
7	I don't feel disappointed in myself. I am disappointed in myself.**2** I am disgusted with myself. **3** I hate myself.	14	I don't feel that I look any worse than I usedto. I am worried that I am looking old orunattractive. I feel that there are permanent changes in my appearance that make me look unattractive. I believe that I look ugly.
15	**0** I can work about as well as before. **1** It takes an extra effort to get startedat doing something. I have to push myself very hard todo anything. I can't do any work at all.	19	I haven't lost much weight, if any, lately. I have lost more than five pounds. I have lost more than ten pounds. I have lost more than fifteen pounds. (Score 0 if you have been purposely trying tolose weight.)
16	I can sleep as well as usual. I don't sleep as well as I used to. **2** I wake up 1-2 hours earlier than usual and find it hard to get back tosleep. **3** I wake up several hours earlier thanI used to and cannot get back to sleep.	20	I am no more worried about my health than usual. I am worried about physical problems suchas aches and pains, or upset stomach, or constipation. I am very worried about physical problems,and it's hard to think of much else. I am so worried about my physical problemsthat I cannot think about anything else.
17	**0** I don't get more tired than usual. **1** I get tired more easily than I usedto. I get tired from doing almostanything. I am too tired to do anything.	21	I have not noticed any recent change in my interest in sex. I am less interested in sex than I used to be. I am much less interested in sex now. I have lost interested in sex completely.
18	**0** My appetite is no worse than usual. **1** My appetite is not as good as it usedto be. My appetite is much worse now.		

		I have no appetite at all anymore.		

Appendix I

Subject Code: ………………………. Date:……………..

Epworth Sleepiness Scale

All aspects of this study have received a favourable ethical opinion from the University of Surrey Ethics Committee.

Use the following scale to choose the most appropriate number for each situation:

0 = would *never* doze or sleep.

1 = *slight* chance of dozing or sleeping

= *moderate* chance of dozing or sleeping

3 = *high* chance of dozing or sleeping

Situation	*Chance ofDozing orSleeping*
Sitting and reading	
Watching TV	

Sitting inactive in a public place

———————————

Being a passenger in a motor vehicle foran hour or more ————

Lying down in the afternoon

———————————

Sitting and talking to someone

———————————

Sitting quietly after lunch (no alcohol)

———————————

Stopped for a few minutes in traffic while driving ————

Total score ————

Appendix J

Appetite and mood rating booklet

Participant:

Time:

Date:

Appendix J

Please answer all the following questions about your APPETITE.

For each question draw a vertical line at the appropriate place through the horizontal line. You should rate how you feel right now.

I feel HUNGRY

NOT AT ALL |————————————————————| EXTREMELY

How strong is your desire to eat?

WEAK |————————————————————| STRONG

How much food do you think you could eat?

NONE |————————————————————| A LARGE AMOUNT
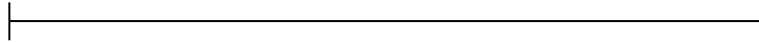
I feel THIRSTY

NOT AT ALL |————————————————————| EXTREMELY

MY STOMACH FEELS FULL
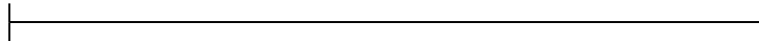
NOT AT ALL |—————————————————————————| EXTREMELY

I feel SATISIFIED
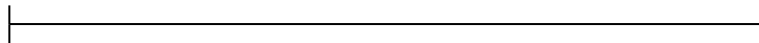
NOT AT ALL |—————————————————————————| EXTREMELY

MY STOMACH FEELS BLOATED / SWOLLEN
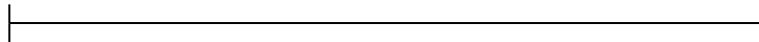
NOT AT ALL |—————————————————————————| EXTREMELY

I feel NAUSEOUS / SICK
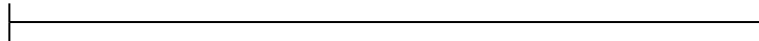
NOT AT ALL |—————————————————————————| EXTREMELY

I have desire to eat something SAVOURY

NOT AT ALL |—————————————————————————| EXTREMELY

I have desire to eat something SWEET

NOT AT ALL |———————————————————————————| EXTREMELY

I feel TENSE / ANXIOUS / NERVOUS / ON EDGE

NOT AT ALL |———————————————————————————| EXTREMELY

I feel MENTALLY ALERT / ATTENTIVE / OBSERVANT

NOT AT ALL |———————————————————————————| EXTREMELY

I feel ELATED

NOT AT ALL |———————————————————————————| EXTREMELY

Karolinska Sleepiness Scale – Please circle to indicate which level best reflects your psycho-physical sate experienced in the last 10 min

Extremely Alert	**1**
Very alert	**2**
Rather alert	**3**
Alert	**4**
Neither alert nor sleepy	**5**
Some signs of sleepiness	**6**
Sleepy, but no effort to keep awake	**7**
Sleep, but some effort to keep awake	**8**
Very sleepy, great effort to keep awake, fighting sleep	**9**
Extremely sleepy, can't keep awake	**10**

Appendix K

Correction of blood markers to DLMO using the 25% method

Hourly samples in chapters 4 and 5 were corrected for dim-light melatonin onset, which is a reliable phase marker of the central circadian clock. Relative thresholds (e.g., 25% of maximum) can be calculate when overnight or 24-hour melatonin data available. Calculation of relative thresholds allows for normalisation of differences in rhythmic amplitude, thereby enabling between group comparisons.

The first step in this process is to simply calculate 25% of each individual's peak concentration.

Peak concentration	25 % of peak
138.9 pg/mL	34.7 pg/mL

Next, visually inspect the data to identify the two time points between which this value would occur (mins from first sample) – highlighted in yellow.

Clock Time	Time (mins)	Concentration
19:00	660	5.0
20:00	720	5.0
21:00	780	5.0
22:00	840	5.0
23:00	900	22.0
00:00	960	60.7
01:00	1020	95.8

Following this, plot a line graph of the two identified points with Time (mins from first sample) on the x-axis and melatonin concentration on the y-axis. Display the equation of the line between these two points on the graph and solve for x (time of 25 % peak melatonin) with known y (25 % peak melatonin concentration: 34.7 pg/mL) (see figure below).

$Y = 0.645x - 558.5$

$34.7 = 0.645x - 558.5$

$x = (34.7 + 558.5)/0.645$

Time of DLMO = x = 920 mins from first sample = 23:20
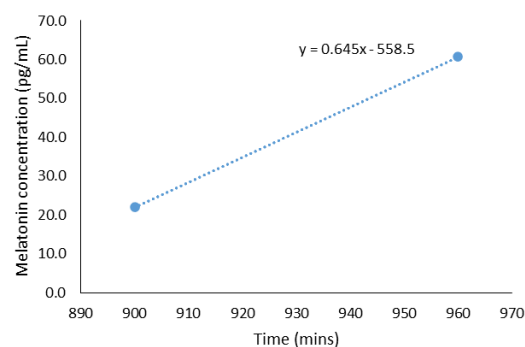
The difference between the time of DLMO and midnight is then calculated (40 mins) and the participants values for each respective hourly outcome are shifted accordingly.

For example:

Original data

Time	12:00	13:00	14:00
Concentration	5.75	6.23	6.33

Shifted data

Time	11:20	12:20	13:20
Concentration	5.75	6.23	6.33

When plotting the shifted data, the resulting x-values were binned around half past the hour with average y-values plotted accordingly.

For example:

Shifted data

Time	11:20	12:20	13:20
Concentration	5.75	6.23	6.33

Binned data

Time	11:30	12:30	13:30
Concentration	5.75	6.23	6.33

Appendix L

Confidence interval calculation

Error bars displayed on figures in Chapters 6 and 7 are confidence intervals corrected to remove between subject variation according to the methods described by Loftus & Masson (1994). These CI were calculated using SPSS statistical software as demonstrated below:

Begin with a data set to be compared, if comparing multiple points over time then take the data from a given time point in each condition e.g.

Participant	Trial 1	Trial 2	Trial 3
1	5.63	5.65	6.05
2	5.59	5.39	5.75
3	5.37	4.5	6.32
4	4.89	4.77	7.25
5	4.81	5.09	6.49
6	5.35	5.00	5.89

Paste these data into SPSS in columns as laid out above and perform a general linear model (GLM):

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Trial	Sphericity Assumed	5.161	2	2.580	9.895	.004
	Greenhouse–Geisser	5.161	1.330	3.881	9.895	.014
	Huynh–Feldt	5.161	1.629	3.169	9.895	.008
	Lower–bound	5.161	1.000	5.161	9.895	.026
Error(Trial)	Sphericity Assumed	2.608	10	.261		
	Greenhouse–Geisser	2.608	6.648	.392		
	Huynh–Feldt	2.608	8.143	.320		
	Lower–bound	2.608	5.000	.522		

When 3 or more treatments are to be compared, the F ratio calculated using a general linear model will only be accurate when sphericity can be assumed. The degree of sphericity can be assessed through referring to the Greenhouse-Geisser epsilon. If this value is <0.75 then, similar to hypothesis testing, the Greenhouse-Geisser corrected MSsxc and df should be used. If the Greenhouse-Geisser epsilon is >0.75 then the Huynh-Feldt corrected MSsxc and df are used. In the current example the Greenhouse-Geisser epsilon was used.

Note the mean square for error (0.392) and the corresponding degrees of freedom (df = 10).

Use a t distribution table to determine a t-value using the degrees of freedom. For the current example df = 10, so t = 2.228.

α / df	0.25	0.1	0.05	0.025	0.01	0.005	0.0025	0.001	0.0005
1	1.000	3.078	6.314	12.706	31.821	63.656	127.321	318.289	636.578
2	0.816	1.886	2.920	4.303	6.965	9.925	14.089	22.328	31.600
3	0.765	1.638	2.353	3.182	4.541	5.841	7.453	10.214	12.924
4	0.741	1.533	2.132	2.776	3.747	4.604	5.598	7.173	8.610
5	0.727	1.476	2.015	2.571	3.365	4.032	4.773	5.894	6.869
6	0.718	1.440	1.943	2.447	3.143	3.707	4.317	5.208	5.959
7	0.711	1.415	1.895	2.365	2.998	3.499	4.029	4.785	5.408
8	0.706	1.397	1.860	2.306	2.896	3.355	3.833	4.501	5.041
9	0.703	1.383	1.833	2.262	2.821	3.250	3.690	4.297	4.781
10	0.700	1.372	1.812	2.228	2.764	3.169	3.581	4.144	4.587
11	0.697	1.363	1.796	2.201	2.718	3.106	3.497	4.025	4.437
12	0.695	1.356	1.782	2.179	2.681	3.055	3.428	3.930	4.318
13	0.694	1.350	1.771	2.160	2.650	3.012	3.372	3.852	4.221
14	0.692	1.345	1.761	2.145	2.624	2.977	3.326	3.787	4.140
15	0.691	1.341	1.753	2.131	2.602	2.947	3.286	3.733	4.073
16	0.690	1.337	1.746	2.120	2.583	2.921	3.252	3.686	4.015
17	0.689	1.333	1.740	2.110	2.567	2.898	3.222	3.646	3.965
18	0.688	1.330	1.734	2.101	2.552	2.878	3.197	3.610	3.922
19	0.688	1.328	1.729	2.093	2.539	2.861	3.174	3.579	3.883
20	0.687	1.325	1.725	2.086	2.528	2.845	3.153	3.552	3.850
21	0.686	1.323	1.721	2.080	2.518	2.831	3.135	3.527	3.819
22	0.686	1.321	1.717	2.074	2.508	2.819	3.119	3.505	3.792
23	0.685	1.319	1.714	2.069	2.500	2.807	3.104	3.485	3.768
24	0.685	1.318	1.711	2.064	2.492	2.797	3.091	3.467	3.745
25	0.684	1.316	1.708	2.060	2.485	2.787	3.078	3.450	3.725
26	0.684	1.315	1.706	2.056	2.479	2.779	3.067	3.435	3.707
27	0.684	1.314	1.703	2.052	2.473	2.771	3.057	3.421	3.689
28	0.683	1.313	1.701	2.048	2.467	2.763	3.047	3.408	3.674
29	0.683	1.311	1.699	2.045	2.462	2.756	3.038	3.396	3.660
30	0.683	1.310	1.697	2.042	2.457	2.750	3.030	3.385	3.646
40	0.681	1.303	1.684	2.021	2.423	2.704	2.971	3.307	3.551
60	0.679	1.296	1.671	2.000	2.390	2.660	2.915	3.232	3.460
120	0.677	1.289	1.658	1.980	2.358	2.617	2.860	3.160	3.373

A normalised confidence interval can now be calculated using the following formula:

$$CI = \sqrt{\frac{MS_{SxC}}{n}} \times [\text{criterion } t(df_{SxC})]$$

Where:

MS_{SxC} = Mean squared error

n = number of subjects in each condition

criterion t = t distribution value

Therefore, for the current example:

$$= \sqrt{(0.392/6)} \times 2.228$$

$$= 0.57$$

Therefore, the overall CI that will be plotted about each mean at that time point will be:

	Trial 1	Trial 2	Trial 3
Mean ± CI	5.27 ± 0.57	5.07 ± 0.57	6.29 ± 0.57

Interpretation of normalised CI is not entirely objective and is not intended to correspond with traditional null hypothesis testing techniques. As a general rule, however, plotted means whose confidence intervals overlap by no more than half the distance of 1 side of an interval are likely to be deemed statistically different via t-test.

When applied to within-subjects designs, calculating confidence intervals using this method does not infer that there is a 95% probability that the mean of the general population lies within that interval (as is convention). Alternatively, the CI calculated in the current study illustrate the probability that the pattern of recorded means is reflective of the general population (i.e., statistical significance). The reason that this relationship does not always correspond with typical statistical methods is that these CI also provide additional information regarding the statistical power of each comparison.

Appendix M

Autophagy by flow cytometry

This document provides details of the reagents, consumables, and equipment needed to process blood samples so that isolated neutrophils can be assessed for autophagy using flow cytometry.

Reagents:

Item	Brand/Supplier	Code	Use	Location/Notes
Miltenyi Neutrophil Separation kit	Miltenyi Biotec	130-104-434	Neutrophil isolation	JT Fridge 1.
MACSxpress® Whole Blood Neutrophil Isolation Isolation Cocktail, human – lyophilized	Miltenyi Biotec	130-104-434	Neutrophil isolation	JT Fridge 1.
MACSxpress® Buffer A	Miltenyi Biotec	130-104-434	Neutrophil isolation	JT Fridge 1.
MACSxpress® Buffer B	Miltenyi Biotec	130-104-434	Neutrophil isolation	JT Fridge 1.
RPMI	Gibco	21875-034	Making up media.	Aliquots in JT Freezer 2. Spare in
Human AB Serum	Corning Thermofisher	15333691	To make up media.	JT Freezer 1.
Penstrepto	Gibco	15140-22	To make up media.	JT Freezer 1.
FlowCellect Kit	Millipore			JT Fridge 1.
20X Anti-LC3 FITC clone 4E12	Millipore	CS208214	Evaluation of autophagy.	JT Fridge 1.
Autophagy reagent A	Millipore	CS208212	Autophagy Inhibitor.	JT Freezer 1.
Autophagy reagent B	Millipore	CS208215	Evaluation of autophagy.	JT Fridge 1.
5X Assay Buffer	Millipore	CS202124	Evaluation of autophagy.	JT Fridge 1.
Benzonase Nuclease	Sigma	E1014-25KU /1002748882	Permeabilization.	JT Freezer 1, bottom drawer.
PBS	Gibco	14190-144	Washing samples.	Aliquots in JT Freezer 1. Spare in JT Fridge 2.
Sterile Water	Sigma	7732-18-5	Making up/diluting reagents.	JT Fridge 2. Nestle section.
Antibody CD45Pb	Beckman Coulter	A74763	Cell staining	JT Fridge 1. Right hand drawer.
Antibody CD16PE	Beckman Coulter	A07766	Cell staining	JT Fridge 1. Right hand drawer.
Antibody CD66b A647	Biolegend	305110	Cell staining	JT Fridge 1. Right hand drawer.

Consumables:

Item	Brand/Supplier	Code	Use	Location/Notes

14 mL PP/PS sterile tubes mod falcon w/cap	Greiner.	187-261	Sample handling. FACS.	In working box on top of JT locked cabinet. Sterile.
5 mL PS sterile tubes	Falcon.	352052	Sample handling	Sterile. Drawers in JT Locked cabinet.
15 mL Falcon Tubes	Falcon.	525-0150	Sample handling.	In working box on top of JT locked cabinet. Purple caps.
96 well V bottom plates	Corning.	3894	Sample handling	In working box on top of JT locked cabinet. Sterile.
5/10 mL Serologic pipettes	Falcon-Corning	356543	Sample handling	Sterile. Next to JT Locked Cabinet.
Micropipette tips (10 µL -1000 µL)	Eppendorf.	Any	Sample handling	In working box on top of JT locked cabinet. Sterile. Filter tips.

Equipment:

Item	Example	Location/Notes
Biological Safety Cabinet	Any	6WS Biochemistry Lab.
Miltenyi MacsXpress magnet	Any	JT Locked cabinet.
MACS Tube rotator	Any	JT Locked cabinet.
Serological pipette controller	Corning	6WS Biological safety cabinet. Sterile.
Centrifuge	See notes	6WS Biochemistry Lab. Plates = Under microscope. Tubes = next to sink.
Microscope for counting cells	Zeiss	6WS Biochemistry Lab.
FACS Aria	Any	3West Bio-imaging suite.

Tasks to perform to establish the assay protocol

All aliquoting should be done in sterile conditions under the biological safety cabinet.

1. **Heat inactivation of Human AB Serum**
   - Remove from freezer and leave to defrost for 4-5 hours on a piece of blue roll at room temperature.
   - With 1 hour remaining of defrost time, make sure there is enough fluid in the water bath to cover the total volume of serum in the bottle (but not so far as to touch the cap), turn on the water bath to 56⁰C and leave for 1 hour.
   - When Serum has completely defrosted, carefully place in water bath using the red bottle weight by the biological safety cabinet to prevent the bottle from tipping.
   - Cover the water bath and heat the serum for exactly 30 minutes.
   - Remove serum from the water bath, place on a piece of blue roll and allow to return to room temperature (~1-2 hours).
   - Store in fridge at 2-8⁰C
   - Aliquot within 1-2 days of heat inactivation.
   - Store aliquots at -20⁰C

2. **Defrost PenStrep**

- Remove from freezer and allow to defrost on blue roll at room temperature for 4-5 hours.
- Once defrosted, place in fridge at 2-8ºC.
- Aliquot within 1-2 days of defrosting.

3. Prepare Millipore Autophagy Reagent A
This material is supplied in a lyophilized vial.
This is the autophagy inhibitor and is used in step 8 (8 uL)
Instructions
- Add 250 µL deionized water to one vial of lyophilized pellet
- Make 15 µL aliquots from this.
- Store aliquots at -20 ºC

Volume per timepoint (µL)	*8*
Volume Millipore A per aliquot (µL)	*15*
Aliquots/sample	*1*
Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*10*
Total Aliquots	*50*

4. RPMI Aliquots
- Used to make up media
- Aliquot 20 mL RPMI into 50 mL falcon tubes.
- Store aliquots at -20 ºC

Volume per time point (mL)	*20*
Volume RPMI per aliquot (mL)	*10*
Aliquots/sample	*2*
Aliquots/participant	*4*
Participants	*20 (max)*
Required Aliquots	*80*
Extra	*10*
Total Aliquots	*90*

5. Human AB Serum Aliquots
- Used at stage 4/5 to re-suspend cells.
- Add 2 mL aliquots to 2mL eppendorfs
- Store aliquots at -20 ºC

Volume per Sample (mL)	*2*
Volume AB Serum per aliquot (mL)	*2.1*
Aliquots/sample	*1*

Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*5*
Total Aliquots	**45**

6. PBS Aliquots

- Used at stages 2 (12 mL), 14 (800 µL), 19 (600 µL), and in PBS/BSA (1%).
- Aliquot 20 mL PBS into a 50 mL falcon tube.
- Store aliquots at -20 ⁰C

Volume per sample (mL)	*~20*
Volume PBS aliquot (mL)	**20**
Aliquots/sample	*1*
Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*10*
Total Aliquots	**45**

7. Penstrep Aliquots

- Used to make up media
- Aliquot 350 µL into 0.5 mL eppendorfs

Volume per sample (µL)	*200*
Volume Penstrep per aliquot (µL)	**350**
Aliquots/sample	*1*
Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*10*
Total Aliquots	**50**

8. PBS/BSA 1% Aliquots

- This is used at stage 17 to make up the antibody mix.
- Dissolve 0.2 g BSA in 20 mL PBS
- Aliquot 350 µL into 0.5 mL eppendorfs.
- Store aliquots at -20 ⁰C

Volume per sample	*280*
Volume PBS/BSA 1% per aliquot (µL)	**350**
Aliquots/sample	*1*

Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*10*
Total Aliquots	*45*

9. Deionized Water aliquots

- Used to dilute Millipore authophagy reagent A,Millipore autophagy reagent B, Millipore Assay Buffer, and benzonase at stage 9.
- Aliquot into 1.5 mL Eppendorf.
- Store aliquots at -20 ºC

Volume/sample (mL)	*1-1.5*
Volume Water per aliquot (mL)	*1.5*
Aliquots/sample	*1*
Aliquots/participant	*2*
Participants	*20 (max)*
Required Aliquots	*40*
Extra	*10*
Total Aliquots	*45*

Tasks prior to blood collection

1. Defrost the following aliquots
- Serum (~1 hour)
- RPMI (~30 mins in water bath)
- PBS (~1.5 hours)
- PBS/BSA 1% (~10 mins or immediately before using heat from hand)
- Water (~1 hour)

2. Check water bath level and turn on ~30 mins before use.

3. Prepare Millipore 5X Assay Buffer
Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. (1:5)
- Add 1600 µL of assay buffer to 6400 µL of deionized water (this gives us enough buffer for both samples).
- Make up in 15 mL Falcon Tube.
- *Note: Prepared 1X Assay Buffer is stable up to one year.*
- *Store at 2 - 8ºC.*

4. Make up RPMI/10% AB Serum mix for re-suspension in step 10 and dilution of Millipore reagent A at step 12.

- Add 36 mL RPMI to a 50 mL falcon tube
- To make it 10% AB Serum, add 4 mL of AB Serum to 36 mL of RPMI
- Add Pen-strep to 10% of the volume of AB Serum (e.g. if adding 4 mL AB serum, add 400 µL of pen-strep)

5. Make up FACS lyse
- Add 3600 µL deionized water to a 15 mL falcon tube
- Add 400 µL of FACS lyse to the 3600 µL deionized water.

6. Make sure the MACSXPress magnet and tube rotator are both charged and/or plugged in.

Assay Procedure

1. Separate the neutrophils from whole blood (total time 25 min).

   *-Reconstitute the MACS lyophilized pellet with 2ml of MACS Buffer A using a P1000 pipette. **Mix gently pipetting up and down 3-4 times**. (You can store the reconstitute pellet for 1 week)*

   -Prepare the final cocktail by mixing appropriate volumes of the reconstituted pellet and Buffer B.

   -As a general principle: To process 1 volume of whole blood mix 0.25 volumes of the MACS reconstituted pellet and 0.25 volumes of MACS buffer B in a greiner tube (typically we will work with 8 mL):

Volume of whole blood	Volume of MACS reconstituted pellet	Volume of MACS buffer B
8 mL	*2 mL*	*2 mL*
6 mL	*1.5 mL*	*1.5 mL*
4 mL	*1 mL*	*1 mL*

   *-Mix gently (UP-DOWN 3-4X) and incubate for **5 min** using the MACSmix rotator. Make sure to place at a gentle angle to ensure proper mixing. Press this symbol to run at 20 rpm:*

   -Place the tube in the magnet for 15 min

   - Harvest exactly 3.5 mL of the supernatant into a 15 mL falcon tube using P1000 tips (change tip each time)
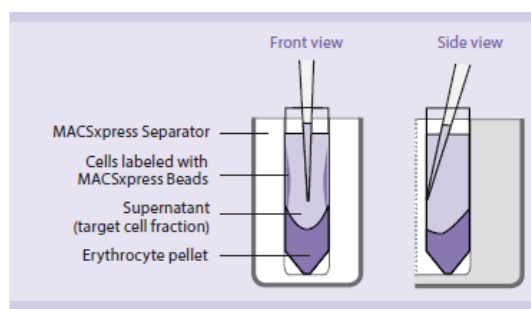
Figure 1: Front and side view of the MACSxpress Separator containing a separated blood sample in a 50 mL tube.

2. Dilute supernatant 1:1 with PBS (e.g. 2 mL + 2 mL)

3. Add 10 uL of trypan blue to a single well in a 96 well plate (found in cell culture drawer)

4. Add 10 uL of diluted neutrophil plasma to the well with trypan blue in and mix gently

5. Take 10 uL of the trypan blue and neutrophil mix and pipette into a counting slide ensuring there are no air bubbles.

6. Count the cells in the top left (or most representative) 4x4 grid 2 times.

7. Calculate the average of the two cell counts (e.g. 61)

- Multiple by 2 for the PBS dilution (e.g. 61 x 2 = 122)
- Multiply by 2 for the trypan blue dilution (e.g. 122 x 2 = 244)
- This number is $x10^4$ cells/mL (e.g. 244 x 10^4 cells/mL)
- Easier to express as $x10^6$ cells (e.g. 2.44 x 10^6 = 2,440,000)
- Multiple by the number of mL plasma harvested to get cells in the total volume (e.g. if we have harvested 3.5 mL then 2,440,000 x 3.5 = 8,540,000 cells total)
- You now need to divide the cells you have by the cells you want (e.g. 8,540,000 total / 500,000 per mL = 17.08)
- This value (e.g. 17.08) is how many times too many cells we have (e.g. 17.08 times too many cells)

2000h	0000h
x2 =	x2 =
x2 =	x2 =
$x10^4$	$x10^4$
$x10^6$	$x10^6$
x =	x =
/500,000 =	/500,000 =

8. Top up collected supernatant to the top of the falcon tube with PBS using a 25 mL serologic pipette tip.

9. Centrifuge **@200g** for **5min** at **ROOM TEMP** (Centrifuge next to the sink)
   - *Make sure the brake and acceleration speeds are slow as to not damage cells (~setting 5 for each).*
   - *Pour off, dab, and agitate*

10. Resuspend cells in RPMI solution containing 10% AB serum
    - Using the value from the end of the calculations in step 7:
    - Multiply this value by 1000 uL (e.g. 17.08 * 1000 = 17,080 uL)
    - Re-suspend the cell pellet in this volume to give us our 500,000/mL (e.g. 17,080 uL)
    - **NB: If the cell count is very high, multiply by a fraction of 1000 (e.g. 250) add 250 uL of cell suspension to each greiner tube and then top up with RPMI-10% AB Serum to achieve 500,000 cells per 1 mL**

11. Add **1 ml** of cell suspension **per tube** to **8** separate greiner polypropilene tubes (6 for flow cytometry and 2 to be stored for Mass Spectrometry)
    - **NB: If the cell count is very high, multiply by a fraction of 1000 (e.g. 250) add 250 uL of cell suspension to each greiner tube and then top up with RPMI-10% AB Serum to achieve 500,000 cells per 1 mL**

12. Dilute Millipore Autophagy Reagent **A** (already made up in 15 µL aliquots – defrost at time as small volume) 1:49 in RPMI-10% AB Serum
    - *We need 400 µL total to cover all 4 replicates plus any extra.*
    - *Therefore at a 1:49 ratio = 8 µL: 392 µL – P10 and P200*

13. Add 50ul of the Millipore autophagy reagent A dilution to four replicates (i.e .add on top of 1 mL cell suspension)

14. Incubate for 30min @37°C/5%CO2.

15. During these 30 minutes, prepare the whole-blood and compensation controls for flow cytometry.

    Mix the following in separate FACS tubes:

	Volume/tube µL
Blood	30
FACS Lysis Buffer	3000

Tube number	Fluorophore/ Surface Marker	Total volume (*µL*)	Positive Beads	Negative Beads
1	FITC- LC3B	5	20 uL	20 uL
2	Pblue- CD45	2	20 uL	20 uL
3	PE- CD16	2	20 uL	20 uL

| 4 | A647- CD66b | 1 | 20 uL | 20 uL |
| 5 | Blank | N/A | 20 uL | 20 uL |

Incubate for 15-20 min then add 2 mL PBS to each FACS tube
Centrifuge @200g for 5 min
Pour of supernatant and re-suspend each tube in 300 uL PBS

Make up an additional FACS tube with *20 uL* of rainbow beads and 150 uL of 1X assay buffer

Also in this 30 mins: Prepare the Millipore Autophagy reagent B w/Benzonase for 8 wells (6+2 in excess)

Reagent	Volume (µL)
10X Millipore Autophagy Reagent B	80
Sigma sterile Water	720
Benzonase Nuclease	2
Sigma sterile Water	123

- **Dilute 80 µL of 10X Millipore Autophagy Reagent B in 720 µL of Sigma sterile water (1:10 dilution in a 1.5ml Eppendorf)**– *P100 & P1000*

- **Add 2 µL of Benzonase (stored @-20°C in glycerol) to 123 µL of distilled water in a 500ul Eppendorf tube** – *P10 & P200*

- *Bezonase is 250 units/µL so 2 µL in 125 µL = 500 units in 125 µL = 4 units/µL = 4000 units/mL*

- **Add 10 µL of diluted Benzonase to 790 µL of the now 1X Millipore autophagy reagent B**

- *We are taking 10 µL out of a solution with 4 units/µL (4000 units/mL) = 40 units/10 µL.*

- *We add this 10 µL to 790 µL of B solution to get 800 µL total (8 fold dilution) = 40 units in 800 µL = 0.05 units/ µL = 50 units/mL.*

16. Centrifuge the tubes @ 200g for 5 min (centrifuge by the sink).

17. Whilst the samples are being centrifuged, label 2*1.5 mL eppendorfs for the MS replicates. Make sure one clearly states which has received Millipore autophagy reagent A and which hasn't.

18. Label the lid of a 96 V-well plate highlighting which wells are being used. Make sure to include which wells have had Millipore autophagy reagent A added and which haven't

19. Discard the supernatants (pour and blot) and transfer the cell pellets for flow cytometry into the labelled wells of a 96 V-well plate (put everything from the tube into the well). – P100

20. For MS replicates, add 100 µL PBS in a circular motion to each tube, and then transfer into the labelled 1.5 mL eppendorf. Repeat this step by adding another 100 µL PBS to the tube and transferring into the appropriate Eppendorf. – P100 - Freeze replicates at -20°C

21. Add 100 µL PBS to each well – P100.

22. Centrifuge @ 200g for 5 min (select program #1 on the well-plate centrifuge) and discard supernatant by flicking off and gently dabbing on clean white tissue (Centrifuge for plates below microscope – make sure the balance has 200 µL of fluid in matching wells to the experimental plate)

23. While centrifuging, make up the antibody mix in a 500 µL eppendorf::

Fluorophore/Reagent	Surface Marker	Total volume for 6+2 replicates (*µL)*
*PBS/BSA 1%**	*N/A*	*280*
Pblue	*CD45*	*8*
PE	*CD16*	*8*
A647	*CD66b*	*4*

Already made up.

24. Add 50 µL antibody mix for each well (1 µL CD45 Pblue, 1 µL aCD16 PE, 0.5 µL CD66b A647 in 50ul PBS/BSA 1% *per well*).

25. Cover with lid and incubate @ RT for 10 min in the dark

26. Add 100ul PBS to each well

27. Centrifuge @ 200g for 5 min (select program #1 on the well-plate centrifuge). – P100.

28. Flick off and gently dab supernatant

29. Add 100ul of diluted Millipore autophagy reagent B containing Benzonase to each well. P100.
Gently mix once with the pipette (UP-DOWN).
As this is permeabilizing the cells take extra care with the samples from this stage onwards.

30. <u>Immediately Centrifuge @ 200g for 5 min (select program #1 on the well-plate centrifuge).</u>

31. While centrifuging make up FITC antibody mix

Reagent	Volume/*well µL*	Total Volume needed (*µL)*
Millipore 1X Assay Buffer*	95	760
Millipore 20X LC3B FITC	5	40

*Already made up

32. Remove supernatant (pour off) and incubate with 100ul of 20X LC3B FITC diluted to 1X in 1X Assay buffer. - P1000 & P50.

33. Cover with plate lid and incubate @RT for 20 min in the dark

34. Add 100 µL of Millipore 1X assay buffer to **each well** as prepared earlier. –P100.

35. Centrifuge @200g for 5 min (select program #1 on the well-plate centrifuge) (Flick off).

36. Whilst this is centrifuging, label a FACS tube for each replicate, make sure it is clear which ones have received Millipore Autophagy reagent A and which haven't.

37. Re-suspend in assay buffer (300 μl) in a labelled FACS tube for each replicate. – P200.

    Add 150 µL of assay buffer to each well, GENTLY MIX (UP-DOWN-UP). Then add to FACS tube.
    Add another 150 µL of assay buffer to each well, GENTLY MIX (UP-DOWN-UP). Then add to FACS tube.

38. Prepare samples for transport
    Places tubes in a FACS rack and cover in tin foil.

Estimated total time of the procedure: **3hrs**

FACS Aria III set-up and experimental set-ups:

Cytometer set-up checks:	Voltages:
- Ensure 1.5 black side scatter filter insert is in - Ensure 85micron filter is in - FSC Threshold: 12, 0000 - Drop 1: 200-250 - Pixel width: 14 - Sweet spot to lock the stream	- FSC – 150 - SSC – 280 - FITC – 450 - PE – 400 - APC – 520 - PBlue - 460

1. Turn on the computer and turn on the machine using the big green button on the side.

2. Run fluidics start up → follow instructions on screen

3. Optimise the stream to achieve a stable gap of 14 by manually adjusting the amplitude. When optimized, hit the sweet spot button at the top of the stream window to lock the settings.

4. Select all fluorophores under the parameter tab in the cytometer window and amend voltages to those outlined in the table above.

5. Prior to compensation, check FSC and SSC voltages are correct.

    - IMPORTANT: at this stage ensure Area (A) and height (H) are selected for FSC to allow for doublet gating

6. To check these, run the tube containing whole lysed blood.

    - Make a new specimen and tube and check using a dot plot in a global worksheet that granulocytes, mononuclear cells, and lymphocytes are easily detectable with room to spare towards the end of the X axis as shown in the figure below. DO NOT SAVE THS DATA.

7. Set up compensation;

    - New work book → ensure settings are correct (consult the bottom of this document for cytometer settings).
    - Go to the menu bar, click EXPERIMENT, then go down to COMPENSATION SETUP and then click CREATE COMPENSATION CONTROLS. Make sure a tick is next to the "include blank" (keep labels as 'generic').
    - Make a gate around the beads, right click and apply to all sheets.
    - Run through all once to quickly 'eye' data to find any obvious voltage errors.
    - Begin compensation at 5,000 events/s → complete all tubes → go to 'Experiment' tab→compensation → check and adjust all gates to get all positive cells→calculate compensation→link and save this.

- Create new specimen → right click compensation check → new cytometer settings → right click cytometer setting created and link set-up to that saved on first run.
- Create all 9 compensation tubes manually and label accordingly. Run all tubes again, checking each fluorophore against the other – create a new global worksheet to view these and new dot plots (one for FSC vs SSC, and one for the fluorophores)
- If there is an error with compensation (e.g., under or over compensation) then the newly calculated compensation that is visible in the CYTOMETER window needs to be adjusted (increased or decreased) for the fluorophores exhibiting problems. These numbers should be written down ready to edit into the compensation matrix. Do this, see the next step. (Tip: X-% from Y = matrix X axis against matrix Y axis).
- To edit the compensation matrix (if required), create your new experiment folder→create a new specimen→create new cytometer settings→link setup→click 'EDIT'→select the spectral overlap tab→adjust as required then save and link to the new specimens to follow.
- Then apply these compensation settings.
- You are ready to begin experimental tubes

7.   Once compensation has been set up, create new specimen, label the tubes accordingly then go into 'labels' tab and place the surface marker protein label next to the antibody it is represented by (i.e., FITC = LC3B).

8.   Run all samples. When recording event, make a gate around the cells relevant to that tube (neutrophils), this is P3, record 10,000 events in this gate, specifically.

a.   ALWAYS ensure the tube doesn't run low on fluid or else the stream will be disrupted.

9.   Once you have done all experimental tubes, run the rainbow beads through at 5,000 events (stopping gate all events).

Troubleshooting:

-      If the stream is disrupted, unload the tube, stop the stream, remove the 85micron nozzle and dab with paper towel. If this doesn't resolve the issue complete a backwash step (Cytometer→Cleaning→Backwash).

-      A line clean can also be found in the same menu bar if also required.

-      If cells are not appearing on the chart, unload the tube, give another vortex and reload.

-      If the stream values are disrupted, stop the stream, adjust the amplitude as required (typically 16-18) and sweet spot again.

Shut down:

-      To shut down, save all work to a USB or return later→cytometer→fluidics shut down. Follow the instructions on screen (attaching nozzles to EtoH tank, replace closed loop nozzle, run clean, de-pressurize sheath tank, run cleaning solution through, shut down machine, then software, then large green button for whole machine.)