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INTERINDIVIDUAL VARIABILITY IN PERCEIVED APPETITE AND APPETITE-RELATED HORMONE RESPONSES TO EATING AND EXERCISE IN HUMANS

by

Fernanda Reistenbach Goltz

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy of Loughborough University

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ABSTRACT

As obesity rates continue to rise worldwide, scientific interest in the area of appetite regulation has increased in an attempt to identify strategies that can prevent energy overconsumption and body weight gain. Appetite regulation is complex and involves many different physiological and psychological factors, allowing for great interindividual variability. Recently, some studies assessing appetite and energy intake responses to meal or exercise interventions have shifted the focus on presenting findings exclusively as group means to assessing individual responses and exploring interindividual variability. However, important methodological limitations may have impaired the detection of true interindividual variability, and gold standard study design and statistical approaches that address these limitations have been recently suggested. Therefore, this thesis aimed to assess the reproducibility and quantify the interindividual variability in appetite responses to acute exercise and to a standardised meal, and to explore the influence of genetic, physiological and behavioural characteristics on fasting and postprandial appetite-related outcomes. To achieve this, a total of 145 healthy men and women were recruited into four experimental studies.

The first experimental study (Chapter 4) demonstrated, using a replicated crossover design, that young men exhibited reproducible appetite responses after 60-min of fasted treadmill running at 70% peak oxygen uptake. True interindividual variability was observed in acylated ghrelin, total peptide YY (PYY) and perceived appetite responses over and above any random within-subject variability and measurement error, even after adjustment for individual baseline measurements. In the second experimental study (Chapter 5), the fat mass and obesity-associated gene (FTO) was not significantly associated with fasting or postprandial perceived appetite, acylated ghrelin, total PYY, insulin, glucose and leptin in healthy men and women, with or without the addition of physiological and behavioural covariates in the statistical models. While fasting leptin, glucose and insulin and postprandial insulin concentrations were associated with adiposity outcomes, the associations between fasting and postprandial acylated ghrelin, total PYY and general or abdominal adiposity were small. The third experimental study (Chapter 6) employed a replicated crossover design to demonstrate that the reproducibility of appetite responses to a standardised meal (5025 kJ) is generally good in healthy men. True interindividual variability was present in perceived appetite, acylated ghrelin, total PYY, insulin and glucose responses to the meal beyond any random within-

subject variation over time, but the magnitude of change in postprandial appetite responses was not influenced by the FTO gene. The final experimental study (Chapter 7) consisted of a pilot study which showed no significant association between brown adipose tissue activity assessed with thermal imaging, FTO genotype and fasting and postprandial acylated ghrelin, total PYY, insulin and glucose in healthy males.

Collectively, these studies demonstrate that appetite responses to acute exercise and to eating are reproducible in healthy men, and true interindividual variability exist in these responses. However, the FTO genotype was not significantly associated with fasting and postprandial perceived appetite and appetite-related hormones, and further studies are warranted to investigate other individual characteristics that may moderate the observed interindividual variability. These findings highlight the importance of exploring individual differences in appetite responses in the context of the prevention and/or management of obesity.

Keywords: Acute exercise, acylated ghrelin, appetite, brown adipose tissue, FTO, glucose, hormones, insulin, interindividual variability, leptin, peptide YY, replicated crossover design, reproducibility.

ACKNOWLEDGEMENTS

I should probably start by apologising for the length of this section, but this is surely my favourite part of this thesis as I am given the chance to try my best to put into words how grateful I am for having some very special people by my side during this crazy journey called PhD. If someone had told me ten years ago that I would end up completing a PhD in England, I would have found it very hard to believe. Not because I did not feel capable of it, but because it felt like a very distant dream. I have no doubt that the wonderful opportunities I have had in my life, including this one, only happened because of the amazing people I was lucky enough to cross paths with. The PhD has not been an easy journey, I must say, and it felt rather endless at times. Nevertheless, it was also full of great moments, achievements and I feel like I have never learned and grown so much in my life. I would like to thank each of the following people, who continually supported me and kept me going through these forty-six months, from moving to a new country to delivering this thesis.

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PREFACE

Peer-reviewed publications

Goltz FR, Thackray AE, Varela-Mato V, King JA, Dorling JL, Dowejko M, Mastana S, Thompson J, Atkinson G, Stensel S. Exploration of associations between the FTO genotype, fasting and postprandial appetite-related hormones and perceived appetite in healthy men and women. Appetite. 2019;142:104368 [Epub ahead of print].

Goltz FR, Thackray AE, Atkinson G, Lolli L, King JA, Dorling JL, Dowejko M, Mastana S, Stensel S. True interindividual variability exists in postprandial appetite responses in healthy men but is not moderated by the FTO genotype. J Nutr. 2019;149(7):1159-69.

Goltz FR, Thackray AE, King JA, Dorling JL, Atkinson G, Stensel DJ. Interindividual responses of appetite to acute exercise: a replicated crossover study. Med Sci Sports Exerc. 2018;50(4):758-68.

Conference presentations and published abstracts

Goltz FR, Thackray AE, Varela-Mato V, King JA, Stensel DJ. Acylated ghrelin response to a standardised meal is associated with the glucose response, but not with visceral adipose tissue or liver fat levels in healthy men and women. 25th European Congress on Obesity 2018, Vienna, Austria. Abstract published in Obesity Facts 2018;11(suppl 1).

Goltz FR, Thackray AE, King JA, Dorling JL, Atkinson G, Stensel DJ. Inter-individual variability in the appetite, acylated ghrelin and total peptide YY response to acute exercise in young men: a replicated cross-over study. BASES Conference 2017, Nottingham, UK. Abstract published in Journal of Sports Sciences 2017;35(suppl 1).

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LIST OF ABBREVIATIONS

The following abbreviations are used throughout this thesis. All abbreviations are defined in the first instance they appear in each chapter.

^{18}F-FDG: ^{18}F-fluorodeoxyglucose

AA: Fat mass and obesity-associated gene homozygous minor allele

AT: Fat mass and obesity-associated gene heterozygous allele

AUC: Area under the curve

BAT: Brown adipose tissue

BMI: Body mass index

CI: Confidence interval

DNA: Deoxyribonucleic acid

EAT: Exercise activity thermogenesis

EDTA: Ethylenediaminetetraacetic acid

EE: Energy expenditure

EI: Energy intake

ELISA: Enzyme-linked immunosorbent assays

ES: Effect size

FFM: Fat-free mass

FM: Fat mass

FTO: Fat mass and obesity-associated gene

GH: Growth hormone

IRT: Infrared thermography

MCID: Minimal clinically important difference

MRI: Magnetic resonance imaging

MVPA: Moderate-to-vigorous physical activity

NHS: National Health Service

PAEE: Physical activity energy expenditure

PET-CT: Positron emission tomography – computed tomography

PFC: Prospective food consumption

PYY: Peptide tyrosine-tyrosine

REE: Resting energy expenditure

RMR: Resting metabolic rate

RPE: Ratings of perceived exertion

SCV: Supraclavicular

SD: Standard deviation

SE: Standard error

SNP: Single-nucleotide polymorphisms

TDEE: Total daily energy expenditure

TEF: Thermic effect of food

TT: Fat mass and obesity-associated gene homozygous major allele

UCP-1: Uncoupling protein 1

$\dot{V}O_2$: Oxygen uptake

CHAPTER 1

Introduction

The rapid increase in the prevalence of obesity has been a major concern worldwide, given the well-known association of high levels of body fat mass with metabolic complications such as type 2 diabetes, hypertension, dyslipidaemia, and coronary artery disease (Kopelman, 2007). Such medical conditions have a meaningful negative impact both on people's quality of life and on healthcare costs. In 2016, 39% of adults in the world were overweight and 13% were obese, meaning that worldwide obesity has nearly tripled since 1975 (World Health Organization, 2018). In England, 40% of men and 30% of women are overweight, and a further 26% of men and 27% of women are obese (National Health Service, 2018). The annual cost of obesity and overweight for the National Health Service (NHS) is estimated to be approximately £6.1 billion (Public Health England, 2017), and 617,000 admissions in NHS hospitals have obesity as a primary or secondary diagnosis per year (NHS, 2018). Predictions show that England could be a mainly obese nation by 2050 (Foresight Report, 2007).

Major efforts have been made in an attempt to understand the causes of the 'obesity epidemic' in order to propose effective interventions capable of changing the current scenario. People with obesity are often blamed for their own condition, as obesity is considered mainly an issue of lack of individual willpower, where people are eating too much food and doing too little exercise (Foresight Report, 2007). It should be noted, however, that it is unlikely that people today have less willpower than previous generations, and it is clear that our current society and living environments present major changes when compared to previous decades. The ‘obesity epidemic’ coincides with the increasing availability of highly palatable food with high energy density (Wren and Bloom, 2007), and part of the blame could be directed to the obesogenic environment, where food consumption is continuously promoted and physical activity is decreasing significantly (Blundell, 2011).

Obesity is a result of a continuous excess of energy intake over energy expenditure, and research investigating the mechanisms involved in the regulation of energy balance has gained increasing attention. Of note, the number of scientific publications on ‘appetite’ in the United States National Library of Medicine from the National Institutes of Health (PubMed.gov) has increased from 142 publications per year in 1979 to 1840 publications per year in 2018. Energy

homeostasis is a complex process, in which organs such as the stomach, intestine, pancreas, and adipose tissue send and receive information from the brain (Wren and Bloom, 2007). These organs are responsible for sending hormone signalling from the periphery via vagal afferents to the hindbrain and the hypothalamus, where signals are integrated with information from other brain regions (Murphy and Bloom, 2006; MacLean et al. 2017). An increasing number of studies have been investigating the peptide hormone signals originating from the gut and their role in appetite regulation, nutrient intake and metabolism, in an attempt to develop strategies to help in weight control (MacLean et al. 2017).

Physical activity and exercise are associated with a variety of health benefits and play an integral part in energy balance (i.e. via increasing energy expenditure). Furthermore, exercise can influence appetite-related hormones to some extent (Deighton and Stensel, 2014), and therefore, possibly impact on eating behaviour. Public health strategies to combat the global rise in obesity typically encourage higher physical activity levels or structured exercise programs in order to increase energy expenditure and achieve weight loss (Pontzer et al. 2016). However, increasing physical activity or exercise levels may not always lead to a negative energy balance and weight loss, due to possible compensatory responses which can be behavioural, such as increased energy intake, or metabolic, such as reduced resting metabolic rate (King et al. 2007; Pontzer et al. 2016).

It is important to highlight that any intervention targeting weight loss is likely to show variability among individuals and the success of an intervention is dependent upon individual appetite physiology, motivation and previous experiences (Senior et al. 2016). This individual variability should be taken into consideration and the average response of a group must be interpreted with caution as it may mask the true variation in the results observed in intervention studies (Blundell, 2011). Assessing biological and behavioural responses to interventions at the individual level can help to understand why some individuals are successful in achieving the expected results from varied interventions while others are not. Likewise, not everyone gains weight living in a strongly obesogenic environment, which raises the hypothesis of the existence of susceptible and resistant phenotypes. Certain individuals may possess predispositions that make them vulnerable to overeating (Blundell, 2011). In this regard, it has been suggested that interindividual variability exists in appetite and energy intake responses to a session of exercise, suggesting some individuals are more likely to increase energy intake after exercising than others (Finlayson et al. 2009; Hopkins et al. 2014). However, other studies

found that energy intake after a bout of exercise varies across occasions (Unick et al. 2015) and, in most cases, the interindividual variability in appetite and energy intake responses to acute exercise can be explained by normal day-to-day variation in measurements (King et al. 2017).

Before interindividual variability and any influencing factors are investigated in the responses to an intervention, it is crucial to examine whether the observed responses are reproducible across separate occasions, and to quantify the impact of normal day-to-day variation and measurement errors on the assessment of the outcomes of interest. The use of a replicated crossover study design, where all study participants perform at least two intervention and two control conditions, together with appropriate statistical analyses quantifying participant-by-response interactions, has been suggested as a gold-standard for the quantification of interindividual variability (Senn et al. 2011; Senn, 2016; Atkinson et al. 2018). If true interindividual variability can be detected, one important objective is to determine the factors that contribute to the variability seen between individuals.

Genetic approaches are a valuable tool that can help to understand possible causes of obesity and the large variability observed between individuals regarding food intake and weight control, as well as differences seen in the success of various interventions. The role of genetics in the aetiology of obesity has been recognized for a long time, but the identification of genes contributing to body weight gain has been relatively slow (Rankinen et al. 2010). It is evident that 'common obesity' is not caused by rare loss of function mutations previously revealed to be the responsible for severe obesity cases, but rather through the interaction between environmental factors and the individual susceptibility to these factors determined by genetics (Hess and Brüning, 2014). Genetic variants have been identified having a much smaller effect on weight, considering their individual effects, when compared with the aforementioned mutations. The 12 genes most strongly associated with obesity are estimated to account for only 1–2% of the variance in body weight, and each additional active allele from these genes contributes about 440 g of extra body weight (Blundell, 2011). However, summing of their effects, these variations define an individual's predisposition to gain weight in the face of environmental changes, involving multiple physiological and behavioural mechanistic pathways affected by each of the active alleles (Blundell, 2011; Hess and Brüning, 2014). Understanding how each of these genes contributes to weight gain is crucial to decipher the exact role of genetics in the causes of 'common obesity' (Hess and Brüning, 2014).

The fat mass and obesity-associated (FTO) gene is the strongest common genetic determinant of body weight identified so far; however, the mechanistic pathways and the contribution of the polymorphisms to the success of weight loss after an intervention are still controversial and require further study (Sailer et al. 2016). Available evidence suggests individuals with the at-risk FTO genotype possess an attenuated postprandial suppression of appetite (Karra et al. 2013), which may predispose to chronic energy overconsumption and lead to fat mass accumulation in the long term. Additionally, studies in rodents suggest brown adipose tissue may contribute to the obesity risk associated with the FTO genotype (Tews et al. 2013; Ronkainen et al. 2016), possibly through alterations in both energy expenditure and appetite regulation (Chondronikola et al. 2017; Li et al. 2018). A question with major public health relevance now is how the FTO genotype influences weight gain and responses to weight loss therapy. Given the alarming increase in obesity rates, it is crucial to understand whether true interindividual variability exists in appetite regulation and to identify potential influencing factors, so that the feasibility of targeted interventions with increased efficacy for weight loss or prevention of weight gain can be assessed.

Therefore, the aims of the experimental studies described in this thesis were three-fold:

1. To assess the reproducibility and quantify the interindividual variability in appetite responses to acute exercise;
2. To assess the reproducibility and quantify the interindividual variability in appetite responses to a standardised meal;
3. To explore the association between genetic, physiological, behavioural (i.e. physical activity and sedentary behaviour) characteristics and fasting and postprandial appetite-related outcomes.

It was hypothesized that true variability exists between individuals, over and above any random variability, in their appetite responses to acute exercise and to a standardised meal. Furthermore, it was hypothesized that FTO genotype, adiposity level and brown adipose tissue contribute to the variability observed between individuals in appetite-related outcomes.

A comprehensive review of the literature to date on the topics explored in the subsequent experimental chapters is presented in Chapter 2. The general methods employed in the experimental studies presented in this thesis are described in Chapter 3. The first experimental study is presented in Chapter 4, which employed a replicated crossover study design in order

to assess the reproducibility and interindividual variability in appetite responses to acute aerobic exercise in healthy young males. Chapter 5 presents the second experimental study, which consisted of a cross-sectional study investigating the associations between the FTO genotype, fasting and postprandial appetite-related hormones and perceived appetite in healthy men and women. The third experimental study is presented in Chapter 6 and consisted of a replicated crossover study assessing the reproducibility and interindividual variability in appetite responses to a standardised meal, as well as any moderating influence of the FTO genotype, in healthy males. Chapter 7 presents the fourth and final experimental study, consisting of a pilot study to explore the association between brown adipose tissue activity, FTO genotype and appetite-related blood parameters in healthy males. Finally, a general discussion is presented in Chapter 8, where the main findings from the research presented in this thesis are reflected upon and future research directions are highlighted.

CHAPTER 2

Literature review

2.1 Introduction

This chapter initially summarises the key mechanisms involved in the maintenance of energy balance, with special attention given to the regulation of appetite. Evidence relating to both physiological and psychological factors influencing appetite and energy intake is reviewed. This review then explores the effect of exercise on appetite and the potential influence exercise exerts on energy balance. This is followed by a detailed review of methodological approaches to examine reproducibility and to quantify interindividual variability in responses to an intervention, with a summary of the available evidence on reproducibility and interindividual variability of appetite and energy intake responses to exercise and to eating. The review ends by exploring the potential role played by the fat mass and obesity-associated (FTO) gene on appetite regulation, eating behaviour and energy balance.

2.2 Energy balance

Energy balance is defined as the status in which no substantial difference exists between energy intake and energy expenditure, where body weight remains stable. Energy intake entails the consumption of foods and drinks containing carbohydrates, protein, fat and alcohol, which will be digested in order to provide energy which can be utilised by the body. Energy expenditure combines the energy needed for the vital body functions i.e. resting metabolic rate, the energy used to digest food, and the energy expended when performing any type of physical activity. In a simplistic summary, when energy intake exceeds energy expenditure, the excess energy is stored by the body in the form of adipose tissue, which in the long term, can lead to overweight and obesity. When the opposite occurs, i.e. energy expenditure exceeds energy intake, body energy depots are mobilized in order to provide energy, which typically results in body weight loss.

However, maintaining energy balance is more complex in practice. Energy balance is a dynamic, non-linear process, rather than a static process (Manore et al. 2017). Energy balance, and consequently body weight, is regulated by genetic, metabolic, environmental, social, and behavioural factors, influencing both energy intake and expenditure in varied proportions

according to the individual and the circumstances (Manore et al. 2014). Additionally, energy expenditure is influenced by energy intake, diet macronutrient composition and energy density, as well as the timing of food intake (Manore et al. 2017). The effect of physical activity and exercise on energy expenditure and type of fuel used can also vary significantly, depending on the type, intensity and duration of the activity (Manore et al. 2017). When energy balance is disturbed, hormonal and neuroendocrine systems act together via central and peripheral signals to restore homeostasis (Moreno and Lanni, 2016).

The human body is equipped with various physiological mechanisms to protect energy stores, which were essential for preserving life in the past when humans experienced starvation for longer periods as a result of the challenges faced to acquire food on a daily basis. While the environment in which most humans live today has changed dramatically, with high-energy foods readily available at any time, the physiological mechanisms to protect energy stores seem to be preserved. In addition, major changes in lifestyle are observed, leading to drastic reductions in physical activity energy expenditure. As a result, it is not surprising that obesity rates continue to rise worldwide (World Health Organization, 2018), and the currently available strategies for the prevention and treatment of obesity do not seem effective enough to combat the obesity epidemic.

Weight loss resultant of strategies focusing on reducing energy intake, increasing energy expenditure, or a combination of both, is often less than expected, with some individuals showing more success than others (Manore et al. 2014). It is often expected that a deficit of 500 kcal per day will result in weight loss of 1 lb (or 0.45 kg) per week. This estimation originated from a calculation assuming exclusive loss of adipose tissue, which is not realistic (Hall, 2008). Furthermore, when one component of energy balance is altered, it is likely that co-ordinated responses in other factors influencing energy balance will happen in an unpredictable way, reducing the gap between energy intake and expenditure (Manore et al. 2014; Casanova et al. 2019). Compensatory metabolic and behavioural responses to energy deficit are likely to occur, such as reduced resting metabolic rate, increased muscular efficiency and increases in energy intake, which may undermine weight loss and also stimulate weight regain (Pontzer et al. 2016; Casanova et al. 2019).

Understanding the mechanisms that regulate energy balance, and consequently body weight, and how they are affected by individual and environmental factors is essential for the development of strategies aiming to prevent weight gain and to treat overweight and obesity.

2.3 Appetite regulation

Appetite is influenced by both physiological and psychological factors. The physiological regulation of appetite involves many neuroendocrine inputs that have an impact on hunger and satiety, with a number of tissues, organs and hormones sending and receiving signals to and from the brain (MacLean et al. 2017). The gastrointestinal tract is the largest endocrine organ in the body of lean individuals, and gut hormones have a prime role in signalling nutrient intake according to the pattern of eating (Neary and Batterham, 2009). Many gut peptides are believed to play unique roles in hunger and satiety signalling. Satiation, or meal termination, is likely initiated by neural input from the stomach to the brain signalling gastric distension after food intake, followed by the release of gut hormones able to sense absorption of nutrients and signal satiety i.e. post-meal inhibition of eating (Stensel, 2010). These hormones include cholecystokinin (secreted from the duodenum and jejunum), glucagon-like peptide 1, oxyntomodulin and peptide YY (PYY) (secreted from the small and large intestines) and pancreatic polypeptide (secreted from the pancreas). Gut hormones act as episodic signals because they are released in harmony with episodes of eating. They can signal satiation and satiety to the brain via the vagus nerve or via blood perfusing the hypothalamus (Stensel, 2010). A brief summary of the main gut hormones and their effects on appetite on a meal-to-meal basis is presented in Table 2.1.

Table 2.1 Peripheral effects of selected appetite-regulating gut hormones (adapted from Perry and Wang, 2012).

Gut hormone	Site of synthesis	Peripheral effect on food intake
Cholecystokinin	Intestinal L-cells	Decrease
Ghrelin	Stomach	Increase
Glucagon-like peptide 1	Intestinal L-cells	Decrease
Oxyntomodulin	Intestinal L-cells	Decrease
Pancreatic polypeptide	Pancreas/colon	Decrease
Peptide YY	Intestinal L-cells	Decrease

Tonic hormonal signals, on the other hand, regulate energy balance over the long term, indicating the level of energy storage in the body i.e. the degree of adiposity, such as insulin (released from the pancreas) and leptin (released from adipose tissue) (Stensel, 2010; Perry and Wang, 2012). Fat-free mass also modulates appetite, as it is the primary determinant of resting metabolic rate, which consists of 60 to 70% of total energy expenditure. The exact mechanism through which fat-free mass communicates energy requirements to the brain in order to influence food intake is still unknown. Additionally, an increasing body of evidence points to the important role played by the gut microbiome and bile acids, in a complex interplay with gut hormones, to regulate appetite (MacLean et al. 2017).

2.3.1 Ghrelin

In contrast to all the remaining appetite-related gut hormones identified to date, which act as satiety signals, ghrelin is the only known appetite-stimulating peptide hormone. Ghrelin was first discovered in 1999 (Kojima et al. 1999) and its important role in the regulation of appetite and food intake was discovered shortly after (Tschöp et al. 2000). Over the years, ghrelin has been suggested to have many other physiological actions such as stimulation of gut motility and gastric acid secretion, modulation of sleep, taste sensation and reward seeking behaviour, regulation of glucose metabolism, suppression of brown fat thermogenesis, modulation of stress and anxiety, protection against muscle atrophy, and improvement of cardiovascular functions such as vasodilatation and cardiac contractility (Müller et al. 2015).

Ghrelin is the natural ligand of growth hormone (GH) secretagogue receptor released predominately from the gastric cells within the stomach. Its blood levels are increased with hunger sensations and its receptor is located in the hypothalamic neurons responsible for regulating food intake (Müller et al. 2015). Two different forms of the hormone have been described in the circulation: acylated and non-acylated. Acylated ghrelin results from the addition of an acyl group to serine-3 and makes up only 10 to 20% of total ghrelin but seems to be the responsible to increase appetite and feeding, as it is able to bind to the GH secretagogue receptor, cross the blood-brain barrier, and therefore exert its effects at the hypothalamic level (Adams et al. 2011). Ghrelin is often called “hunger hormone” as it acts as a meal initiation signal, informing the gastrointestinal energy status to the central nervous system in order to regulate food intake and energy expenditure (Müller et al. 2015). Peripheral ghrelin administration induces hunger and food intake in humans (Wren et al. 2001), and postprandial changes in ghrelin are well correlated to changes in perceived hunger under

natural feeding circumstances (Gibbons et al. 2013). Additionally, ghrelin concentrations before an *ad libitum* meal have been positively associated with energy intake during the meal (Gibbons et al. 2013).

The regulation of ghrelin release is a complex process involving both the sympathetic nervous system and the gastrointestinal tract (Wren et al. 2001). Food intake is the main factor influencing circulating ghrelin levels, which increase shortly before meal initiation and fall back to baseline levels within the first hour after eating (Cummings et al. 2001; Müller et al. 2015). Ghrelin increases hunger and energy intake in the short- and long-term and has an effect in both homeostatic and reward-related feeding, shifting food preference toward diets rich in fat (Cummings, 2006; Karra et al. 2013). Additionally, studies in rodents indicate ghrelin increases adiposity through the stimulation of enzymes promoting fatty acid storage and decreasing fat oxidation, which occurs independently from changes in food intake or energy expenditure (Müller et al. 2015). The magnitude of ghrelin postprandial suppression is proportional to the energy and macronutrient content of the meal (Müller et al. 2015). When meals composed primarily of carbohydrates, proteins or lipids were ingested, lipids were shown to be the least effective suppressors of ghrelin (Monteleone et al. 2003; Foster-Schubert et al. 2008).

Available evidence suggests ghrelin acts against prolonged energy deficiency to maintain long-term energy balance (Müller et al. 2015). Circulating ghrelin levels have been shown to increase after diet-induced weight loss (Kotidis et al. 2006; Iepsen et al. 2016); however, recent evidence shows that ghrelin concentrations gradually return to baseline values after 1 year of weight-loss maintenance (Iepsen et al. 2016). Fasting plasma ghrelin concentrations are inversely correlated with body mass index (BMI) (Tschöp et al. 2001; Lindeman et al. 2002; Katsuki et al. 2004; Sondergaard et al. 2009). Ghrelin levels are low in obesity, which suggests high levels of adipose tissue affect ghrelin concentrations, rather than ghrelin causing overeating which led to obesity (Shiiya et al. 2002). Conversely, ghrelin levels are high in individuals with cachexia or anorexia nervosa, suggesting a link between adiposity and ghrelin regulation, although the exact mechanisms involved are still to be determined (Müller et al. 2015). Additionally, it has been suggested that visceral fat might influence circulating ghrelin levels more than total body fat, and this is likely to be caused indirectly via altered levels of metabolites or hormones, but further evidence is needed to confirm this hypothesis (Sondergaard et al. 2009).

2.3.2 Peptide YY

Peptide tyrosine-tyrosine (peptide YY or PYY) was first discovered in porcine intestine in 1982 (Tatemoto, 1982), but its role in energy homeostasis was only revealed in 2002 (Batterham et al. 2002). PYY is the most studied gut peptide hormone known to induce satiety, as it has a potent anorectic effect and influences the overall intake of food during a meal predominantly by influencing central appetite-regulating circuits and brain regions involved in food reward (Batterham et al. 2007; Kullmann et al. 2016). The key areas where PYY acts in order to mediate its anorectic effects are the hypothalamic arcuate nucleus and brainstem regions (Manning and Batterham, 2014). PYY and ghrelin are likely to have complementary effects in modulating appetite through both homeostatic and reward centres (Manning and Batterham, 2014).

PYY is synthesized and released from L-cells found predominantly within the distal gastro-intestinal tract and is released into the circulation in a nutrient-dependent manner (Karra et al. 2009). Meals with higher fat content promote higher circulating PYY levels, in comparison with meals high in carbohydrates (Essah et al. 2007; Gibbons et al. 2013). PYY levels are low in the fasting state and rapidly increase in response to meal ingestion, in proportion to the energy content, reaching a peak 1–2 h after a meal and remaining elevated for several hours (Karra et al. 2009; Manning and Batterham, 2014). PYY exists in human blood in two forms, PYY_{1-36} and PYY_{3-36}, the latter being the predominant circulating form which preferentially binds to the inhibitory presynaptic Y2 receptors expressed in the appetite regulatory centre of the arcuate nucleus within the hypothalamus (Kanaley et al. 2014). Increases in PYY concentration were shown to be correlated with increased perceived feelings of satiety in some studies (Guo et al. 2006; Le Roux et al. 2006), but not others (Gibbons et al. 2013). It has been suggested that PYY is involved in energy balance regulation by a combination of two actions i.e. reducing food intake and increasing energy expenditure, although the mechanisms underlying the latter remain to be elucidated (Guo et al. 2006; Karra et al. 2009).

Circulating PYY has shown to be negatively associated with adiposity levels (Batterham et al. 2003; Guo et al. 2006). Individuals with obesity show lower levels of circulating PYY, and exogenous administration of PYY reduces food intake in both healthy people and in people with obesity, indicating that individuals with obesity remain sensitive to the anorectic actions of the hormone (Batterham et al. 2003). The mechanisms involved in the reduced PYY levels observed in people with obesity remain to be elucidated. Perturbations in energy balance

caused, for example, by very low energy diets, have been shown to reduce both fasting and postprandial circulating PYY levels, which persists for long periods (Sumithran et al. 2011), suggesting a role of PYY in the relapse often observed among people who lose weight. However, recent evidence shows that successful weight maintenance after 1 year of diet-induced weight loss is accompanied by increased postprandial responses of PYY_{3-36}, indicating that an increase in appetite-inhibiting mechanisms may contribute to the success of a weight loss intervention (Iepsen et al. 2016).

2.3.3 Glucose and insulin

Glucose is the most important fuel for the brain and its availability is sensed by neurons mainly located in the hypothalamus and brainstem. Neurons modulate the release of anorexigenic and orexigenic neuropeptides according to glucose availability. Similarly, circulating glucose concentrations are sensed in the body periphery and reported to the brain (Schultes et al. 2016). While many studies suggest appetite is directly influenced by circulating glucose concentrations, with meal initiation and satiation being influenced by glucose availability, a meta-analysis of test meal studies failed to find any association between blood glucose and perceived appetite in individuals of normal weight and individuals who were overweight (Flint et al. 2007). The precise role of neuronal glucose sensing in the regulation of appetite and eating behaviour in humans under normal physiological conditions is still controversial. Fluctuations in circulating glucose might influence appetite regulation by concomitant changes in the secretion of insulin, which itself affects appetite regulation (Schultes et al. 2016).

Insulin was discovered in 1922 as a potential therapeutic option to reverse type 1 diabetes and its role in non-diabetic individuals was determined subsequently (Flier and Maratos-Flier, 2017). Insulin is produced in the β-cells of the pancreas and is the primary factor responsible for glucose uptake in most peripheral tissues and for the suppression of glucose secretion by the liver, lowering glucose in the blood (Woods et al. 2006). Plasma insulin is low during fasting and increases during and immediately after meal consumption or glucose administration, as a result of blood glucose increases. Insulin enters the brain where it reacts with insulin receptors on neurons, triggering diverse effects on energy homeostasis, such as reduction in food intake and body weight (Woods et al. 2006; Filippi et al. 2013). Insulin receptors are expressed in many areas of the brain, especially in the arcuate nucleus in the mediobasal hypothalamus (Woods et al. 2006; Filippi et al. 2013).

Administration of intranasal insulin has been shown to decrease food intake and increase satiety in humans (Benedict et al. 2008; Hallschmid et al. 2012), and insulin increases the brain sensitivity to short-term satiety signals such as gut appetite-related hormones (Woods et al. 2006). Additionally, the postprandial increase in insulin has been consistently negatively associated with perceived hunger and positively associated with perceived satiety in normal weight individuals (Flint et al. 2007). Nevertheless, when dynamic fluctuations in blood glucose and insulin were induced by intravenous glucose infusion, no effect on perceived hunger, satiety or fullness was observed, challenging the role played by circulating glucose and insulin in the short-term regulation of appetite (Borer et al. 2009; Schultes et al. 2016).

The amount of insulin secreted into the blood changes in parallel with body weight changes, as an adiposity signal to the brain, with lean individuals having lower levels than individuals with obesity. Additionally, insulin is directly correlated with visceral fat, consisting of a risk factor for metabolic syndrome (Woods et al. 2006). Higher insulin levels are perceived by brain regions in order to adjust key neural circuits of energy balance, interacting with many other factors to increase the sensation of satiety (Woods et al. 2006; MacLean et al. 2017). However, under chronic conditions of positive energy balance, the central and peripheral resistance to the action of insulin reduce its influence on appetite regulation (MacLean et al. 2017). The insulin receptor-facilitated transport through which insulin enters the brain is reduced in obesity (Woods et al. 2006), and postprandial changes in insulin are not associated with perceived appetite in individuals with overweight (Flint et al. 2007). After acute and long-term fat mass loss, circulating levels of insulin are reduced and insulin sensitivity is improved (Iepsen et al. 2016).

Apart from signalling in the hypothalamus in order to regulate energy homeostasis, central insulin mediates non-homeostatic feeding, i.e. eating for pleasure, by signalling within mesolimbic reward circuits, which mediate different aspects of reward (Tiedemann et al. 2017). The palatability of foods is decreased in the fed state and this effect seems to be resultant of the increased insulin release after food intake, possibly in an attempt to prevent overconsumption of palatable foods (Tiedemann et al. 2017). Additionally, increases in insulin concentration after meal ingestion suppress the motivation to eat and the motivation for physical activity, and may be related to the display of somnolence after the meal (Borer, 2010).

2.3.4 Leptin

The identification of the obese gene in 1994, named ‘ob’ in rodents and ‘lep’ in humans, led to the characterization of the key actions of its encoded protein leptin in energy balance and metabolism (Zhang et al. 1994). Leptin is a well-characterized peptide hormone synthetized and secreted into the blood mainly from adipocytes, reflecting long-term body energy reserves. Leptin is one of the major adipokines and its circulating levels are positively correlated to the level of body adiposity (MacLean et al. 2017; Rostás et al. 2017). The gastric mucosa also secrets large amounts of leptin into the gastric juice, independently from the regulation of the secretion from the adipose tissue (Cammisotto et al. 2010). After secretion by the gastric mucosa, leptin connects to receptors on the intestinal epithelial cells and reach the central nervous system to control food intake and nutrient absorption on a meal-to-meal basis, in combination with the action of other appetite-related hormones. The secretion of adipose and gastric leptin is coordinated in order to manage food processing and energy storage (Cammisotto et al. 2010).

Higher leptin concentrations convey messages to brain regions such as the hypothalamic arcuate nucleus in order to reduce food intake and increase energy expenditure (Borer et al. 2009; MacLean et al. 2017). Of note, leptin has been shown to increase energy expenditure through the activation of non-shivering thermogenesis in brown adipose tissue (Fruhwürth et al. 2018). However, the tonic influence on energy homeostasis has a stronger influence under conditions of adipose tissue depletion, where leptin acts as a signal in order to protect the organism from critical reductions in fat mass that threaten reproductive capacity and survival (Rosenbaum and Leibel, 2014). On the contrary, when energy overconsumption is sustained for longer periods, central and peripheral resistance to the action of leptin reduces its influence on appetite regulation and food intake (MacLean et al. 2017; Fruhwürth et al. 2018), meaning that the major function of leptin in humans is to signal inadequate energy stores rather than to prevent the storage of excessive body fat mass (Rosenbaum and Leibel, 2014; Flier and Maratos-Flier, 2017). In addition to its homeostatic actions, leptin also modulates neural circuits of motivation and reward via the mesolimbic dopaminergic system to control the motivation to seek and consume food (Fruhwürth et al. 2018).

Congenital absence of leptin leads to hyperphagia and morbid obesity very early in childhood, which is reversed by leptin administration (Borer et al. 2009; Cammisotto et al. 2010). High levels of leptin are also an important etiological factor of cardiometabolic syndrome and

inflammatory disorders, and exercise training has been suggested to be able to reduce circulating leptin levels even when only small changes in body weight occur (less than 5%) (Rostás et al. 2017). Additionally, lean body mass has been suggested to be negatively associated with leptin concentrations, independently of fat mass (Marshall et al. 2000). The administration of leptin can promote a modest reduction in energy intake in weight-reduced individuals but is less effective in promoting weight loss on its own. Nevertheless, the efficacy of leptin in reducing energy intake during weight loss is much less than the effects of leptin repletion in individuals aiming to sustain a reduced body weight (Rosenbaum and Leibel, 2014).

2.3.5 Hedonic aspects of appetite

Energy overconsumption and weight gain cannot be explained solely by the failure of homeostatic mechanisms to maintain a healthy body weight, as food intake is also influenced by psychological and behavioural aspects. It is clear that humans do not eat only when feeling physiological hunger, and the current obesity epidemic proves that homeostatic body weight regulatory mechanisms can be overridden by other factors (Borer, 2010; Berthoud, 2011). Appetite is rather controlled by a psychobiological system that signals hunger, satiation and satiety, which are translated into food intake (Simon et al. 2017; Beaulieu et al. 2018). Hedonic eating refers to all factors which affect eating behaviour but are not considered to be part of the homeostatic control of appetite, including cognitive, reward and emotional factors (Berthoud, 2011). Psychological aspects such as cognitive restraint and disinhibition are robust predictors of energy intake, alongside physiological factors (Beaulieu et al. 2018; Hopkins et al. 2019). Food hedonics reflect the process of liking, i.e. the degree of sensory pleasure obtained from foods, and wanting, i.e. the motivation and attraction for foods (Beaulieu et al. 2018).

Environmental factors such as food availability, portion sizes, energy density, palatability, variety, and presence of food cues also play a big role in influencing eating behaviour. Equally, agricultural policies, pricing strategies, socioeconomic status, level of education, and stress vulnerability can influence food choices (Zheng et al. 2009). Environmental circumstances enhance the motivation for food seeking when energy deprived and after body fat loss but also facilitate overeating of highly palatable and energy dense foods in normal conditions (Borer, 2010). A combination of physiological and psychological factors is likely to determine individuals who become obese and individuals who can maintain a healthy body weight in the

modern environment with constant access to energy dense foods and little energy expenditure through physical activity.

As opposed to metabolic feedback signals and neural systems, which are located mainly in the brainstem and hypothalamus, the neural pathways and functions responsible for hedonic eating are located mostly in corticolimbic structures, and have similarities to addiction mechanisms (Berthoud, 2011; Simon et al. 2017). As occurs with other behaviours, the feelings of satisfaction and well-being generated by eating certain foods result in strong motivation to repeat the same pattern of behaviour (Zheng et al. 2009). People with obesity often report higher preference for high-fat and high-sugar foods, which may indicate a decreased sensitivity to sweet and fatty tastes, but whether this is the cause or consequence of obesity remains unclear (Andriessen et al. 2018). It has been suggested that exercise-induced changes in the hedonic response to food is associated with compensations in energy intake after exercise and may partially explain why some individuals fail in losing weight through exercise interventions (Finlayson et al. 2009). On the other hand, it was recently shown that substantial weight loss induced by low-calorie dieting can change postprandial appetite and food preferences in favour of a decreased food intake (Andriessen et al. 2018).

Importantly, a considerable functional overlap between homeostatic and hedonic mechanisms of appetite control and energy balance has been reported, as appetite-related hormones such as leptin, insulin, PYY and ghrelin also act to modulate the wanting of food and reward processing (Zheng et al. 2009; Simon et al. 2017; Beaulieu et al. 2018). Thus, the measurement of perceived appetite and desire for food, together with measurements of appetite-related physiological factors, such as appetite-related hormones, is crucial for the holistic understanding of the effect of any intervention on the regulation of appetite and/or energy intake. Recently, a randomised-controlled double-blinded experiment revealed that healthy individuals receiving placebo treatments showed altered subjective feelings of appetite and satiety in the suggested direction, and the appetite-enhancing placebo intervention was able to increase circulating ghrelin levels in women (Hoffmann et al. 2018). These findings highlight the important interplay between psychological and physiological mechanisms in the regulation of appetite which should be considered in research and clinical settings.

2.4 Effects of exercise on appetite

Exercise can be a powerful tool in weight management interventions as it typically increases

daily energy expenditure both by the energy needed to perform the exercise and by increasing muscle mass and, as a consequence, increasing resting metabolic rate. Therefore, exercise can contribute to creating the negative energy balance needed to achieve weight loss. Indeed, the combination of diet and exercise was shown to provide significantly greater weight loss compared to diet only interventions (Wu et al. 2009). However, if an increase in energy intake occurs as a response to exercise, which has been termed 'compensatory eating', the energy balance might not be challenged (Hopkins et al. 2014). Depending on the magnitude of the compensatory response, it can even lead to a positive energy balance which, in turn, will generate weight gain. Thus, in order to have a meaningful impact on energy balance, exercise depends on its ability to increase energy expenditure, but also on its effect on appetite and energy intake (Manore et al. 2017). Therefore, understanding the effect of exercise on appetite regulation and energy intake is crucial for planning effective strategies targeting weight loss or preventing weight gain.

The first observation that exercise is able to suppress appetite, with the suggestion of the term 'exercise-induced anorexia', was made 25 years ago (King et al. 1994), although the first evidence showing an effect of exercise on appetite-related gut hormones is more recent (Martins et al. 2007). It has been shown that when individuals face acute energy deficits by food restriction, compensatory responses in plasma acylated ghrelin, PYY_{3-36}, perceived appetite and *ad libitum* energy intake occur. However, an equivalent energy deficit produced by exercise did not produce such compensatory responses, suggesting exercise-induced anorexia may blunt increases in appetite produced by energy deficits (King et al. 2011).

Results from a meta-analysis investigating the acute effects of exercise on subsequent energy intake suggest that exercise does not affect energy intake, and consequently, is likely to induce an acute negative energy balance (Schubert et al. 2013). Another meta-analysis published more recently by the same research group concluded that acute exercise leads to a small to moderate suppression of appetite, stimulating three of the known anorexigenic hormones (PYY, glucagon-like peptide 1 and pancreatic polypeptide) and suppressing acylated ghrelin (Schubert et al. 2014). This suppression of appetite is temporary, with the hormonal concentrations tending to return to normal levels within 30 minutes. This has been observed during a variety of exercise modes, mainly with strenuous intensities (Deighton and Stensel, 2014). Running, jumping rope and other high-intensity exercises seem to have the greatest negative impact on appetite, and although producing a transient effect, these activities can

potentially delay initiation of the next meal (Manore et al. 2017). Although the precise mechanisms involved in exercise-induced anorexia remain unknown, delayed gastric emptying observed during high-intensity exercise, resulting in prolonged gastric distention, is likely to contribute to the appetite suppression (Horner et al. 2015). While many studies support the short-term suppressive effect of acute exercise on appetite, the effect of chronic exercise on appetite is less clear and further studies are warranted.

Similar to the findings from acute exercise studies, a systematic review found limited evidence of exercise training having an effect on energy intake (Donnelly et al. 2014). It is unlikely, however, that individuals will maintain a negative energy balance and continue to lose weight over prolonged periods when the daily energy expenditure is increased by exercise. It seems obvious, therefore, that either energy intake will increase, tracking energy expenditure, or energy expenditure will be reduced via other compensatory mechanisms, i.e. reduced daily physical activity or basal metabolism (Whybrow et al. 2008; Pontzer et al. 2016). However, the mechanisms involved and the exact manner that energy homeostasis is maintained over long periods with exercise training is still unknown. A small compensatory response is observed in energy intake when exercise is continued for several days; however, it only partially balances the energy expenditure from exercise, and it is greatly variable between individuals (Whybrow et al. 2008).

Of note, recent evidence shows that activity energy expenditure, which includes daily physical activity plus any planned exercise, is an independent predictor of daily energy intake in healthy weight-stable individuals, alongside with resting metabolic rate and fat mass (Hopkins et al. 2019). Most exercise training studies report reductions in leptin concentrations, although the findings for insulin, acylated ghrelin and PYY are not consistent. Insulin concentrations have been reported to be reduced or unchanged, whereas acylated ghrelin and PYY concentrations were increased or unchanged (Dorling et al. 2018). The effect of exercise training on appetite regulation seems to involve an increase in the overall drive to eat and a concomitant increase in post-prandial satiety (King et al. 2009). These two processes do not operate with the same strength in all individuals, and the relative strength of them may determine whether individuals lose weight with exercise or whether compensatory eating responses will undermine the weight loss (King et al. 2009).

A recent systematic review suggests that habitually active individuals have increased sensitivity to the energy density of foods, in comparison with inactive individuals (Beaulieu et

al. 2016). Additionally, a higher energy flux, i.e. maintained higher energy expenditure and matching energy intake, may be key to successful weight maintenance as it allows for more appropriate energy intake control (Manore et al. 2017). On the contrary, physical inactivity may lead to a dysregulation of appetite and subsequent overconsumption, with energy intake not reflecting energy expenditure. It has been suggested that the relationship between physical activity level and energy intake follow a J-shaped curve, where the lowest levels of physical activity show an unexpected high energy intake (Beaulieu et al. 2016). The mechanisms responsible for this effect are not yet known, although differences in body composition, gastric emptying, insulin sensitivity, appetite-related hormones and resting metabolic rate might be involved (Beaulieu et al. 2016). Interestingly, when previously active people become sedentary and therefore reduce their daily energy expenditure, the energy intake is not proportionally reduced, suggesting a lack of automatic regulation and leading to a positive energy balance (Stubbs et al. 2004). Becoming or remaining sedentary may expose people to eating behaviours more strongly influenced by sensory and environmental factors (Blundell, 2011).

Exercise may influence appetite through its impact on biological inputs, but its overall impact is variable and complicated by compensatory eating behaviours. A greater understanding of the mechanisms involved in exercise-induced compensatory eating, and why it only appears to affect some individuals and not others, is still needed. The lack of conclusive evidence for behavioural adjustments in response to a negative energy balance caused by exercise can be partially attributed to the focus on average group values in most studies published to date, which ignores the large individual variability in responses reported in some studies (Drenowatz, 2015). The substantial variability in appetite, appetite-related hormone and energy intake responses to exercise likely reflects the many factors involved in appetite regulation and deserves further investigation (Dorling et al. 2018).

2.5 Reproducibility and interindividual variability of appetite-related outcomes

Most studies conducting interventions targeting changes in appetite, food intake or body weight report their results using the group mean or any measure of central tendency, which by its nature, does not represent the true effect of the intervention as the variability in responses observed at the individual level within the study sample is not reported (MacLean et al. 2017). In fact, interindividual variability has been commonly treated as statistical noise without the recognition of its biological significance. For example, if the mean result of a weight loss intervention shows successful weight reduction, but the intervention consistently caused

weight gain in some individuals within the study sample, the mean result should not be interpreted and communicated as indicative of the effectiveness of the intervention for the whole population (Senior et al. 2016). It is possible that this traditional research methodology has led the scientific community to miss many important factors in understanding human appetite and eating behaviour. The diversity of responses to an intervention should be taken into consideration and the exploration of underlying reasons for such variability should be encouraged (Senior et al. 2016; MacLean et al. 2017).

In the obesogenic environment that most people live currently, the observation that not everyone becomes overweight or obese leads to the hypothesis of susceptible and resistant phenotypes and the interest for interindividual variability in appetite regulation has increased considerably (MacLean et al. 2017). Personalised medicine approaches continue to gain attention and, concomitantly, more studies aiming to assess and report interindividual variability in response to a certain intervention are being published. Appetite regulation involves a complex interaction between factors such as epigenetic, genetic variability, overall health and disease processes, environmental and behavioural stressors. Additionally, the great number of nutrients sensed along the gastrointestinal tract generate a wide variety of signals, activating areas in the brain involved in both homeostatic and hedonic eating behaviours. Thus, it is not surprising that great interindividual variability is observed in the self-regulation of eating behaviour (MacLean et al. 2017).

The assessment of variability across different interventions can result in targeting phenotypes with specific weight loss interventions (Senior et al. 2016). Of note, marked variability has been observed in response to every form of treatment of obesity, which is an unexplained observation to this point (Bray et al. 2018). It is clear that the success of any weight loss intervention depends on appetite physiology, motivation and previous experiences, and the variability of these factors between individuals should be assessed (Senior et al. 2016). Likewise, it is commonly acknowledged that the magnitude of the effect of an exercise intervention can vary significantly among individuals, regardless of the outcome of interest. Multiple factors are associated with this variation, including the characteristics of the training regimen, environmental conditions, habitual physical activity, fitness levels, physiological, genetic, social and psychological factors (Garber et al. 2011). The variability in appetite and food intake produced by exercise reflects a dynamic regulatory system in which physiological mediators can act as drivers of behaviour (Hopkins et al. 2014).

Interindividual variability has been suggested to exist in perceived appetite and energy intake responses to acute exercise in healthy individuals (Finlayson et al. 2009) and in individuals who are overweight and obese (Hopkins et al. 2014). While the publication of studies focusing on assessing interindividual variability on appetite-related topics, rather than merely looking at group means, is of great relevance, some concerns about the true significance of the findings exist. The two aforementioned studies estimated the interindividual variability using a single pair of trials, i.e. one control and one exercise condition. Such study design makes it possible to calculate the difference between outcomes in the control and intervention conditions at a single point of time; however, there is no guarantee that the observed effect of the intervention will be seen when the outcomes are assessed on a second occasion. The repetition of similar conditions to enable the assessment of the reproducibility of the observed findings is key as a first step in determining whether true interindividual variability exists in responses to an intervention (Senn, 2016). The reproducible individual responses can be defined as those that can be explained by differences between subjects due to stable characteristics or traits, whereas the random responses can be attributed to changes in subject characteristics or any external factor between the repeated assessments (Hopkins, 2015).

In regard to the reproducibility of findings in appetite and energy intake outcomes, available evidence has shown good reproducibility of *ad libitum* energy intake, cholecystokinin, glucose, insulin (Nair et al. 2009; Horner et al. 2014), and appetite perceptions after test meals (Flint et al. 2000; Gonzalez et al. 2012; Horner et al. 2014). On the other hand, poor reproducibility was recently reported at the individual level in perceived appetite after the consumption of liquid meals (Gonzalez et al. 2017). When exercise interventions were performed, good reproducibility of *ad libitum* energy intake after aerobic exercise, resistance exercise and resting control conditions in young, active adults was reported (Laan et al. 2010). However, when the difference between energy intake between exercise and control interventions was calculated, the reproducibility of findings was rather low (Unick et al. 2015; Brown et al. 2012).

Two key methodological issues can be highlighted in some of the aforementioned studies. First, when no control condition was included in the study design (Gonzalez et al. 2017), it becomes impossible to differentiate the true effect of the intervention from other sources of variability such as measurement errors and random day-to-day variability (Atkinson and Batterham, 2015; Senn, 2016). Second, where a control condition was included but the difference between the results from the intervention and the control conditions was not calculated (Laan et al. 2010),

the variability occurring in the control condition is equally not being taken into consideration when assessing the effect of the intervention. Of note, when the same study reported the reproducibility of energy intake after exercise in an isolated manner and also the reproducibility of the difference between energy intake in the exercise and the control condition, good reproducibility was reported on the former but not on the latter (Unick et al. 2015).

Published studies to date have often employed statistical approaches such as confidence intervals or Bland-Altman analyses in order to assess the reproducibility of responses to an intervention (Bland and Altman, 1986). While these methods can be appropriate choices for such purpose, they only serve as good tools to quantify the agreement of findings between two observations; however, the subject-by-condition interaction cannot be quantified. The employment of a replicated crossover study design has been suggested as the gold-standard for the determination of interindividual variability, as it makes it possible to assess reproducibility in the first place due to its replicated nature (Atkinson and Batterham, 2015; Senn, 2016). Additionally, appropriate statistical models should be employed for the assessment of the subject-by-condition interaction, where the extent to which the effects of treatments vary from subject to subject can be appropriately quantified (Senn, 2016). Replication is key in identifying this interaction and the difference between measurements taken on the intervention and control conditions should be calculated for the determination of the true effect of the intervention (Senn, 2016). The standard deviation of change scores should also be compared between intervention and control conditions, where a significantly larger standard deviation of change scores in the intervention condition is indicative of interindividual variability caused by the intervention *per se* (Hopkins et al. 2015).

No previous studies have examined the interindividual variability in perceived appetite or appetite-regulatory hormone responses to eating or exercising including the quantification of subject-by-condition interaction in a replicated crossover design. Before any attempt of classifying subjects as ‘responders’ and ‘non-responders’ to a given intervention, and the consequent implied call for investigation of the underlying causes of such variation in responses, one should ensure the employment of a robust study design and statistical analyses in order to determine the existence or not of true interindividual variability of responses.

2.6 Fat mass and obesity-associated gene

Individual susceptibility to gain weight is thought to be determined by interactions between an

individual's genetics, behaviour and the environment (Scuteri et al. 2007). The genetic factors predisposing to weight gain and obesity are still poorly understood and attempts to identify gene variants predisposing to common obesity often show controversial results (Frayling et al. 2007). In 2007, genome-wide association studies identified single-nucleotide polymorphisms (SNPs) in the FTO gene region on chromosome 16 strongly associated with BMI (Frayling et al. 2007; Scuteri et al. 2007). The FTO gene is the first common variant identified that influences obesity risk, being widely expressed in human tissues, with the highest expression in the hypothalamus within the brain, which is known to play a key role in the control of energy homeostasis (Gerken et al. 2007).

The most studied SNP in the FTO gene is rs9939609 and each additional copy of the risk A allele is associated with an increase of ~0.4 kg/m^2 on BMI. This association was present in adults of all ages, with no difference between males and females (Frayling et al. 2007). The FTO risk allele is common, with 74% of Europeans, 76% of African-Americans and 28-44% of Asians carrying at least one copy of it (Kilpelainen et al. 2011). Individuals homozygous for the A allele represent 16% of the population and have a 1.4-fold increased risk for overweight and a 1.7-fold increased risk for obesity, compared with those homozygous for the low-risk T allele, which represent 37% of the population (Frayling et al. 2007).

The association with body weight seems to be mainly due to higher food intake, with increased intake of dietary fat, increased appetite and reduced satiety (Loos and Yeo, 2014). On the contrary, FTO genotype does not seem to affect energy expenditure through resting metabolic rate or physical activity levels (West et al. 2018). Even though FTO genotype does not exert an effect on physical activity levels, the association of the FTO rs9939609 risk variant with BMI and with the odds of obesity was shown to be reduced by ~30% in physically active compared with inactive adults (Kilpelainen et al. 2011). Furthermore, while the risk variant of FTO has been associated with lower cognitive restraint and higher disinhibition and hunger, suggestive of poorer eating behaviours, this was not observed in a sample of physically active individuals, where AA individuals showed higher cognitive restraint and similar disinhibition and hunger scores to TT individuals, suggesting a protective effect of physical activity (West et al. 2018).

Despite increasing evidence that the FTO gene is associated with increased BMI, the mechanisms by which it may lead to higher energy intake are not yet known. Karra et al. performed studies in normal-weight, adiposity-matched individuals with FTO rs9939609

obesity-risk AA or low-risk TT genotype. Interestingly, the results suggest that AA subjects may have attenuated postprandial suppression of hunger and circulating acylated ghrelin levels (Karra et al. 2013). Furthermore, significant differences in neural responsivity to food cues in brain regions linked to reward and behavioural control, as well as in neural responsivity to circulating acylated ghrelin, were observed between AA and TT subjects (Karra et al. 2013). This was the first evidence suggesting possible mechanisms involved in the association between FTO genotype and eating behaviour. Further evidence is needed to understand how the gene modulates appetite, energy balance and body weight. Additionally, studies assessing the effect of FTO genotype within exercise interventions targeting weight loss have shown mixed results (Sailer et al. 2016) and there is still no evidence to elucidate if FTO genotype influences the effect of exercise on appetite regulation.

Recently, brown adipose tissue (BAT) has been suggested to be involved in the link between FTO genotype and obesity risk. BAT is a highly metabolically active tissue which uses glucose and free fatty acids to produce heat when activated by cold exposure, resulting in increased energy expenditure independently from shivering (Cannon and Nedergaard, 2004; Nedergaard et al. 2007). BAT is typically found in infants, where it exerts the important function of preserving body temperature, but there is now evidence of its presence in most human adults (Chechi et al. 2014; Sidossis and Kajimura, 2015). The volume and activity of BAT is inversely related to BMI and body fat mass (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Vijgen et al. 2011). White adipose tissue can also be induced to produce similar thermogenesis as BAT, a process that is typically called ‘browning’, and evidence shows that this process is enhanced in FTO knockout mice, resulting in increased energy expenditure (Tews et al. 2013; Ronkainen et al. 2016). In face of such evidence, it has been hypothesized that individuals with the at-risk FTO genotype present impaired browning of white adipocytes and reduced energy expenditure, which could lead to an increased risk of fat mass accumulation over the course of life (Tews et al. 2013). However, to date, this hypothesis has not been tested in humans.

2.7 Summary

Energy balance is a dynamic process and is influenced by a wide range of physiological and psychological factors. Physiological factors include hormones secreted by the gut and by adipose tissue, which act both on a meal-to-meal basis and over the long-term to regulate appetite and energy intake. The current obesity epidemic poses a great challenge worldwide, where the current available lifestyle interventions for weight loss do not seem effective in much

of the population. Personalised medicine and targeted interventions for specific phenotypes arise as a promise for more effective strategies to achieve a healthy body weight, where the identification of individual factors which may determine the success or failure of an intervention is the priority. In this respect, major methodological challenges exist for the identification of true interindividual variability and these should be considered in future studies. The fat mass and obesity-associated gene is the first genetic common variant identified that is associated with obesity risk and further evidence is needed to elucidate the mechanisms involved. Therefore, this thesis aims to expand the evidence on the existence of interindividual variability in perceived appetite and appetite-related hormone responses to eating and to exercising in humans, as well as on the potential factors underlying such variability.

CHAPTER 3

General methods

This chapter describes the general methods employed in the experimental studies presented within this thesis. All studies were conducted in the laboratories at Loughborough University and were approved by the University Ethics Approvals Sub-Committee. Written consent was obtained from all participants before any aspect of the research experiments was conducted.

3.1 Participant recruitment

Participants were recruited from Loughborough University and the local area by word of mouth, poster, e-mail and social media advertising. Volunteers attended the laboratory for a preliminary visit to confirm eligibility, where they received information sheets explaining the purpose, protocol and demands of the study, as well as any potential risks and discomforts. After a verbal explanation and the opportunity to ask any questions about the study, volunteers completed an informed consent form (Appendix A) and a health screen questionnaire (Appendix B) before the start of any experimental procedures. Participants also completed questionnaires assessing habitual physical activity (Appendix C; Craig et al. 2003), food preferences to ensure adherence to standardised meals (Appendix D) and dietary habits (Appendix E; Stunkard and Messick, 1985) to identify any atypical eating tendencies.

The inclusion criteria for participation were:

- Aged 18 - 50 years;
- Non-smoker;
- Body mass stable (≤ 3 kg change in the previous 3 months);
- Not dieting;
- No history of cardiovascular or metabolic disease;
- Not taking any medications (except for oral contraceptives);
- No severe dislike or intolerance of any study food.

3.2 Anthropometry

Participants wore light clothing and removed shoes and all items from pockets for anthropometric measurements. Height was measured to the nearest 0.1 cm and body mass was

measured to the nearest 0.1 kg using an electronic measuring station (Seca, Hamburg, Germany). Body mass index was subsequently calculated as body mass (kg) divided by stature squared (m^2). Waist circumference was measured with an inelastic polyfibre tape measure (Seca, Hamburg, Germany) at the end of expiration at the narrowest point of the torso between the lower rib margin and the iliac crest.

Measurements of subcutaneous fat were taken to estimate total body fatness. Skinfold thickness was measured by the same investigator to the nearest 0.2 mm on the right-hand side of the body using Harpenden callipers (Baty International, West Sussex, UK), with the participant standing in a relaxed position. Skinfolds from seven sites (chest, triceps, subscapular, mid-axilla, supraspinale, abdominal and thigh) were assessed in Chapter 4, and from three sites (chest, abdominal and thigh in males, and triceps, supraspinale and thigh in females) in Chapters 5 and 6. Each measurement was taken within two seconds of calliper pressure while maintaining the pinch of the skinfold. Measurements were made by rotating through the anatomical sites to allow time for the skin to regain normal texture and thickness. The median of three measurements at each site was used to estimate body density (Jackson and Pollock, 1978, 1980) and percentage of body fat (Siri, 1961).

3.3 Environmental temperature and humidity

Environmental temperature and humidity were kept constant and assessed periodically throughout all study visits using a wireless weather station (Opes, London, UK).

3.4 Heart rate and rating of perceived exertion

Heart rate was monitored continuously during all exercise tests and interventions using short-range telemetry (Polar A3, Kempele, Finland). The Borg scale was used to record participants' perceived level of exertion at pre-determined intervals during all exercise tests and interventions (Appendix F; Borg, 1973). The scale ranges from 6 indicating no exertion to 20 indicating maximal exertion.

3.5 Expired gas sampling and analysis

3.5.1 Douglas bags

Expired air samples were collected during exercise into 100 L Douglas bags in Chapter 4. Oxygen consumption and carbon dioxide production were determined using a paramagnetic

oxygen analyser and an infrared carbon dioxide analyser (Servomex 1400, East Sussex, UK). Prior to sample analysis, the analysers were calibrated with certified reference gases. The volume of expired air was quantified using a dry gas meter (Harvard Apparatus Ltd., Kent, UK) and the temperature of expired air was measured using a thermometer housed in the dry gas meter during evacuation (Edale Instruments Ltd., Cambridge, UK). All expired air measurements were corrected to standard room temperature and pressure for a dry gas.

3.5.2 Portable metabolic cart with facemask

Expired air samples were monitored continuously during exercise using an online breath-by-breath gas analysis system (Cortex Metalyzer 3B, Leipzig, Germany) in Chapter 5. The analyser was calibrated before each measurement using a bottled gas mixture containing 5.01% carbon dioxide, 16.98% oxygen, and nitrogen (Cranlea Human Performance, Birmingham, UK) and a 3 L syringe (Hans Rudolph, Shawnee, USA). Participants wore a facemask (Hans Rudolph, Shawnee, USA) connected to the online system via a flowmeter before the expired air measurement began. The size of the facemask was selected for each participant as checks for leaks were performed.

3.5.3 Portable metabolic cart with ventilated hood

In Chapter 5, resting metabolic rate was measured using an open circuit indirect calorimetry system (GEM Nutrition Ltd., Cheshire, England). The analyser was calibrated with certified reference gases before each measurement. Participants were asked to lie in a comfortable supine position and were instructed not to talk or sleep, and to move as little as possible during the measurement. The clear hood canopy was placed over the head area, and plastic sheeting attached to the hood was placed around the body to form a seal between the air inside and outside the hood. Oxygen uptake, carbon dioxide production, respiratory exchange ratio and energy expenditure were determined at 30 s intervals over a 30 min period. The first 10 min of data was discarded to account for any initial short-term respiratory artefact.

3.6 Preliminary exercise test

All exercise tests and interventions were conducted on a treadmill (Technogym Excite Med, Cesena, Italy). Participants were familiarised with walking, running and dismounting the treadmill before the exercise tests commenced.

In Chapter 4, participants completed two preliminary exercise tests. The first test involved a 16-min submaximal incremental treadmill protocol divided into 4 x 4 min stages to determine the relationship between treadmill speed and oxygen consumption. The initial running speed was set between 8 and 12 $km \cdot h^{-1}$ depending on each participant's fitness level, and the treadmill speed was increased by 1–1.5 $km \cdot h^{-1}$ at the start of each subsequent stage. Heart rate was monitored continuously, and ratings of perceived exertion were assessed at the end of each stage. Expired air samples were collected into Douglas bags in the final minute of each 4-min stage. After a 20-min standardised rest period, a peak oxygen uptake test was conducted using an incremental uphill treadmill protocol at a constant speed until the participants reached volitional fatigue. The initial incline of the treadmill was set at 3.5% and this was increased by 2.5% every 3 min (Taylor et al. 1955). Peak oxygen uptake was determined from an expired air sample collected in the final minute when participants indicated that they could only continue for an additional 1 min. Heart rate and ratings of perceived exertion were monitored, and verbal encouragement was provided throughout the test. Data from the 16-min submaximal incremental and peak oxygen uptake tests were used to determine the running speed required to elicit 70% of peak oxygen uptake during the experimental exercise conditions. Participants began the treadmill exercise at this speed during the main study visits (Chapter 4) but the treadmill speed was adjusted to account for cardiovascular drift when necessary.

In Chapter 5, during the peak oxygen uptake test, participants ran at a fixed individualised speed chosen as a speed at which each participant felt 'comfortable exercising' (4.5 to 14 $km \cdot h^{-1}$), with the initial gradient of the treadmill set to 0%. Treadmill gradient was increased by 1% every minute until volitional exhaustion. Verbal encouragement was provided throughout the test. Heart rate was monitored continuously, and ratings of perceived exertion were recorded at the end of each minute. Expired air samples were monitored continuously using a breath-by-breath gas analysis system (Cortex Metalyser 3B, Leipzig, Germany). An average of the breath-by-breath oxygen uptake data was taken every 10 s, and peak oxygen uptake was defined as the highest 30 s rolling average.

3.7 Calculation of energy expenditure

For expired gas samples collected during rest and exercise, oxygen consumption and carbon dioxide production values were used to determine substrate oxidation and energy expenditure using the equations of Frayn (1983):

$$\text{Fat oxidation } (g \cdot min^{-1}) = 1.67 \times VO_2 (L \cdot min^{-1}) - 1.67 \times VCO_2 (L \cdot min^{-1})$$

$$\text{Carbohydrate oxidation } (g \cdot min^{-1}) = 4.55 \times VCO_2 (L \cdot min^{-1}) - 3.21 \times VO_2 (L \cdot min^{-1})$$

$$EE (kJ \cdot min^{-1}) = 4.1855 \times ((\text{fat } (g \cdot min^{-1}) \times 9) + (\text{carbohydrate } (g \cdot min^{-1}) \times 4))$$

3.8 Physical activity and dietary control

Participants refrained from alcohol, caffeine, and strenuous physical activity during the 24 h preceding main study visits. In Chapters 4 and 6, participants completed a weighed food record (Appendix G) in the 24 h preceding the first main study visit and were instructed to replicate this feeding pattern before each subsequent visit. In Chapters 4, 5 and 6, participants were given a standardised evening meal and were instructed to consume it between 19:00 and 20:00. Participants were instructed to consume the whole meal without any additional food or drink items except plain water, and compliance was confirmed from the food record completed prior to the first visit (Chapters 4 and 6), and verbally on the remaining visits. After this meal, participants consumed no food or drink except plain water before arriving at the laboratory the next day.

3.9 Standardised meals

3.9.1 Standardised evening meal

In Chapters 4, 5, and 6, participants were given a pizza as a standardised evening meal to consume on the evening preceding each main study visit. The energy and macronutrient intake during standardised evening meals is presented in Table 3.1.

Table 3.1 Energy and macronutrient intake during standardised evening meals.

	Energy (kJ)	**Carbohydrates (%)**	**Protein (%)**	**Fat (%)**
Chapter 4: Sainsbury's pepperoni pizza	4891	48	18	34
Chapter 5: Tesco cheese and tomato pizza	3297	39	21	40
Chapter 6: Tesco margherita pizza	3054	44	22	34

3.9.2 Standardised test meal

In Chapters 5 and 6, participants were given a standardised test meal to consume during specific study visits. The meal consisted of a ham and cheese sandwich, milkshake and chocolate biscuit in Chapter 5 and croissants, butter, chocolate spread, cereal biscuits and milkshake in Chapter 6. The energy and macronutrient intake during standardised test meals is presented in Table 3.2.

Table 3.2 Energy and macronutrient intake during standardised test meals.

	Energy (kJ)	**Carbohydrates (%)**	**Protein (%)**	**Fat (%)**
Chapter 5	4435	41	18	41
Chapter 6	5025	47	9	44

3.10 Assessment of perceived appetite

Ratings of perceived appetite (hunger, satisfaction, fullness and prospective food consumption) were assessed periodically throughout experiments in Chapters 4, 5, and 6 using previously validated 100 mm visual analogue scales (Appendix H; Flint et al. 2000). The scales were anchored by a descriptor at each end defining the extremes of the appetite perception being measured. Participants rated each perceived appetite perception by placing a mark along the horizontal line corresponding to the degree of each perception. These were then quantified by measuring the distance from the left-hand side of the scale to the point on the line indicated by the participant.

3.11 Blood sample collection

Approximately 1 hour before commencing all main conditions including blood samples in Chapters 4, 5 and 6, participants rested in a semi-supine position and a cannula (Becton Dickinson, Helsinborg, Sweden) was inserted into an antecubital vein, from which blood samples were collected periodically. Patency of the cannula was maintained by flushing with 10 mL non-heparinised saline (0.9% sodium chloride, L. E. West International, Barking, UK) after each blood sample. To avoid dilution of subsequent samples, residual saline was drawn

off immediately prior to collection using a 2 mL syringe. To control for postural changes in plasma volume, participants rested in a semi-supine position for the five min prior to each blood sample and remained in this position during the collection.

Venous blood samples were collected into pre-cooled 4.9 or 9 mL ethylenediaminetetraacetic acid (EDTA)-coated monovettes (Sarstedt, Leicester, UK) via a multi-adapter (Sarstedt, Leicester, UK). Samples for the quantification of plasma acylated ghrelin concentrations were collected into pre-chilled 4.9 mL EDTA monovettes containing *p*-hydroxymercuribenzoic acid to prevent the degradation of acylated ghrelin by protease and were centrifuged at 2,383 *g* for 10 min at 4°C (Burkard, Hertfordhire, UK). The plasma supernatant was aliquoted into a storage tube and 100 µL of 1 M hydrochloric acid was added per mL of plasma. Samples were re-centrifuged at 2,383 g for 5 min at 4°C before being transferred into Eppendorf tubes and stored at -80°C for later analysis. Samples for the quantification of plasma total PYY, leptin, insulin and glucose were collected in 9 mL monovettes and centrifuged immediately at 2,383 g for 10 min at 4°C prior to storage at -80°C. In Chapter 5, an additional fasting venous blood sample was collected into a 2 mL EDTA monovette and the whole blood sample was stored at 4°C to undergo deoxyribonucleic acid (DNA) extraction and genotyping at a later date.

At the first and last sampling points in each study visit, duplicate 20 µL blood samples were collected into micropipettes for the determination of blood haemoglobin, and duplicate blood samples were collected into heparinised micro haematocrit tubes for the determination haematocrit concentration.

3.12 Blood sample analysis

3.12.1 Estimation of changes in plasma volume

Haemoglobin concentration was measured in duplicate by the cyanmethaemoglobin method using a spectrophotometer (Shimadzu, Milton Keynes, UK), and haematocrit was measured in duplicate using a microcentrifuge (Hawksley, Sussex, UK). Haematocrit and haemoglobin concentrations were used to estimate plasma volume change relative to baseline (Dill and Costill, 1974), enabling plasma concentration of hormones to be adjusted to account for changes in plasma volume if necessary.

3.12.2 Glucose

Plasma glucose concentrations were determined by enzymatic, colorimetric methods using a benchtop analyser (Horiba Medical Pentra 400, Montpellier, France). To ensure precision of analysis, quality controls of known concentrations were analysed prior to study sample analyses.

3.12.3 Insulin

Commercially available enzyme-linked immunosorbent assays (ELISA) were used to determine the concentrations of plasma insulin (Mercodia, Uppsala, Sweden). To ensure precision of analysis, quality controls (Mercodia diabetic antigen control) with low and high concentrations were analysed in duplicate in each assay plate.

3.12.4 Total PYY

Commercially available ELISAs were used to determine the concentrations of plasma total PYY (Millipore, Billerica, USA). Precision of analysis was ensured by the quantification of quality controls with low and high concentrations, in duplicate, in each assay plate.

3.12.5 Acylated ghrelin

Commercially available ELISAs were used to determine the concentrations of plasma acylated ghrelin (Bertin Technologies, Montigney le Bretonneux, France). Precision of analysis was ensured by the quantification of a quality control of known concentration, in duplicate, in each assay plate.

3.12.6 Leptin

In Chapter 5, commercially available ELISAs were used to determine the concentrations of plasma leptin (R&D Systems, Minneapolis, USA). Precision of analysis was ensured by the quantification of quality controls of low, medium and high concentrations, in duplicate, in each assay plate.

3.12.7 Precision of analysis

To eliminate inter-assay variation, the plasma samples for each participant were analysed in the same run. Additionally, in Chapters 4 and 6, all samples were analysed in duplicate. The

within-batch coefficients of variation for each assay were calculated by repeated measurements of a single plasma sample from 4 to 10 times, depending on the space available in each assay plate in order to optimise ELISA kit utilization. Values of within-batch coefficient of variation for each assay are presented within the methods section of Chapters 4, 5 and 6.

3.12.8 Genotyping

In Chapter 5, genomic DNA was extracted from the whole blood samples using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). The samples were genotyped for the rs9939609 allele within the FTO gene using the Applied Biosystems TaqMan® (Roche Molecular Systems, Pleasanton, USA) genotyping assay and real-time polymerase chain reaction system. Participants were assigned to one of three groups according to their genotype: homozygous major allele, TT; heterozygous allele, AT; or homozygous minor allele, AA.

3.13 Habitual physical activity and sedentary time

In Chapter 5, physical activity and sedentary time of participants were assessed over a 7-day period. Participants wore an ActiGraph GT3X+ accelerometer (ActiGraph, Pensacola, USA) on an elasticated belt on the waist above the mid-line of the thigh on their non-dominant side of the body. The device was initialised at a frequency of 100 HZ and downloaded using ActiLife software v6.11.8 and firmware v2.0.0 (ActiGraph, Pensacola, USA). ActiGraph data were downloaded in 60-second epochs and physical activity was classified as low, light and moderate-to-vigorous. Participants also wore an activPAL3 accelerometer. The activPAL3 was attached directly to the skin on the midline of the anterior aspect of the thigh in line with the ActiGraph GT3X+ accelerometer. The activPAL3 determines posture using information derived from accelerations of the thigh, including the gravitational component, using a triaxial accelerometer (Atkin et al. 2012). The activPAL3 is a valid measure of time spent sitting/lying, standing, and walking in adults (Kozey-Keadle et al. 2011). ActivPAL sitting time data were retrieved and clustered in 60-second epochs using a customized spreadsheet. Participants were advised to wear both devices concurrently and continuously over 7 days. Moderate-to-vigorous physical activity and sitting time data were averaged from the seven-day period, and non-wear time and sleep time were removed from the analysis.

3.14 Magnetic resonance imaging scan

All participants recruited for the study presented in Chapter 4 were invited and agreed to participate in a pilot study which consisted of one magnetic resonance imaging (MRI) scan visit in order to assess visceral adipose tissue, abdominal subcutaneous adipose tissue and liver fat fraction. This pilot study aimed to test the protocol needed for the assessment of the outcomes of interest using the semi-automated tool for the quantification of body fat (AMRA^{TM} Profiler) developed by Advanced MR Analytics (AMRA, Linköping, Sweden) in order to analyse the images acquired from the body scans. This tool has been previously validated against manual quantification methods (Borga et al. 2015).

Each participant underwent an MRI scan in the supine position using a dual-echo Dixon fat and water sequence on a 3-T MRI scanner (GE Healthcare MR750w, Chicago, USA). Seven overlapping image stacks were acquired from the neck to knee with stacks covering the abdomen (stacks 2 to 5) acquired during breath hold of seventeen seconds. Additional abdominal slices were acquired with the IDEAL-IQ sequence to assess proton density fat fraction in the liver. Scans were analysed to quantify visceral adipose tissue, abdominal subcutaneous adipose tissue and liver fat fraction using the AMRA Profiler (AMRA Medical AB, Linköping, Sweden) (Borga et al. 2015; West et al. 2016). The identification of visceral adipose tissue on a single abdominal MRI image slice using the AMRA^{TM} Profiler is demonstrated in Figure 3.1. The exact same protocol was employed in the study presented in Chapter 5.

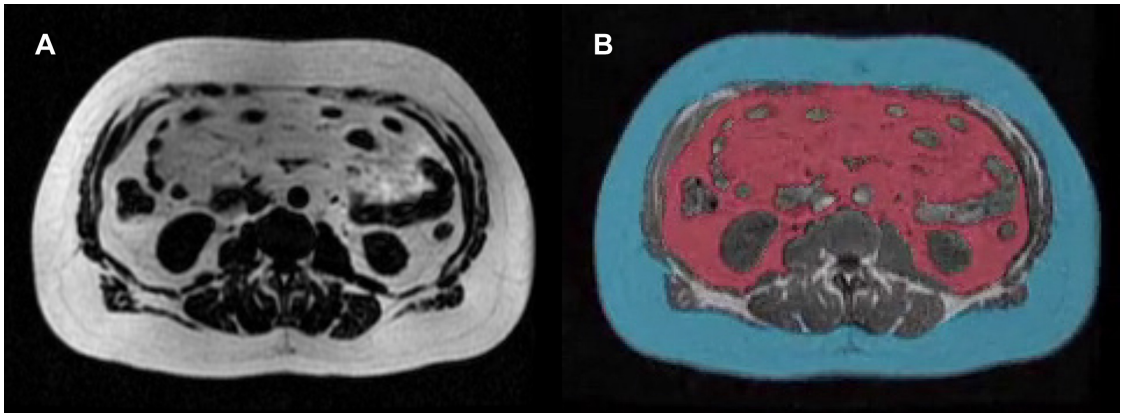


Figure 3.1 Abdominal cross-sectional image slice obtained using magnetic resonance imaging: A) image slice acquired directly from the scan; B) corresponding image slice analysed using the AMRA^{TM} Profiler to identify visceral adipose tissue (in pink) and abdominal subcutaneous adipose tissue (in blue).

3.15 Statistical analysis

Specific statistical analysis employed for each study are described in detail in Chapters 4 to 7. Data were analysed using the IBM SPSS Statistics software for Windows version 23.0 (IBM Corporation, New York, USA) and SAS OnDemand for Academics (https://www.sas.com/en_us/software/on-demand-for-academics.html). Pearson's product-moment correlation coefficients were calculated to explore associations between outcomes of interest. Thresholds of 0.1, 0.3 and 0.5 were used to define small, moderate and large correlation coefficients, respectively (Cohen, 1988). In Chapter 5, multivariable general linear models were used to quantify the differences between genotype groups for each appetite outcome. In Chapters 4, 6 and 7 within-participant linear mixed models were formulated to quantify participant-by-condition interactions for each outcome and/or genotype-by-condition interactions.

In the absence of a robust and precise prognostic anchor for an important difference in appetite-related outcomes, standardised effect sizes (ES) were calculated to support significant findings. An ES of 0.2 denoted the minimum important mean difference for all outcomes, with an ES of 0.5 being moderate and an ES of 0.8 being large (Cohen, 1988). Data are described as mean and standard deviation. Mean differences and correlation coefficients are presented along with respective 95% confidence intervals. *P* values are expressed in exact terms apart from very low values, which are expressed as $P < 0.001$, and statistical significance was accepted as $P < 0.050$.

CHAPTER 4

Interindividual responses of appetite to acute exercise: a replicated crossover study

4.1 Abstract

Background: Acute exercise transiently suppresses appetite, which coincides with alterations in appetite-regulatory hormone concentrations. Individual variability in these responses is suspected, but replicated trials are needed to quantify them robustly. **Objectives:** To examine the reproducibility of appetite and appetite-regulatory hormone responses to acute exercise and to quantify the individual differences in responses. **Methods:** Fifteen healthy, recreationally-active men completed two control (60-min resting) and two exercise (60-min fasted treadmill running at 70% peak oxygen uptake) conditions in randomised sequences. Perceived appetite and circulating concentrations of acylated ghrelin and total peptide YY (PYY) were measured immediately before and after the interventions. Interindividual differences were explored by correlating the two sets of response differences between exercise and control conditions. Within-participant covariate-adjusted linear mixed models were used to quantify participant-by-condition interactions. **Results:** Compared with control, exercise suppressed mean acylated ghrelin concentrations and appetite perceptions (all ES = 0.62 to 1.47, $P < 0.001$), and elevated total PYY concentrations (ES = 1.49, $P < 0.001$). For all variables, the standard deviation (SD) of the change scores was substantially greater in the exercise versus control conditions. Moderate-to-large positive correlations were observed between the two sets of control-adjusted exercise responses for all variables (r = 0.54 to 0.82, $P \leq 0.036$). After adjusting for baseline measurements, participant-by-condition interactions were present for all variables ($P \leq 0.012$), with exception of prospective food consumption ($P = 0.053$). **Conclusion:** Our replicated crossover study allowed, for the first time, the interaction between participant and acute exercise response in appetite parameters to be quantified. Even after adjustment for individual baseline measurements, participants demonstrated individual differences in perceived appetite and hormone responses to acute exercise bouts beyond any random within-subject variability over time.

4.2 Introduction

Understanding the relationship between exercise and appetite control has direct implications regarding the role of exercise in regulating energy homeostasis and weight control (Stensel, 2010; Beaulieu et al. 2016). It is well-documented that circulating concentrations of acylated ghrelin are suppressed and satiety hormones, most notably PYY, are elevated in response to acute bouts of moderate- to high-intensity exercise (Schubert et al. 2014). These hormonal fluctuations coincide with a transient reduction in appetite during and immediately after exercise without stimulating compensatory increases in appetite and *ad libitum* energy intake in the short term (Schubert et al. 2013; Deighton and Stensel, 2014).

The notion of interindividual variability in response to an intervention, within the context of 'personalised' or 'precision' medicine, continues to attract significant scientific attention (Atkinson and Batterham, 2015; Betts and Gonzalez, 2016; King et al. 2017). Whilst the majority of researchers have focussed on main effects and mean group changes, some investigators have attempted to quantify the individual variability in appetite and energy intake responses to acute (Finlayson et al. 2009; Hopkins et al. 2014; Unick et al. 2015) and chronic (Barwell et al. 2009; King et al. 2009) exercise interventions. Some researchers have classified individuals as 'compensators' or 'non-compensators' according to the individual magnitude and direction of change in energy intake they observed after exercise (Finlayson et al. 2009; Hopkins et al. 2014). Although the important issue of interindividual variability has been considered in exercise and appetite regulation studies, recent evidence has recognised that the methodological and statistical approaches for such investigations are challenging and often lacking in some cases (Atkinson and Batterham, 2015; Hecksteden et al. 2015; Hopkins, 2015).

One approach to quantifying "true" individual responses is via the participant-by-response interaction term in a statistical model, which requires replicated intervention and comparator arms with sufficient washout (Senn et al. 2011; Senn, 2016). Previous researchers have reported intra-class coefficients to support claims that pre-to-post changes in *ad libitum* energy intake in response to acute exercise are not consistent within an individual over time (Unick et al. 2015). Interindividual variability in appetite and appetite-regulatory hormone responses to repeated acute exercise exposures are suspected; however, no published studies have confirmed this notion using robust designs (the replicated crossover) and appropriate statistical models.

Therefore, the aims of the present study were to examine the reproducibility of appetite, acylated ghrelin and total PYY responses to acute exercise bouts, and to quantify the magnitude of individual differences in responses using a replicated crossover design. Recent insights have provided a framework for the accurate statistical analyses to quantify true interindividual variability in exercise responses using the SD of the change scores and participant-by-response interaction (Senn et al. 2011; Atkinson and Batterham, 2015; Hecksteden et al. 2015; Hopkins, 2015; Senn, 2016). Using these approaches, it was hypothesised that exercise-induced changes in subjective and hormonal appetite parameters would be reproducible on repeated occasions and true interindividual variability in appetite responses to acute exercise bouts would be observed in healthy, recreationally active men.

4.3 Methods

4.3.1 Ethical approval

This study was conducted in accordance with the Declaration of Helsinki (2013) and all procedures were approved by the local ethics advisory committee. All participants provided written informed consent before taking part in any aspect of the study.

4.3.2 Participants

Fifteen healthy, recreationally active men (mean (SD): age 23 (3) years, body mass 81.9 (11.4) kg, body mass index 24.8 (3.0) $kg \cdot m^{-2}$, waist circumference 84.3 (6.9) cm, body fat percentage 13.1 (5.9)%, peak oxygen uptake ($\dot{V}O2$ peak) 54.9 (6.5) $mL \cdot kg^{-1} \cdot min^{-1}$) participated in the study. The participants' body mass was stable; ≤ 3 kg change in the previous 3 months. Participants were non-smokers, had no history of cardiovascular or metabolic disease, and were not dieting or taking any medications.

4.3.3 Preliminary measurements

Before the main experimental conditions, participants attended the laboratory for a preliminary visit to complete screening questionnaires, and to undergo familiarisation, anthropometric measurements and exercise testing. Specifically, participants completed questionnaires assessing health status, food preferences, habitual physical activity (International Physical Activity Questionnaire) (Craig et al. 2003) and psychological eating tendencies (Three-Factor Eating Questionnaire) (Stunkard and Messick, 1985). Height and body mass were quantified

using an electronic measuring station (Seca, Hamburg, Germany). Waist circumference was measured at the narrowest point of the torso between the lower rib margin and the iliac crest. The sum of seven skinfolds was used to estimate body density (Jackson and Pollock, 1978) and body fat percentage (Siri, 1961).

After familiarisation with walking and running on the treadmill (Technogym Excite Med, Cesena, Italy), participants completed two preliminary exercise tests, as described in detail in Chapter 3. Data from exercise tests were used to determine the running speed required to elicit 70% of peak oxygen uptake during the experimental exercise conditions.

4.3.4 Experimental design

In a replicated, crossover experimental design, participants were randomised to different sequences of four experimental conditions: two control and two exercise (Senn, 2016). Each condition was separated by an interval of at least five days. Participants completed a weighed food record in the 24 h preceding the first experimental condition and were instructed to replicate this feeding pattern before each subsequent condition. Participants refrained from alcohol, caffeine, and strenuous physical activity during the same period. A standardised meal was consumed in the evening before the experimental conditions consisting of a pepperoni pizza (4891 kJ, 48% carbohydrate, 18% protein, 34% fat). Participants were instructed to consume the meal between 19:00 and 20:00, after which they consumed no food or drink except plain water until arriving at the laboratory the next morning.

4.3.5 Main trials

Participants arrived at the laboratory at 08:00 having fasted overnight for a minimum of 12 h. A cannula (Becton Dickinson Venflon, Helsingborg, Sweden) was inserted into an antecubital vein for venous blood sampling, and participants rested for 1 h (~08:00–09:00) to acclimatise to the study environment (Chandarana et al. 2009). During both exercise conditions, participants then completed 60 min of fasted treadmill running at a speed predicted to elicit 70% of peak $\dot{V}O_2$. One-minute expired air samples were collected and analysed every 15 minutes, and the treadmill speed was adjusted if necessary during both exercise conditions to ensure the target exercise intensity was achieved. Heart rate was monitored continuously and rating of perceived exertion was determined after each expired air sample was collected. The exercise energy expenditure and substrate utilisation were subsequently estimated using the

equations of Frayn (Frayn, 1983). Identical procedures were completed during both control conditions except participants rested within the laboratory for the equivalent duration.

4.3.6 Appetite perceptions

Ratings of perceived appetite (hunger, satisfaction, fullness and prospective food consumption (PFC)) were assessed immediately before (0 h) and after (1 h) the exercise and control interventions using 100 mm visual analogue scales (Flint et al. 2000). The scales were anchored by a descriptor at each end defining the extremes of the appetite perception being measured.

4.3.7 Blood sampling and biochemical analysis

Blood samples were collected in the semi-supine position immediately before (0 h) and after (1 h) the exercise and control interventions for the assessment of plasma acylated ghrelin and total PYY concentrations. Plasma acylated ghrelin and total PYY concentrations were quantified from venous blood samples collected following the procedures described in detail in Chapter 3. Measurements of haemoglobin and haematocrit were determined in duplicate at 0 and 1 h in all conditions to calculate the acute change in plasma volume (Dill and Costill, 1974).

Commercially available enzyme immunoassays were used to determine the plasma concentrations of acylated ghrelin (Bertin Technologies, Montigney le Bretonneux, France) and total PYY (Millipore, Watford, UK). All samples were analysed in duplicate. To eliminate inter-assay variation, samples for each participant were analysed in the same run. The within-batch coefficients of variation for acylated ghrelin and total PYY concentrations were 4.1% and 3.6%, respectively.

4.3.8 Statistical analyses

Data were analysed using the IBM SPSS Statistics software for Windows version 23.0 (IBM Corporation, New York, USA) and the PROC MIXED procedure in SAS OnDemand for Academics (https://www.sas.com/en_us/software/on-demand-for-academics.html). The presence of interindividual differences in acylated ghrelin, total PYY and perceived appetite responses to acute exercise bouts were examined according to three recently-reported analytical approaches (Senn et al. 2011; Atkinson and Batterham, 2015; Senn, 2016):

(i) Pearson's correlation coefficients were quantified between the exercise and control pre-to-

post (0 to 1 h) change scores for each appetite parameter on the two occasions (Senn, 2016). The first exercise bout in any participant's sequence was paired to the first control bout in the same individual's sequence. Differences between these trials were correlated with the second exercise-control condition differences in the participant's trial sequence. Thresholds of 0.1, 0.3 and 0.5 were used to define small, moderate and large correlation coefficients, respectively (Cohen, 1988).

(ii) The difference in SDs of the pre-to-post changes between the exercise and control conditions was calculated to represent the true individual response SD using the following equation:

$$SD_R = \sqrt{SD_E^2 - SD_C^2}$$

where SD_R is the SD of the true individual response to the exercise conditions and SD_E and SD_C are the SDs of the pre-to-post change scores for the exercise and control conditions, respectively (Atkinson and Batterham, 2015; Hopkins, 2015). This estimation of the true SD for individual differences in response should be considered a "naïve estimation", since important aspects of the experimental design, e.g. period effects, are not included. Therefore, a modelling approach to this estimation was also adopted (see iii below).

(iii) A within-participant linear mixed model was formulated to quantify any participant-by-condition interaction for each appetite parameter. Condition and period (sequence) were initially modelled as fixed effects. Senn et al. raised the question of whether the participant and participant-by-condition interaction terms should be modelled as fixed or random effects (Senn et al. 2011). Differences between these modelling approaches may exist depending on the distribution of the participant factor and the magnitude of the treatment (exercise effect). Our sample was, in clinical trial terms, relatively small and we expected the general effects of exercise to be substantial. Therefore, we modelled our data with participant and participant-by-condition terms as both fixed and random effects and compared these results as a sensitivity analysis. When the participant-by-condition interaction was considered as a random effect, we used the SAS code supplied by Senn et al. with a modification designed to derive the true individual response variance (also estimated by approach ii) (Senn et al. 2011). This modification involved the adding of a covariate "dummy" variable we called "XVARE" (refer to the SAS code supplied in Appendix I).

It is also relevant to explore the extent to which an individual's response depends on their status at baseline (Atkinson and Batterham, 2015). Therefore, baseline status of the dependent variable was added to the various linear mixed models as a covariate. The mean differences between conditions were also quantified with this same statistical model.

We found that correction of appetite hormone concentrations for acute changes in plasma volume had a negligible influence on our findings. Therefore, the unadjusted plasma concentrations are displayed for simplicity. Absolute standardised effect sizes (ES) were calculated, with a standardised ES of 0.2 denoting the minimum important mean difference for all outcomes, 0.5 - moderate and 0.8 - large (Cohen, 1988). To calculate the minimal clinically important difference (MCID) for individual responses, the threshold of 0.2 for interpreting standardised mean changes (Cohen, 1988) was halved, i.e. 0.1, and multiplied by the baseline between-subject SD (Atkinson and Batterham, 2015; Hopkins, 2015). Pearson's correlation coefficients were quantified between the pooled mean pre-to-post change in appetite-regulatory hormone concentrations and the pooled mean pre-to-post change in appetite perceptions across the four conditions.

Data are described as mean (SD). Mean differences and correlation coefficients are presented along with respective 95% confidence intervals (95% CI). *P* values are expressed in exact terms apart for very low values, which are expressed as $P < 0.001$, and statistical significance was accepted as $P < 0.050$.

4.4 Results

4.4.1 Treadmill exercise responses

Treadmill exercise responses are displayed in Table 4.1. No statistically significant nor practically important differences were observed in any of the treadmill exercise responses between the two exercise sessions ($P \geq 0.130$).

Table 4.1 The various responses during the treadmill exercise for the two exercise conditions.

Variable	Exercise condition 1	Exercise condition 2	95% CI*	ES
Oxygen uptake ($mL \cdot kg^{-1} \cdot min^{-1}$)	38.9 (5.1)	38.5 (4.9)	-4.2 to 3.3	0.09
% peak oxygen uptake	71 (3)	70 (3)	-2 to 0.3	0.31
Heart rate ($beats \cdot min^{-1}$)	176 (10)	176 (13)	-5 to 4	0.04
Rating of perceived exertion	15 (2)	15 (2)	-1 to 0.2	0.13
Respiratory exchange ratio	0.91 (0.03)	0.92 (0.04)	-0.01 to 0.02	0.21
Fat oxidation (g)	29 (12)	26 (14)	-7 to 2	0.22
Carbohydrate oxidation (g)	159 (29)	164 (36)	-6 to 15	0.13
Net energy expenditure (kJ)	3473 (551)	3433 (532)	-104 to 23	0.08

Values are mean (SD). *95% confidence interval for the mean absolute difference between exercise conditions. ES: standardised (to between-subjects SD) effect size.

4.4.2 Acylated ghrelin

A moderate positive correlation of 0.57 (95% CI 0.08 to 0.84, $P = 0.025$) was observed between the two sets of control-adjusted exercise responses for acylated ghrelin (Figure 4.1A). The within-trial SD for acylated ghrelin was substantially greater for the exercise than control conditions (Table 4.2). Baseline-adjusted linear mixed models for acylated ghrelin concentrations revealed a significant main effect of condition ($P < 0.001$) and a significant participant-by-condition interaction ($P < 0.001$). The mean acylated ghrelin concentration was 51 $pg \cdot mL^{-1}$ lower (95% CI -59 to -43 $pg \cdot mL^{-1}$, ES = 0.62) in the exercise versus control conditions. The magnitude of change in individual replicated mean responses after exercise for acylated ghrelin ranged from -141 to -9 $pg \cdot mL^{-1}$, with 100% (n = 15) of participants demonstrating a suppression beyond the MCID (±8.20 $pg \cdot mL^{-1}$) (Figure 4.1B).

4.4.3 Total PYY

A small positive correlation of 0.27 (95% CI -0.28 to 0.69, $P = 0.339$) was observed between the two sets of control-adjusted exercise responses for total PYY (Figure 4.2A). Based on the recommendations of Hopkins et al. (2009), an outlier was identified who exhibited a PYY response greater than 3.5 residual SDs from the mean predicted value (Hopkins et al. 2009). After removal of the outlier, the correlation for total PYY increased to 0.71 and became significant (95% CI 0.31 to 0.90, $P = 0.003$) (Figure 4.2B). The within-trial SD for total PYY was substantially greater for the exercise than control conditions (Table 4.2). Baseline-adjusted linear mixed models for total PYY concentrations revealed a significant main effect of condition ($P < 0.001$) and a significant participant-by-condition interaction ($P = 0.012$). The mean total PYY concentration was 56 pg·mL^{-1} higher (95% CI 44 to 68 pg·mL^{-1}, ES = 1.49) in the exercise versus control conditions. The magnitude of change in individual replicated mean responses after exercise for total PYY ranged from 3 to 112 pg·mL^{-1}, with 93% ($n = 14$) of participants demonstrating an increase beyond the MCID (±3.75 pg·mL^{-1}) (Figure 4.2C).

4.4.4 Appetite ratings

Moderate-to-large positive correlations were observed between the two sets of control-adjusted exercise responses for hunger (r = 0.82, 95% CI 0.53 to 0.94, $P < 0.001$), satisfaction (r = 0.74, 95% CI 0.37 to 0.91, $P = 0.002$), fullness (r = 0.55, 95% CI 0.05 to 0.83, $P = 0.035$) and PFC (r = 0.54, 95% CI 0.04 to 0.82, $P = 0.036$) (Figure 4.3). The within-trial SD was substantially greater for the exercise than control conditions for hunger, satisfaction, fullness and PFC (Table 4.2).

Baseline-adjusted linear mixed models for all ratings of perceived appetite revealed a main effect of condition ($P < 0.001$). Participant-by-condition interactions were observed for ratings of hunger, satisfaction and fullness ($P < 0.001$), but not PFC ($P = 0.053$). The main effect of condition identified suppressed appetite in the exercise compared with control conditions. The mean ratings of hunger and PFC were 26 mm (95% CI -29 to -22 mm, ES = 1.47) and 19 mm (95% CI -25 to -13 mm, ES = 1.05) lower in the exercise versus control conditions, respectively. The mean ratings of satisfaction and fullness were 15 mm (95% CI 11 to 20 mm, ES = 0.95) and 14 mm (95% CI 8 to 21 mm, ES = 0.88) higher in the exercise versus control conditions, respectively. The magnitude of change in individual replicated mean responses after exercise ranged from -65 to 10 mm for hunger, -13 to 72 mm for satisfaction, -23 to 89

mm for fullness and -96 to 7 mm for PFC. Ninety-three percent (n = 14) of participants demonstrated a response beyond the MCID for hunger (±1.76 mm; 13% above, 80% below) and satisfaction (±1.62 mm; 60% above, 33% below), 87% (n = 13) for fullness (±1.64 mm; 53% above, 33% below) and 100% (n = 15) for PFC (±1.82 mm; 33% above, 67% below) (Figure 4.4).

A sensitivity analysis with the participant factor entered into the statistical model as a random, rather than a fixed, effect also resulted in participant-by-condition interactions for all appetite parameters (Table 4.2, P = 0.013–0.077).

4.4.5 Correlations

A large positive correlation was observed between the pre-to-post change in acylated ghrelin and the change in both hunger (r = 0.72, 95% CI 0.33 to 0.90, P = 0.002) and PFC (r = 0.63, 95% CI 0.17 to 0.86, P = 0.011). There were no significant correlations between the pre-to-post change in PYY and appetite perceptions ($P \geq 0.129$) (Table 4.3).

Table 4.2 Unadjusted mean and standard deviations (SD) of the pre-to-post change scores for the exercise and control conditions and the true individual differences SD.

Variable	Exercise change	Control change	Estimate 1[a]	Estimate 2[b]	
	Mean (SD)	Mean (SD)	Individual differences SD	Individual differences SD (SE)	*P* value
Acylated ghrelin (pg·mL^{-1})	-41.9 (33.1)	4.8 (13.0)	30.4	30.9 (19.7)	0.014
Total PYY (pg·mL^{-1})	40.7 (35.5)	-10.7 (23.1)	27.0	25.7 (19.3)	0.077
Hunger (mm)	-13.6 (26.8)	10.5 (7.5)	25.7	24.5 (15.5)	0.013
Satisfaction (mm)	6.5 (25.1)	-7.7 (8.9)	23.5	23.2 (14.8)	0.015
Fullness (mm)	3.6 (34.8)	-8.3 (9.8)	33.4	31.6 (20.1)	0.013
Prospective food consumption (mm)	-9.9 (27.7)	7.7 (9.6)	26.0	23.7 (15.5)	0.019

[a] Estimate 1: Individual differences SD estimated using $SD_R = \sqrt{SD_E^2 - SD_C^2}$ where SD_R is the SD of the true individual response, and SD_E and SD_C are the SDs of the pre-to-post change scores for the exercise and control conditions, respectively (Atkinson and Batterham, 2015; Hopkins, 2015).

[b] Estimate 2: Individual differences SD estimated using a random effects statistical model based on Senn et al. (2011). The SD was derived from the SAS model participant-by-condition interaction term (as a random effect). The *P* value shown is also for this interaction term.

SE, standard error.

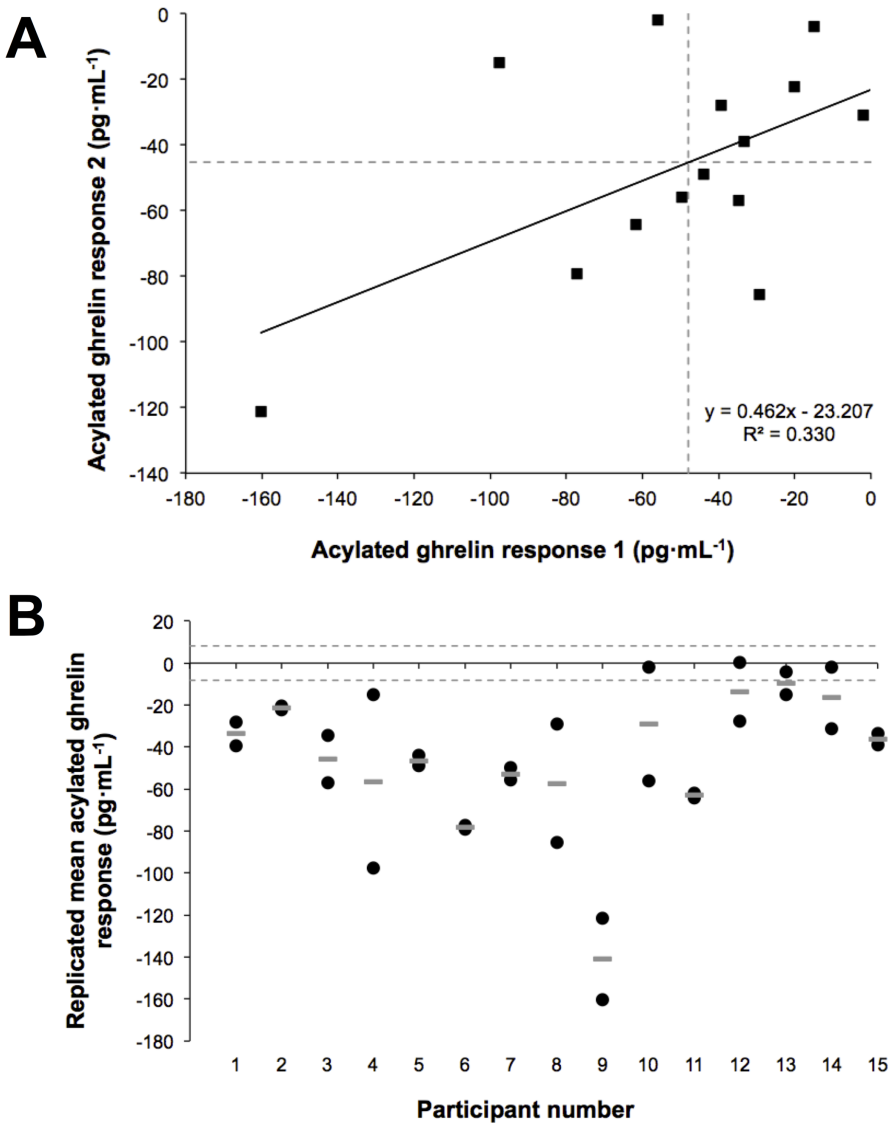


Figure 4.1 (A) Relationship between exercise and control pre-to-post (0 to 1 h) change scores on the two occasions for acylated ghrelin. 'Response 1' corresponds to the first pair of conditions (exercise 1 minus control 1) and 'Response 2' to the second pair of conditions (exercise 2 minus control 2). Dashed lines represent the mean responses. (B) Individual changes in acylated ghrelin between the exercise and control conditions (exercise minus control). Black circles (●) indicate pre-to-post change scores for 'response 1' and 'response 2' for each participant. Grey lines (—) represent each participants' replicated mean response. Dashed lines indicate the standardised minimal clinically important difference calculated as 0.1 multiplied by the baseline between-subject SD (Atkinson and Batterham, 2015).

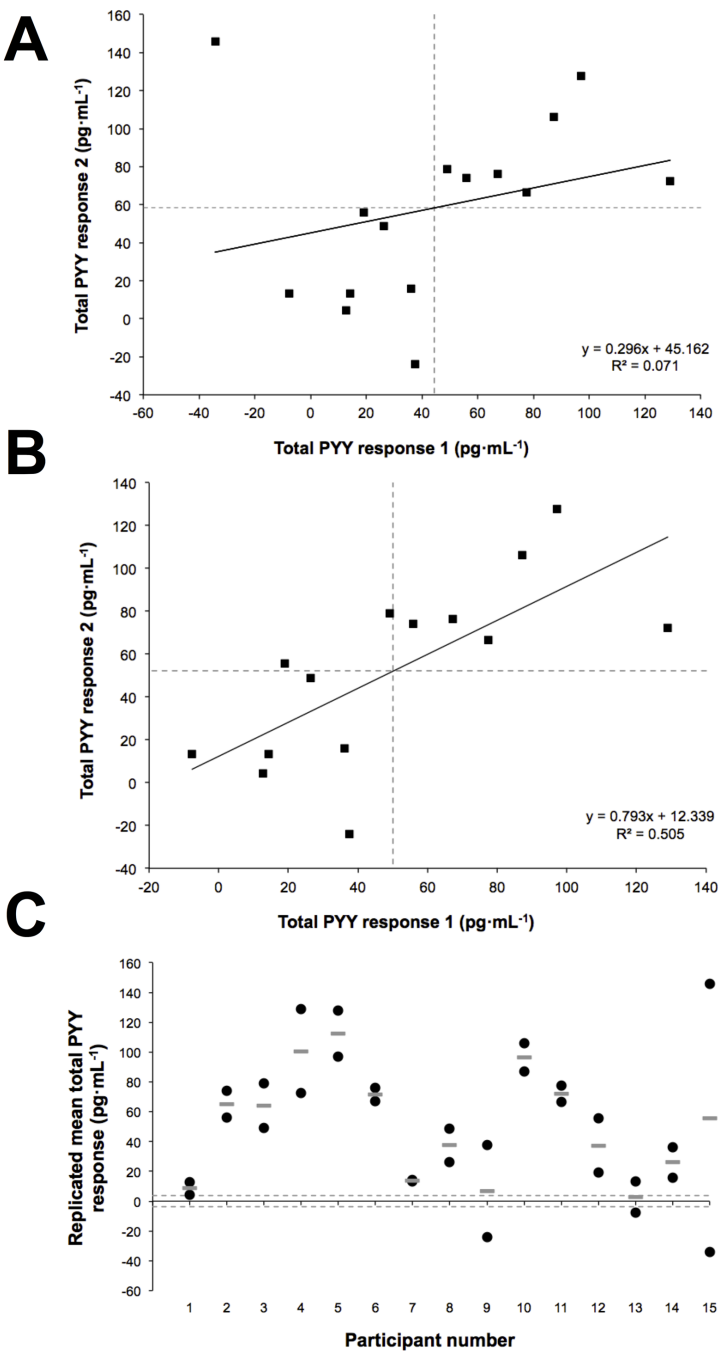


Figure 4.2 Relationship between exercise and control pre-to-post (0 to 1 h) change scores on the two occasions for total PYY before (A) and after (B) the removal of a substantial outlier. 'Response 1' corresponds to the first pair of conditions (exercise 1 minus control 1) and 'Response 2' to the second pair of conditions (exercise 2 minus control 2). Dashed lines represent the mean responses. (C) Individual changes in total PYY between the exercise and control conditions (exercise minus control). Black circles (●) indicate pre-to-post change scores for 'response 1' and 'response 2' for each participant. Grey lines (—) represent each participants' replicated mean response. Dashed lines indicate the standardised minimal clinically important difference calculated as 0.1 multiplied by the baseline between-subject SD (Atkinson and Batterham, 2015).

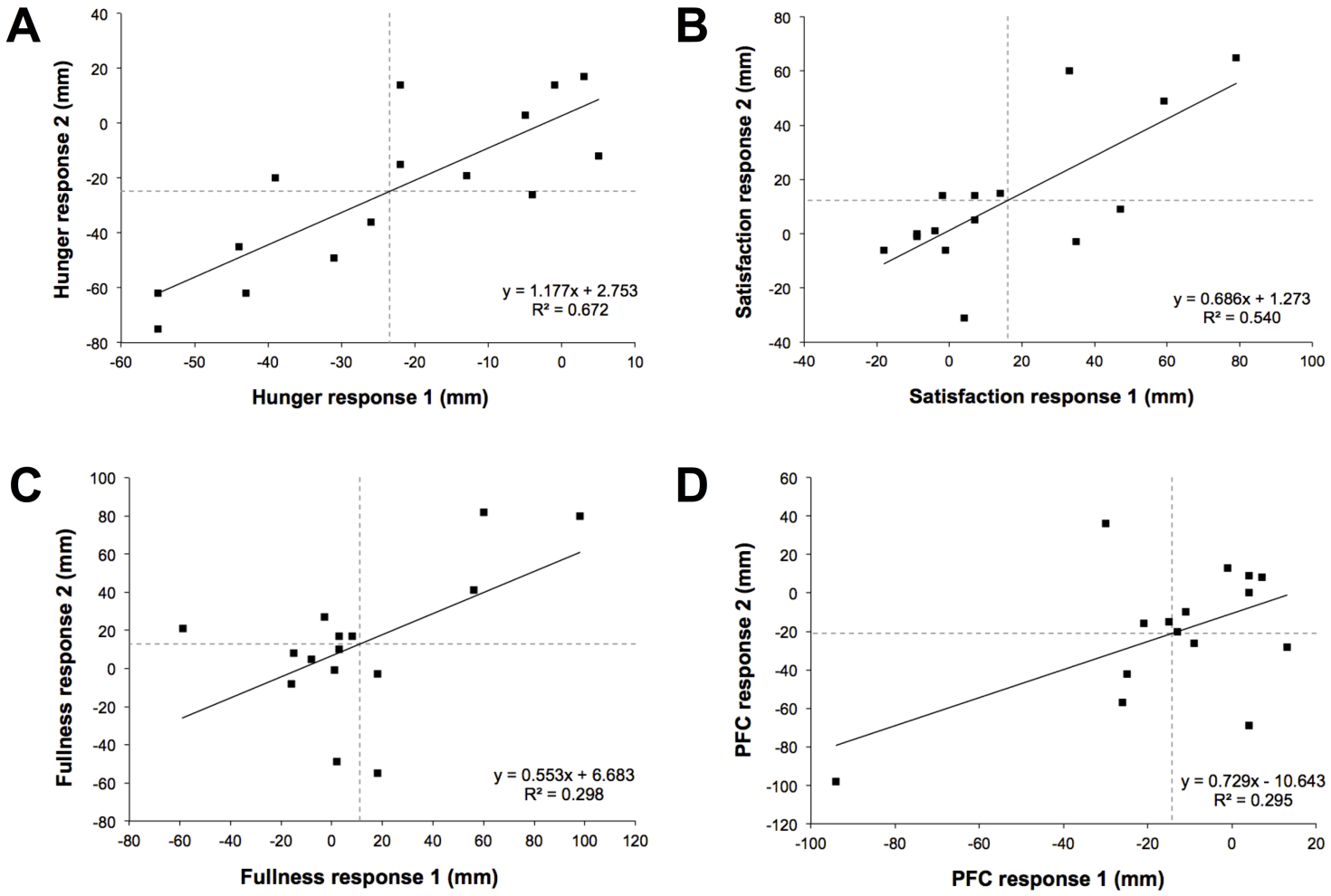


Figure 4.3 Relationship between exercise and control pre-to-post (0 to 1 h) change scores on the two occasions for (A) hunger, (B) satisfaction, (C) fullness, and (D) prospective food consumption (PFC). 'Response 1' corresponds to the first pair of conditions (exercise 1 minus control 1) and 'Response 2' to the second pair of conditions (exercise 2 minus control 2). Dashed lines represent the mean responses.

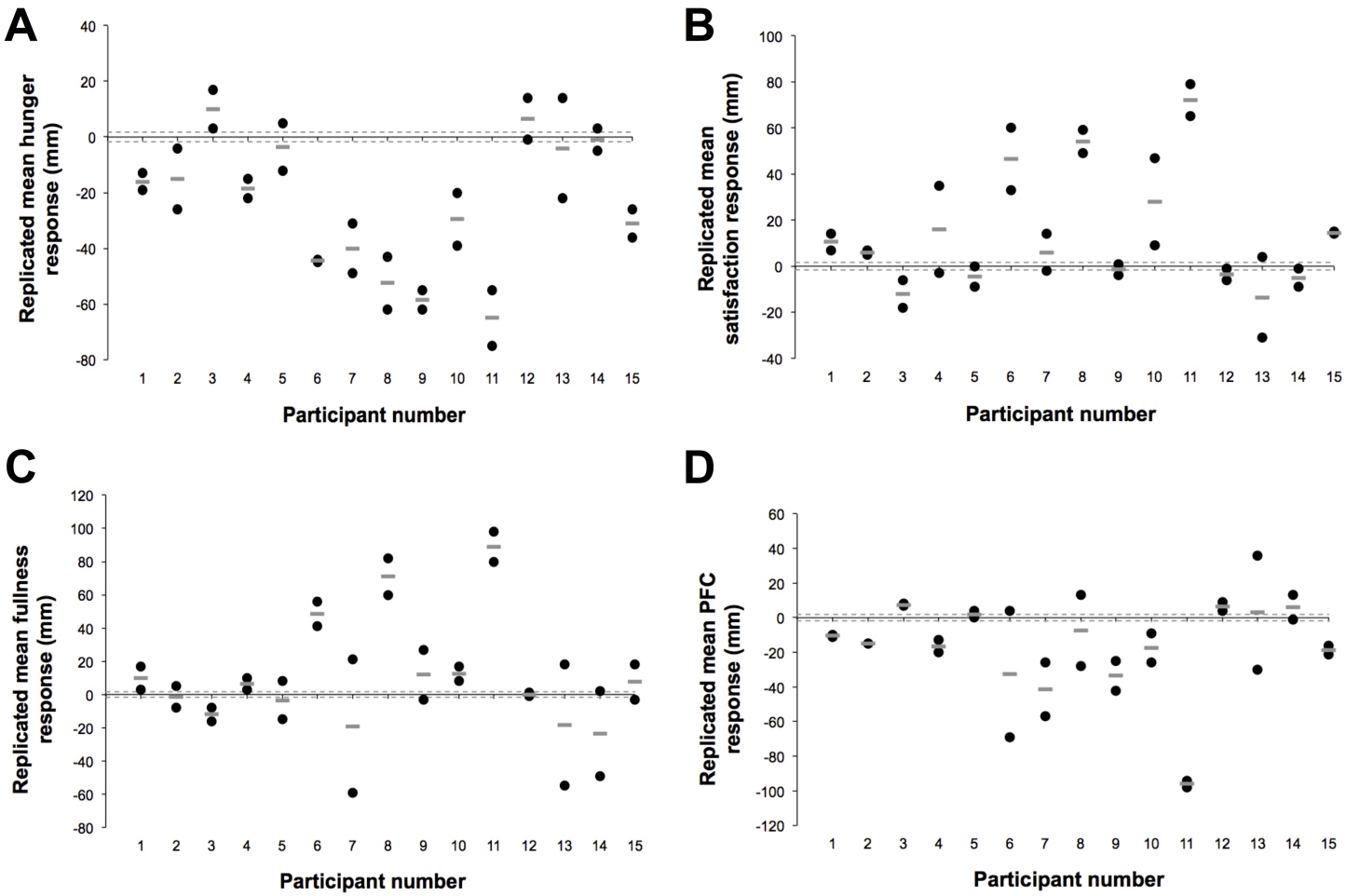


Figure 4.4 Individual changes in each perceived appetite ratings between the exercise and control conditions (exercise minus control): (A) hunger, (B) satisfaction, (C) fullness, (D) prospective food consumption (PFC). Black circles (●) indicate pre-to-post change scores for 'response 1' and 'response 2' for each participant. Grey lines (—) represent each participants' replicated mean response. Dashed lines indicate the standardised minimal clinically important difference calculated as 0.1 multiplied by the baseline between-subject SD (Atkinson and Batterham, 2015).

Table 4.3 Pearson's correlation coefficients between the pooled mean pre-to-post change in appetite-regulatory hormone concentrations and the pooled mean pre-to-post change in appetite perceptions across the four conditions (two exercise and two control).

	Total PYY (pg·mL^{-1})	Hunger (mm)	Satisfaction (mm)	Fullness (mm)	Prospective food consumption (mm)
Acylated ghrelin (pg·mL^{-1})	r = -0.55 *P* = 0.033 95% CI* = -0.83 to - 0.05	r = 0.72 *P* = 0.002 95% CI* = 0.33 to 0.90	r = -0.46 *P* = 0.084 95% CI* = -0.79 to 0.07	r = -0.39 *P* = 0.151 95% CI* = -0.75 to 0.15	r = 0.63 *P* = 0.011 95% CI* = 0.17 to 0.86
Total PYY (pg·mL^{-1})		r = -0.41 *P* = 0.129 95% CI* = -0.76 to 0.13	r = 0.33 *P* = 0.229 95% CI* = -0.22 to 0.72	r = 0.20 *P* = 0.475 95% CI* = -0.35 to 0.65	r = -0.37 *P* = 0.175 95% CI* = -0.74 to 0.17
Hunger (mm)			r = -0.80 *P* < 0.001 95% CI* = -0.93 to -0.49	r = -0.71 *P* = 0.003 95% CI* = -0.90 to -0.31	r = 0.85 *P* < 0.001 95% CI* = 0.60 to 0.95
Satisfaction (mm)				r = 0.83 *P* < 0.001 95% CI* = 0.55 to 0.94	r = -0.66 *P* = 0.007 95% CI* = -0.88 to -0.22
Fullness (mm)					r = -0.57 *P* = 0.026 95% CI* = -0.84 to -0.08

* 95% confidence interval.

PYY, peptide YY.

4.5 Discussion

The primary finding from our replicated crossover trial of appetite responses to exercise was that true interindividual variability exists in the appetite, acylated ghrelin and total PYY responses to acute exercise bouts beyond any measurement error and random within-subject variability over time. A further finding was the moderate-to-large positive correlations observed between the exercise and control pre-to-post change scores on two occasions, indicating good reproducibility for exercise-induced changes in appetite parameters.

Our study supports previous literature by confirming the appetite suppressing effect of acute exercise (Schubert et al. 2014; Deighton and Stensel, 2014). In this regard, the grand mean changes at the sample level indicated a suppression of acylated ghrelin and perceived appetite, and an increase in total PYY after the exercise session. The correlation coefficients quantified between the exercise and control pre-to-post change scores on the two pairs of conditions were positive, significant and moderate-to-large for perceived appetite and acylated ghrelin. Although the correlation for total PYY was small and non-significant, closer examination of the change scores revealed that one participant presented two very opposite responses to exercise. Specifically, the change score between the first pair of trials indicated a suppression in total PYY (-34 $pg \cdot mL^{-1}$) and the second pair of trials showed a very strong increase in total PYY levels (146 $pg \cdot mL^{-1}$) (Figure 4.2A, 4.2C). The reason for this disparity is unclear and removal of this apparent outlier resulted in a larger correlation of similar magnitude to the other appetite-related outcomes measured in our study. Overall, responses to exercise were similar on repeated occasions, providing evidence to support the reproducibility of changes in appetite parameters after acute exercise.

While no previous researchers have quantified the reproducibility of perceived appetite or appetite-regulatory hormone responses to acute exercise, the reproducibility of post-exercise energy intake has received more attention (Laan et al. 2010; Brown et al. 2012; Unick et al. 2015). Specifically, Laan et al. reported good reproducibility for *ad libitum* energy intake after duplicate aerobic exercise, resistance exercise and resting control conditions in young, active adults (Laan et al. 2010). However, the difference in *ad libitum* energy intake between the exercise and control conditions was not calculated (Laan et al. 2010). Therefore, it can be said that within-subject variations were not taken into account and the possibility of the observed responses to exercise being exclusively due to measurement errors and random variability cannot be excluded (Atkinson and Batterham, 2015; Hopkins, 2015). Although energy intake

appears reproducible when considering repeated resting and exercise conditions in isolation (Laan et al. 2010; Unick et al. 2015), the reproducibility of the difference in *ad libitum* energy intake between exercise and control interventions appears low when assessed with the use of intra-class coefficients (Brown et al. 2012; Unick et al. 2015).

Alongside the good reproducibility of appetite responses to acute exercise, our data show that individuals differ in the general magnitude of this response (the mean of the replicated trials, Figures 4.1B, 4.2C and 4.4). A statistically significant participant-by-condition interaction was observed for all appetite parameters, even after adjusting for baseline values. Although previous studies have reported individual variability in perceived appetite and energy intake responses to acute exercise in healthy (Finlayson et al. 2009) and overweight and obese women (Hopkins et al. 2014), this variability was estimated using a single pair of trials, i.e. one control and one exercise condition. Repeated administrations of treatment in a crossover fashion with a comparator arm (control condition) are required to assess individual variability in response to short-term or acute interventions from the participant-by-condition interaction term (Hopkins, 2015). We are not aware of previous studies assessing individual variability in appetite and appetite-regulatory hormone responses to acute exercise using a replicated crossover design and the statistical methods employed in the present study.

The SD of the change scores is a good indication of individual variability in the responses to an intervention. If the SD of the change scores does not differ substantially between control and intervention conditions, the change originated by the intervention could be explained by random within-subject variation and measurement error (Atkinson and Batterham, 2015; Hopkins, 2015). The true individual response SD (using both estimates 1 and 2) was relatively large compared with the mean response for all appetite-related variables measured in this study (Table 4.2). For example, while the mean unadjusted exercise response (versus control change) for acylated ghrelin was approximately 47 $pg \cdot mL^{-1}$, the true individual response SD was approximately ± 30 $pg \cdot mL^{-1}$ (Table 4.2). This SD indicates the presence of substantial true interindividual differences in the acylated ghrelin response to exercise; this interpretation also applies to the other appetite parameters we assessed.

Furthermore, we also highlight that the vast majority of participants showed appetite responses that exceeded the MCID we selected. Therefore, very few participants were identified as "non-responders", but some were "very large responders" while others were "small responders" according to the magnitude of change in acylated ghrelin, total PYY and appetite perceptions

after single bouts of exercise (Figures 4.1B, 4.2C, 4.4). Specifically, all participants demonstrated replicated mean responses beyond the MCID for circulating acylated ghrelin indicating an exercise-induced suppression of this hormone, and 93% of participants experienced an increase in circulating total PYY beyond the MCID. The direction of the replicated mean responses was more variable for the perceived appetite ratings. Of the participants that demonstrated replicated mean responses beyond the MCID, 53–80% of participants reported suppressed appetite after exercise (i.e., lower hunger and PFC, higher satisfaction and fullness), whereas 13–33% of participants reported higher perceived appetite after exercise (i.e., higher hunger and PFC, lower satisfaction and fullness).

Although some studies report concomitant changes in appetite-regulatory hormones and appetite perceptions in response to acute exercise at the group level (Broom et al. 2007; King et al. 2010), exercise-induced changes in these parameters do not always occur simultaneously (Deighton et al. 2013; Sim et al. 2014; Martins et al. 2015). The present study extends these findings by demonstrating that the majority of participants exhibited corresponding exercise-induced changes in acylated ghrelin, total PYY and appetite perceptions, and is further supported by the meaningful positive relationships observed between the pre-to-post change in acylated ghrelin and the change in hunger and PFC. However, some participants demonstrated divergent subjective and hormonal appetite responses to exercise. It is well established that appetite regulation is a complex process involving the interaction of many physiological and psychological factors (Stensel, 2010). Therefore, perceived appetite in some participants could have been more strongly affected by other variables not assessed in the present study. In this regard, several other anorexigenic gut peptides are involved in the acute regulation of appetite including cholecystokinin, oxyntomodulin, pancreatic polypeptide and glucagon-like peptide-1. Indeed, the absence of significant correlations between the pre-to-post change in total PYY and appetite perceptions may reflect the notion that PYY acts synergistically with these other satiety signals to suppress appetite. Furthermore, appetite control is influenced by a variety of non-homeostatic factors such as neuronal responses, hedonic processes and cognitive/behavioural cues (Blundell et al. 2010). Future studies should consider the aforementioned appetite parameters to provide a more holistic scientific understanding of the variability in appetite responses after acute exercise.

A potential source of variability in this study concerns the measurement of acylated ghrelin and total PYY concentrations from venous blood samples collected from an antecubital vein.

Recent studies suggest that compared to arterialised blood, venous blood provides lower concentrations of glucagon-like peptide-1 (Asmar et al. 2017) as well as lower glucose concentrations and higher insulin sensitivity (Edinburgh et al. 2017). Although limited evidence in patient populations suggests that fasting ghrelin concentrations are comparable between venous and arterialised blood (Goodyear et al. 2010; Martin et al. 2011), direct comparisons of acylated ghrelin and total PYY between arterialised and venous blood after exercise have not been investigated. Nevertheless, the findings of the present study are relevant to the wider exercise and appetite regulation literature where blood sampling from an antecubital vein is commonplace for quantifying appetite-regulatory hormone concentrations.

The strengths of our study include the replicated crossover design and the use of recently published robust statistical analyses for individual variability quantification. Moreover, the detailed standardisation protocol followed by all participants during the 24 h preceding each laboratory visit and the precise replication of the exercise sessions add credibility to our results. However, it should be highlighted that our results cannot be generalized to other populations such as females, overweight or obese, and older individuals who may present different results (Alajmi et al. 2016; Douglas et al. 2017). It is also possible that different exercise modes, intensities, or session durations would elicit different responses (Deighton et al. 2013; Deighton and Stensel, 2014; Broom et al. 2017). Therefore, further research is needed to assess the reproducibility and individual variability of exercise-induced changes in appetite-regulatory hormones and appetite perceptions in other populations and with different exercise protocols. The publication of more studies investigating individual variability in appetite responses to exercise may stimulate the development of more efficient weight management strategies by determining whether an exercise intervention is likely to be beneficial, ineffective or detrimental for different individuals. This information would help to identify individuals who may achieve more favourable appetite responses through alternative exercise and/or nutritional interventions, but further work is required to examine this chronically.

In conclusion, healthy, young men exhibited reproducible appetite responses to acute exercise, and true individual variability exists in acylated ghrelin, total PYY and perceived appetite responses over and above any random within-subject variability and measurement error. Individual variability in appetite responses to acute exercise needs to be considered when interpreting study results so that misleading conclusions can be avoided.

CHAPTER 5

Exploration of associations between the FTO genotype, fasting and postprandial appetite-related hormones and perceived appetite in healthy men and women

5.1 Abstract

Background: The fat mass and obesity-associated gene (FTO) has been associated with obesity risk. Although the exact mechanisms involved remain unknown, it has been reported that the at-risk AA genotype is associated with an impaired postprandial suppression of appetite. **Objectives:** To explore the association between the FTO genotype, fasting and postprandial appetite-related hormones and perceived appetite in a heterogeneous sample of men and women. **Methods:** 112 healthy men and women completed three laboratory visits for the assessment of FTO genotype, body composition, aerobic fitness, resting metabolic rate, visceral adipose tissue, liver fat, fasting leptin, and fasting and postprandial acylated ghrelin, total PYY, insulin, glucose and perceived appetite. Participants wore accelerometers for seven consecutive days for the assessment of physical activity and sedentary behaviour. Multivariable general linear models quantified differences between FTO genotype groups for fasting and postprandial appetite outcomes, with and without the addition of *a priori* selected physiological and behavioural covariates. Sex-specific univariable Pearson's correlation coefficients were quantified between the appetite-related outcomes and individual characteristics. **Results:** 95% confidence intervals for mean differences between FTO groups overlapped zero in the unadjusted and adjusted general linear models for all the fasting ($P \geq 0.278$) and postprandial ($P \geq 0.186$) appetite-related outcomes. Eta^2 values for explained variance attributable to FTO group were < 5% for all outcomes. An exploratory correlation matrix indicated that associations between fasting and postprandial acylated ghrelin, total PYY and general or abdominal adiposity were also small (r = -0.23 to 0.15, $P \geq 0.090$). Fasting leptin, glucose and insulin and postprandial insulin concentrations were associated with adiposity outcomes (r = 0.29 to 0.81, $P \leq 0.033$). **Conclusions:** Associations between the FTO genotype and fasting or postprandial appetite-related outcomes were weak in healthy men and women.

5.2 Introduction

The scientific understanding of appetite control has increased considerably in recent decades, which has been helpful in elucidating the complex nature of energy balance and weight control. Central components of the homeostatic control of appetite comprise signals from adipose tissue and peptide hormones secreted from the digestive tract, which act acutely and/or chronically on central neural pathways to influence hunger, satiety and subsequent energy intake (MacLean et al. 2017). These signals and hormones include the tonic signals leptin and insulin that regulate long-term changes in energy balance and adiposity status, as well as a variety of episodic gut signals, which mediate hunger and satiety on a meal-by-meal basis (Blundell et al. 2008, 2015a; MacLean et al. 2017). Notable among the episodic mediators of appetite and energy intake are acylated ghrelin and peptide YY (PYY) which exert orexigenic and anorexigenic effects, respectively, to facilitate meal initiation and termination (Neary and Batterham, 2009).

Over the last 16 years, our laboratory has measured circulating concentrations of appetite-related hormones in response to meal ingestion in many studies. A consistent observation from this body of work is the degree of variability in responses observed between participants studied under identical conditions. Furthermore, using the gold standard replicated crossover study design (Atkinson and Batterham, 2015; Senn, 2016), we have demonstrated recently the presence of true interindividual heterogeneity in appetite perceptions and circulating concentrations of acylated ghrelin, total PYY, insulin and glucose in response to a standardised meal, over and above any random within-subject variability and measurement error (Goltz et al. 2019; Chapter 6). Similar findings were also observed in acylated ghrelin, total PYY and perceived appetite responses to replicated single bouts of aerobic exercise (Goltz et al. 2018; Chapter 4).

The factors responsible for interindividual variability in appetite-related hormone concentrations are not fully understood, but it is plausible that differences in individual characteristics and behaviours may contribute to the variability observed. In this regard, the fat mass and obesity-associated gene (FTO) has been associated with obesity risk, with individuals homozygous for the A allele (AA) of FTO rs9939609 having a 1.7-fold higher obesity risk than individuals homozygous for the T allele (TT) (Frayling et al. 2007). Although the exact mechanisms through which FTO influences fat mass accumulation remain unknown, it has been suggested that FTO exerts its effect on food intake rather than on energy expenditure

(Speakman et al. 2008). Furthermore, AA individuals have been shown to exhibit an attenuated postprandial suppression of hunger and acylated ghrelin compared with TT individuals, which may predispose AA individuals to higher energy intake and, consequently, higher fat mass (Karra et al. 2013). However, the study by Karra and colleagues was performed in young healthy weight males and it is not known whether this influence of the FTO gene on postprandial appetite regulation is observed in a heterogenous sample of men and women.

Beyond genetic influence, it has been speculated that other individual factors may affect appetite regulation. Data from previous studies have indicated that women exhibit higher fasting concentrations of acylated ghrelin than men (Alajmi et al. 2016; Douglas et al. 2017). Furthermore, an inverse relationship between general adiposity levels and fasting ghrelin levels has been suggested, possibly because of elevated insulin or leptin levels (Tschöp et al. 2001; Shiiya et al. 2002; Sondergaard et al. 2009). Individuals who are obese also exhibit a reduced postprandial suppression of ghrelin (Le Roux et al. 2005) and blunted postprandial increases in PYY (Le Roux et al. 2006). Limited evidence has also suggested an inverse association between visceral adipose tissue and fasting ghrelin levels, likely caused by substances secreted by visceral adipocytes, such as tumour necrosis factor α and leptin (Sondergaard et al. 2009). Moreover, fat-free mass, as the largest contributor to resting metabolic rate, has been identified as a key driver of appetite and energy intake (Blundell et al. 2015b). Physical activity has also been suggested to alter the sensitivity of the appetite control system by enhancing meal-induced satiety which may facilitate energy balance over the long term (Beaulieu et al. 2016). Together, these findings highlight the importance of investigating the effect of the FTO gene on appetite parameters in a sample of males and females with a wide range of age, adiposity and physical activity levels, including physiological and behavioural characteristics as covariates in the analyses.

The primary aim of this study was to use objective assessment methods in order to explore the association of the FTO genotype with fasting and postprandial appetite-related hormones and perceived appetite in a sample of healthy men and women. The secondary aim was to explore potential associations between fasting and postprandial appetite outcomes and physiological and behavioural characteristics.

5.3 Methods

5.3.1 Participants

With the approval of the University Ethics Advisory Sub-Committee, a total of 121 participants (57 men, 64 women) aged 18 to 50 years provided written informed consent before taking part in the study. All participants were deemed to be stable in their body mass (≤ 3 kg change in the previous 3 months), non-smokers, habitual breakfast eaters, had no history of cardiovascular or metabolic disease, and were not dieting or taking any medications known to influence the outcome measures. Female participants were premenopausal and postmenopausal and not pregnant. Nine participants withdrew from the study before completing all study measurements due to time constraints. Therefore, data are presented for 112 participants (56 men, 56 women). The study sample self-reported ethnicity distribution was as follows: 93% white Europeans, 6% Asians and 1% black.

5.3.2 Visit 1: Preliminary testing

Participants attended the laboratory for a preliminary visit to confirm eligibility, and to undergo familiarisation, anthropometric measurements and determination of peak oxygen uptake ($\dot{V}O_2$ peak). The eligibility assessment included screening questionnaires to assess health status and food preferences and/or restrictions. Stature was measured to the nearest 0.1 cm and body mass to the nearest 0.1 kg using an electronic measuring station (Seca, Hamburg, Germany), and body mass index (BMI) was calculated. The sum of three skinfolds (chest, abdomen and thigh for men, and triceps, suprailiac and thigh for women) was used to estimate body density (Jackson and Pollock 1978, 1980) and body fat percentage (Siri, 1961). Waist circumference was measured as the narrowest point between the lower rib margin and the iliac crest.

Participants were familiarised with walking and running on the treadmill (Technogym Excite Med, Cesena, Italy) before completing an incremental uphill treadmill protocol to determine $\dot{V}O_2$ peak, as described in detail in Chapter 3.

5.3.3 Visit 2: Magnetic resonance imaging (MRI) scan

Each participant underwent an MRI scan in the supine position using a dual-echo Dixon fat and water sequence on a 3-T MRI scanner (GE Healthcare MR750w, Chicago, USA). A

detailed description of the protocol has been reported previously (Borga et al. 2015; West et al. 2016) and a summary of the procedures is provided in Chapter 3.

5.3.4 Visit 3: Resting metabolic rate and test meal

All premenopausal female participants completed the test meal during the follicular phase of the menstrual cycle (days 6-12) to avoid potential hormonal influences on appetite parameters. Participants were asked to refrain from caffeine, alcohol, and strenuous exercise during the 24 h before the visit. A standardised evening meal (3297 kJ, 40% fat, 39% carbohydrate, 21% protein) was consumed the evening before the visit and only plain water was permitted after the meal until participants arrived at the laboratory the next day.

Participants reported to the laboratory at 08:00 after fasting overnight for 12 h. A cannula (Becton Dickinson Venflon, Helsingborg, Sweden) was inserted into an antecubital vein for venous blood sampling, and participants rested for 60 min to eliminate any stress effects in response to the cannula (Chandarana et al. 2009). During this time, resting metabolic rate was measured using an open circuit indirect calorimetry system (GEM Nutrition, Cheshire, England). Participants were asked to lie in a comfortable supine position and were instructed not to talk or sleep, and to move as little as possible during the measurement, which was described in detail in Chapter 3.

A fasting venous blood sample and rating of perceived appetite were taken 60 min after the insertion of the cannula. Participants then consumed a standardised breakfast within 15 min marking the start of the postprandial assessment period (09:00; 0 h). Breakfast consisted of a ham and cheese sandwich, milkshake and chocolate biscuit which provided 4435 kJ of energy (41% carbohydrate, 18% protein, 41% fat). Subsequent venous blood samples and ratings of perceived appetite were taken at 0.5, 1 and 2 h after the start of the breakfast whilst the participants rested in a semi-supine position.

5.3.4.1 Appetite perceptions

Appetite perceptions (hunger, satisfaction, fullness, prospective food consumption) were assessed using 100 mm visual analogue scales (Flint et al. 2000). An overall appetite rating was calculated as the mean value of the four appetite ratings once satisfaction and fullness were reverse-scored (Stubbs et al. 2000).

5.3.4.2 Blood sampling and biochemical analysis

Venous blood samples were collected into pre-chilled ethylenediaminetetraacetic acid (EDTA) monovettes (Sarstedt, Leicester, UK) for the determination of plasma acylated ghrelin, total PYY, leptin, insulin and glucose concentrations, as described in detail in Chapter 3. Haemoglobin concentration and haematocrit were quantified in duplicate at 0 and 2 h to estimate the acute change in plasma volume (Dill and Costill, 1974).

Commercially available enzyme-linked immunosorbent assays were used to determine the concentrations of plasma acylated ghrelin (Bertin Technologies, Montigney le Bretonneux, France), total PYY (Millipore, Billerica, USA), leptin (R&D Systems, Minneapolis, USA) and insulin (Mercodia, Uppsala, Sweden). Plasma glucose concentrations were determined by enzymatic, colorimetric methods using a benchtop analyser (Horiba Medical Pentra 400, Montpellier, France). The within-batch coefficient of variation for acylated ghrelin, total PYY, leptin, insulin and glucose concentrations were 4.3%, 5.1%, 8.3%, 4.7%, 0.4%, respectively.

An additional fasting venous blood sample was collected into a 2.7-mL EDTA monovette (Sarstedt, Leicester, UK) and the whole blood sample was stored at 4°C to undergo deoxyribonucleic acid (DNA) extraction and genotyping for the rs9939609 allele within the FTO gene, as described in Chapter 3. Participants were assigned to one of three groups according to their genotype: homozygous major allele, TT (36%; males n = 23, females n = 17); heterozygous allele, AT (45%; males n = 22, females n = 29); or homozygous minor allele, AA (19%; males n = 11, females n = 10).

5.3.4.3 Habitual physical activity and sedentary time

Participants wore an ActiGraph GT3X+ accelerometer (ActiGraph, Pensacola, USA) on an elasticated belt on the waist above the mid-line of the thigh on their non-dominant side of the body and an activPAL3 accelerometer (PAL Technologies Ltd., Glasgow, UK), attached directly to the skin on the midline of the anterior aspect of the thigh in line with the ActiGraph GT3X+ accelerometer. Moderate-to-vigorous physical activity (MVPA) and sitting time data were collected as described in detail in Chapter 3.

5.3.5 Statistical analyses

We estimated the effect size detection sensitivity given our sample size using NQuery (version 3, Statistical Solutions, Cork, Ireland). For a total sample size of 110 and three study groups, we estimated that a "medium" (Cohen, 1998) Eta^2 value of 0.18 would be detected in a univariable model as statistically significant ($P < 0.050$) with power of 90%.

Postprandial overall appetite and plasma concentrations of acylated ghrelin, total PYY, insulin and glucose are presented relative to baseline concentrations (delta) to minimise the potential influence of day-to-day biological variability (Deighton et al. 2013, 2014). Total area under the curve (AUC) values were calculated using the trapezoidal method. Correction of blood parameter concentrations for acute changes in plasma volume had a negligible influence on our findings and, therefore, the unadjusted plasma concentrations are displayed for simplicity.

Multivariable general linear models were used to quantify the mean differences (and 95% confidence intervals) between FTO genotype groups for each fasting and postprandial appetite outcome. The Eta^2 statistic (with associated 90% confidence interval) was also estimated for each model and each outcome (Kline, 2004; Steiger, 2004). This statistic is interpreted in a similar way as the coefficient of determination, where 100 x Eta^2 gives the explained variance attributable to the FTO groups. A 90% rather than a 95% confidence interval is reported because the Eta^2 statistic can only be positive in sign. The model residuals of the appetite outcome variables were explored for parity to a Gaussian distribution using histograms. The model residuals for fasting acylated ghrelin and insulin concentrations were observed to show a positively skewed distribution so these data were logarithmically-transformed prior to analysis (Bland and Altman, 1996). Three models were used for each of the fasting and postprandial appetite outcomes, as follows:

1. Model I: Univariable models with FTO genotype as single fixed effect;

2. Model II: A multivariable model based on the selection of matched covariates studied by Karra et al. (2013), i.e. age, fat mass and visceral adipose tissue. FTO genotype was entered as a fixed effect and sex, age, fat mass and visceral adipose tissue were entered as covariates;

3. Model III: A multivariable model, where FTO genotype was entered as a fixed effect and sex, age, BMI, $\dot{V}O_2$ peak, resting metabolic rate, visceral adipose tissue,

abdominal subcutaneous adipose tissue, liver fat, sitting time and MVPA were entered as covariates. Rather than the now discouraged use of stepwise selection procedures, these covariates were included based on their hypothesised influence on the outcome variables, while considering the potential that some predictors were mathematically coupled (Flom and Cassell, 2007; Wittingham et al. 2006). For example, total fat mass was excluded from this model because multiple specific adiposity parameters were considered.

The covariates in models II and III were each standardised prior to analysis by dividing each datum by twice the respective SD (Gelman and Pardoe, 2007). In sensitivity analyses, model III was also run with (i) waist circumference replacing BMI; (ii) percentage body fat replacing BMI; and (iii) with a sex-by-genotype interaction term.

Univariable general linear models with FTO genotype as a single fixed effect were used to quantify differences between genotype groups for body mass, BMI and fat mass. Between-sex differences in participant characteristics and appetite-related outcomes in the fasting and postprandial states were assessed using univariable general linear models with sex as a single fixed effect. Sex-specific univariable Pearson's correlation coefficients were quantified between appetite-related outcomes and individual characteristics, and between appetite-related blood parameters and perceived appetite.

95% confidence intervals (95% CI) were quantified for correlation coefficients. *P* values are expressed in exact terms apart from very low values, which are expressed as $P < 0.001$. A threshold of statistical significance was accepted as $P < 0.050$, although we deemed a *P* value of < 0.005 as a stronger indication of potentially more reproducible results in line with recent advice (Benjamin et al. 2017). All statistical analyses were performed in SPSS (v.23, IBM Corporation, New York, USA).

5.4 Results

5.4.1 Missing data

Due to technical issues with the equipment, resting metabolic rate is presented for 107 participants (53 males), sitting time for 96 participants (47 males) and MVPA for 100 participants (49 males). Eleven participants were unable to undertake the MRI scan for safety reasons and, therefore, visceral adipose tissue and abdominal subcutaneous adipose tissue are

presented for 101 participants (50 males). Liver fat could not be quantified from some images due to motion artefacts and, therefore, data is presented for 97 participants (48 males).

5.4.2 Participant characteristics and appetite-related outcomes

Participant characteristics, perceived appetite and appetite-related blood parameters in the fasting and postprandial states are presented in Table 5.1. In summary, statistically significant differences were observed between men and women in stature, body mass, BMI, waist circumference, fat-free mass, $\dot{V}O_2$ peak, resting metabolic rate, visceral adipose tissue, abdominal subcutaneous adipose tissue, liver fat, fasting leptin, fasting glucose, postprandial acylated ghrelin and postprandial overall appetite ($P \leq 0.016$, $Eta^2 = 0.051$ to 0.644). Postprandial delta values for acylated ghrelin, total PYY, insulin and glucose concentrations and perceived overall appetite are presented in Figure 5.1.

Table 5.1 Participant characteristics and appetite outcomes in the fasting and postprandial states.

	All (*n* = 112)	**Range (min to max)**	**Men (*n* = 56)**	**Women (*n* = 56)**	***P***	**Mean difference 95% CI**
Age (years)	34 (9)	18 to 50	35.3 (9.7)	33.5 (9.1)	0.303	-5.4 to 1.7
Stature (cm)	171.0 (9.2)	149.1 to 200.4	178.5 (6.6)	165.3 (6.2)	**< 0.001***	-15.6 to -10.8
Body mass (kg)	74.9 (14.7)	48.5 to 140.4	83.3 (12.9)	66.5 (11.1)	**< 0.001***	-21.2 to -12.2
Body mass index ($kg \cdot m^{-2}$)	25.2 (3.9)	18.4 to 40.3	26.1 (3.7)	24.4 (4.0)	**0.016**	-3.2 to -0.3
Waist circumference (cm)	82.7 (10.8)	62.4 to 125.0	88.4 (9.8)	77.0 (8.7)	**< 0.001***	-14.9 to -8.0
Fat mass (kg)	16.9 (8.4)	3.5 to 47.8	15.5 (9.1)	18.2 (7.4)	0.078	-0.3 to 5.9
Fat-free mass (kg)	58.1 (12.2)	36.8 to 92.6	67.8 (8.8)	48.3 (5.5)	**< 0.001***	-22.2 to -16.8
$\dot{V}O_2$ peak ($mL \cdot kg \cdot min^{-1}$)	44.0 (9.3)	21.0 to 81.0	49.0 (9.3)	39.0 (6.1)	**< 0.001***	-13.0 to -7.1
Resting metabolic rate (kcal) [a]	1617 (322)	889 to 2567	1808 (290)	1430 (232)	**< 0.001***	-478 to -277
Visceral adipose tissue (L) [a]	1.70 (1.26)	0.11 to 6.22	2.27 (1.41)	1.14 (0.75)	**< 0.001***	-1.58 to -0.69
Abdominal subcutaneous adipose tissue (L) [a]	5.39 (3.02)	1.45 to 16.86	4.49 (2.39)	6.27 (3.33)	**0.003***	0.64 to 2.93
Liver fat (%) [a]	2.12 (1.81)	0.46 to 10.45	2.62 (2.19)	1.63 (1.16)	**0.006**	-1.69 to -0.28
Sitting time ($min \cdot day^{-1}$) [a]	509 (85)	256 to 737	513 (73)	504 (95)	0.630	-43 to 26
MVPA ($min \cdot day^{-1}$) [a]	55 (31)	11 to 163	57 (30)	54 (33)	0.706	-15 to 10
Fasting leptin ($ng \cdot mL^{-1}$)	8.62 (8.63)	1.34 to 43.85	4.07 (3.08)	13.16 (9.95)	**< 0.001***	6.33 to 11.84
Fasting acylated ghrelin ($pg \cdot mL^{-1}$)	173.6 (491.8)	12.0 to 4410.6	103.3 (108.8)	243.8 (682.9)	0.131	-42.6 to 323.6
Fasting total PYY ($pg \cdot mL^{-1}$)	117.5 (50.5)	13.6 to 270.0	121.9 (47.9)	113.0 (53.1)	0.353	-27.8 to 10.0
Fasting insulin ($pmol \cdot L^{-1}$)	23.3 (15.0)	2.9 to 97.1	22.9 (14.3)	23.6 (15.8)	0.825	-5.0 to 6.3
Fasting glucose ($mmol \cdot L^{-1}$)	5.24 (0.43)	4.29 to 6.56	5.37 (0.43)	5.12 (0.39)	**0.001***	-0.41 to -0.10
Fasting overall appetite (mm)	70.8 (15.3)	19 to 95	71.2 (13.4)	70.4 (17.1)	0.787	-6.5 to 5.0
Acylated ghrelin delta AUC (2 h, $pg \cdot mL^{-1}$)	-87.9 (126.6)	-1183.5 to 165.8	-51.3 (56.3)	-124.6 (162.6)	**0.002***	-118.9 to -27.8
Total PYY delta AUC (2 h, $pg \cdot mL^{-1}$)	101.6 (61.0)	-26.4 to 340.7	99.0 (62.4)	104.2 (59.9)	0.653	-17.7 to 28.1
Insulin delta AUC (2 h, $pg \cdot mL^{-1}$)	420.6 (236.8)	121.3 to 1485.8	403.9 (256.6)	437.3 (216.3)	0.458	-55.5 to 122.2
Glucose delta AUC (2 h, $pg \cdot mL^{-1}$)	0.77 (1.59)	-2.20 to 5.79	0.54 (1.37)	1.00 (1.77)	0.125	-0.13 to 1.05
Overall appetite delta AUC (2 h, $pg \cdot mL^{-1}$)	-77.4 (34.4)	-150.0 to -14.0	-65.7 (30.9)	-89.1 (34.0)	**< 0.001***	-35.5 to -11.1

Values are mean (SD). *P* values and 95% CI are from univariable general linear models with sex as a single fixed effect.

[a] *n* = 107 (53 males) for resting metabolic rate, 96 (47 males) for sitting time, 100 (49 males) for MVPA, 101 (50 males) for visceral adipose tissue and abdominal subcutaneous adipose tissue, and 97 (48 males) for liver fat.

AUC, area under the curve; CI, confidence interval; MVPA, moderate-to-vigorous physical activity, PYY, peptide YY; $\dot{V}O_2$ peak, peak oxygen uptake.

* $P < 0.005$.

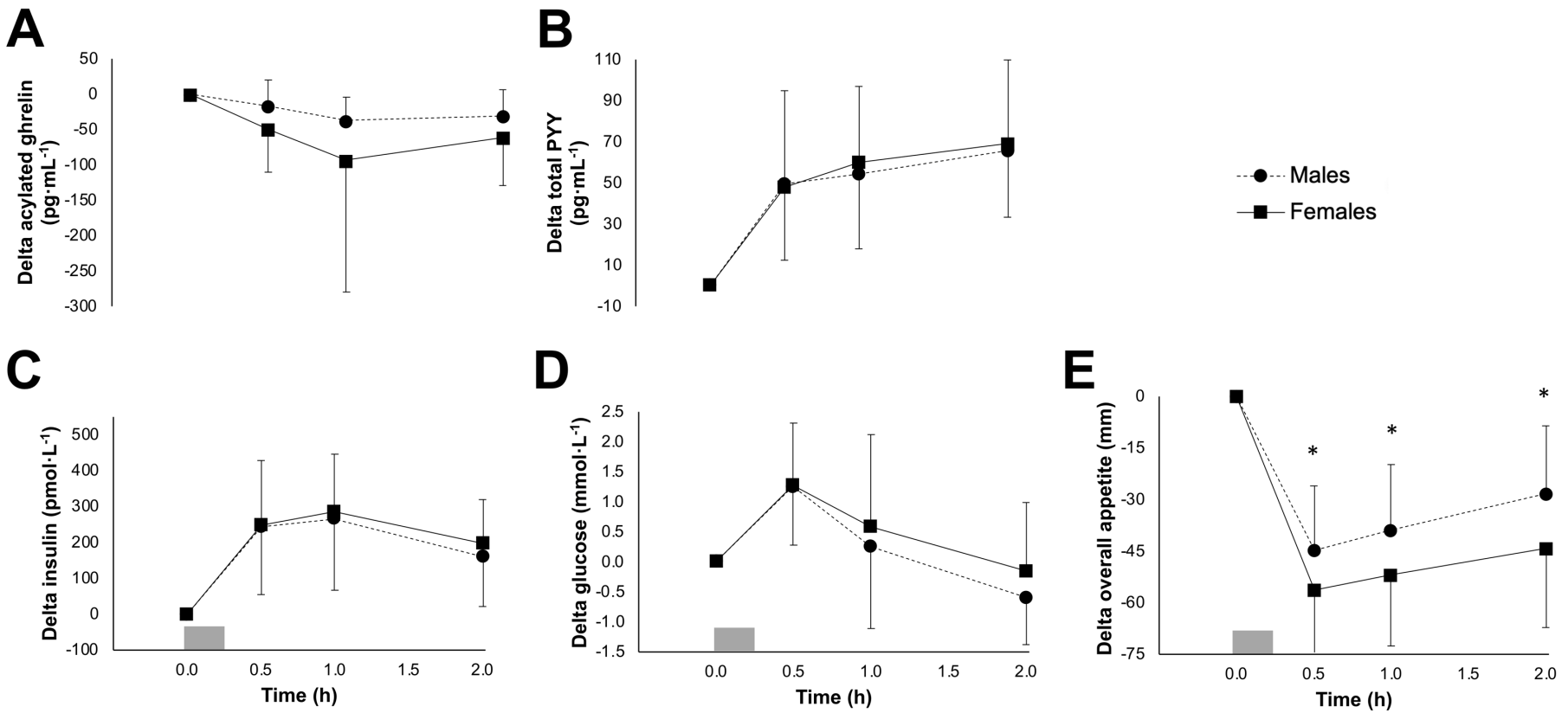


Figure 5.1 Delta postprandial values for acylated ghrelin (A), total peptide YY (PYY) (B), insulin (C), glucose (D) and overall perceived appetite (E) in 56 males and 56 females. Grey rectangles indicate meal consumed within 15 min. Values are presented as mean (SD). Linear mixed models identified main effects of sex for delta acylated ghrelin, delta glucose and delta overall appetite ($P \leq 0.045$), main effects of time for all outcomes ($P < 0.001$) and a sex-by-time interaction for delta appetite ($P = 0.004$).

* $P < 0.001$ for post-hoc analysis of sex-by-time interaction between males and females.

5.4.3 Univariable and multivariable general linear models

No statistically significant influence of the FTO genotype was identified for body mass (Eta^2 = 0.027, P = 0.234), BMI (Eta^2 = 0.003, P = 0.688) or fat mass (Eta^2 = 0.025, P = 0.259).

5.4.3.1 Fasting appetite-related outcomes

Separate univariate modelling (model I) did not reveal any statistically significant influence of the FTO genotype on fasting acylated ghrelin, total PYY, insulin, glucose, leptin or overall appetite ($P \geq 0.501$) (Table 5.2). Similarly, no significant effect of the FTO genotype was detected on fasting appetite-related outcomes in model II ($P \geq 0.098$) or model III ($P \geq 0.453$) (Table 5.2). All Eta^2 values were very low (< 0.05). Replacing BMI with waist circumference, replacing BMI with body fat percentage, and including a sex-by-genotype interaction term in the sensitivity analyses did not result in a significant effect of the FTO genotype on any of the fasting appetite-related outcomes ($P \geq 0.470$, $P \geq 0.437$, $P \geq 0.455$, respectively).

5.4.3.2 Postprandial appetite-related outcomes

Separate univariate modelling (model I) did not reveal any statistically significant influence of the FTO genotype on delta AUC for acylated ghrelin, total PYY, insulin, glucose, leptin or overall appetite ($P \geq 0.322$) (Table 5.3). Similarly, no significant effect of the FTO genotype was detected on delta AUC for any of the appetite-related outcomes in model II ($P \geq 0.271$) or model III ($P \geq 0.186$) (Table 5.3). Again, all Eta^2 values were very low (< 0.05). Replacing BMI with waist circumference, replacing BMI with body fat percentage, and including a sex-by-genotype interaction term in the sensitivity analyses did not result in a significant effect of the FTO genotype on any of the postprandial appetite-related outcomes ($P \geq 0.133$, $P \geq 0.102$, $P \geq 0.206$, respectively).

Table 5.2 Estimated marginal means from the multivariable general linear models used to quantify the differences between FTO genotype groups in each fasting appetite outcome.

	Model I			Model II			Model III		
	AT (*n* = 49)	**AA (*n* = 21)**	**TT (*n* = 40)**	**AT (*n* = 45)**	**AA (*n* = 18)**	**TT (*n* = 37)**	**AT (*n* = 34)**	**AA (*n* = 17)**	**TT (*n* = 28)**
Fasting acylated ghrelin (log pg·mL^{-1})	4.47 (4.25 to 4.69)	4.59 (4.26 to 4.92)	4.51 (4.27 to 4.75)	4.42 (4.18 to 4.65)	4.57 (4.20 to 4.94)	4.57 (4.30 to 4.83)	4.42 (4.20 to 4.64)	4.56 (4.23 to 4.88)	4.29 (4.03 to 4.54)
	Eta^2 = 0.003 (90% CI: 0.000-0.023), *P* = 0.835			Eta^2 = 0.009 (90% CI: 0.000-0.047), *P* = 0.660			Eta^2 = 0.024 (90% CI: 0.000-0.091), *P* = 0.453		
Fasting total PYY (pg·mL^{-1})	110.3 (96.1 to 124.5)	123.5 (101.8 to 145.2)	120.4 (104.7 to 136.2)	109.2 (94.0 to 124.4)	123.6 (100.2 to 147.0)	122.4 (105.7 to 139.1)	114.3 (97.6 to 130.9)	117.2 (93.3 to 141.0)	114.1 (95.0 to 133.2)
	Eta^2 = 0.013 (90% CI: 0.000-0.055), *P* = 0.501			Eta^2 = 0.018 (90% CI: 0.000-0.069), *P* = 0.434			Eta^2 = 0.001 (90% CI: 0.000-0.014), *P* = 0.977		
Fasting insulin (log pmol·L^{-1})	3.00 (2.83 to 3.16)	2.87 (2.61 to 3.12)	2.97 (2.79 to 3.16)	3.03 (2.88 to 3.19)	2.93 (2.70 to 3.17)	2.96 (2.79 to 3.13)	3.01 (2.81 to 3.20)	2.98 (2.70 to 3.27)	2.95 (2.72 to 3.18)
	Eta^2 = 0.007 (90% CI: 0.000-0.038), *P* = 0.699			Eta^2 = 0.007 (90% CI: 0.000-0.041), *P* = 0.716			Eta^2 = 0.002 (90% CI: 0.000-0.028), *P* = 0.935		
Fasting glucose (mmol·L^{-1})	5.23 (5.11 to 5.36)	5.28 (5.09 to 5.47)	5.22 (5.09 to 5.36)	5.27 (5.15 to 5.38)	5.28 (5.11 to 5.46)	5.14 (5.02 to 5.27)	5.24 (5.10 to 5.38)	5.30 (5.10 to 5.51)	5.16 (5.00 to 5.32)
	Eta^2 = 0.002 (90% CI: 0.000-0.016), *P* = 0.882			Eta^2 = 0.027 (90% CI: 0.000-0.087), *P* = 0.278			Eta^2 = 0.018 (90% CI: 0.000-0.078), *P* = 0.553		
Fasting leptin (ng·mL^{-1})	9.17 (6.70 to 11.65)	8.06 (4.27 to 11.84)	7.95 (5.21 to 10.69)	9.77 (8.15 to 11.39)	6.67 (4.17 to 9.17)	7.93 (6.15 to 9.71)	9.76 (7.91 to 11.62)	8.71 (6.05 to 11.37)	8.72 (6.59 to 10.85)
	Eta^2 = 0.005 (90% CI: 0.000-0.030), *P* = 0.779			Eta^2 = 0.049 (90% CI: 0.000-0.122), *P* = 0.098			Eta^2 = 0.010 (90% CI: 0.000-0.057), *P* = 0.713		
Fasting overall appetite (mm)	70.0 (65.7 to 74.4)	69.6 (63.0 to 76.2)	72.2 (67.4 to 77.0)	67.6 (63.0 to 72.3)	70.2 (63.0 to 77.4)	72.4 (67.3 to 77.6)	66.8 (60.9 to 72.7)	68.9 (60.4 to 77.3)	69.3 (62.5 to 76.0)
	Eta^2 = 0.005 (90% CI: 0.000-0.033), *P* = 0.748			Eta^2 = 0.019 (90% CI: 0.000-0.072), *P* = 0.402			Eta^2 = 0.005 (90% CI: 0.000-0.034), *P* = 0.850		

Model I: Univariable model with FTO genotype as single fixed effect. Model II: Multivariable model with FTO genotype as a fixed effect and sex, age, fat mass and visceral adipose tissue as covariates. Model III: Multivariable model with FTO genotype as a fixed effect and sex, age, body mass index, peak oxygen uptake, resting metabolic rate, visceral adipose tissue, abdominal subcutaneous adipose tissue, liver fat, sitting time and moderate-to-vigorous physical activity as covariates.

Values are mean (95% confidence interval (CI)). Eta^2, 90% CI and *P* values are from the fixed effect of the FTO genotype group.

Table 5.3 Estimated marginal means from the multivariable general linear models used to quantify the differences between FTO genotype groups in each postprandial appetite outcome.

	Model I			Model II			Model III		
	AT (*n* = 49)	**AA (*n* = 21)**	**TT (*n* = 40)**	**AT (*n* = 45)**	**AA (*n* = 18)**	**TT (*n* = 37)**	**AT (*n* = 34)**	**AA (*n* = 17)**	**TT (*n* = 28)**
Acylated ghrelin delta AUC (2 h pg·mL^{-1})	-76.0 (-110.8 to -41.2)	-86.3 (-139.5 to -33.1)	-96.3 (-134.9 to -57.8)	-69.5 (-107.1 to -32.0)	-93.1 (-151.1 to -35.0)	-103.2 (-144.5 to -61.8)	-87.4 (-106.9 to -67.9)	-87.0 (-114.9 to -59.0)	-67.8 (-90.2 to -45.4)
	Eta^2 = 0.006 (90% CI: 0.000-0.034), *P* = 0.740			Eta^2 = 0.015 (90% CI: 0.000-0.063), *P* = 0.494			Eta^2 = 0.026 (90% CI: 0.000-0.097), *P* = 0.414		
Total PYY delta AUC (2 h pg·mL^{-1})	101.1 (84.2 to 118.1)	89.7 (63.8 to 115.6)	113.4 (94.7 to 132.2)	98.5 (80.2 to 116.8)	86.5 (58.2 to 114.8)	113.7 (93.5 to 133.8)	103.5 (81.2 to 125.8)	80.4 (48.4 to 112.4)	120.1 (94.4 to 145.7)
	Eta^2 = 0.021 (90% CI: 0.000-0.072), *P* = 0.322			Eta^2 = 0.028 (90% CI: 0.000-0.088), *P* = 0.271			Eta^2 = 0.050 (90% CI: 0.000-0.137), *P* = 0.186		
Insulin delta AUC (2 h pmol·L^{-1})	411 (345 to 476)	404 (303 to 503)	432 (359 to 504)	409 (342 to 477)	415 (311 to 519)	430 (356 to 504)	411 (330 to 492)	429 (313 to 545)	463 (370 to 556)
	Eta^2 = 0.002 (90% CI: 0.000-0.017), *P* = 0.875			Eta^2 = 0.002 (90% CI: 0.000-0.022), *P* = 0.921			Eta^2 = 0.010 (90% CI: 0.000-0.055), *P* = 0.728		
Glucose delta AUC (2 h mmol·L^{-1})	0.66 (0.21 to 1.12)	0.60 (-0.10 to 1.30)	1.01 (0.51 to 1.52)	0.60 (0.19 to 1.02)	0.54 (-0.09 to 1.18)	0.79 (0.34 to 1.25)	0.68 (0.19 to 1.17)	0.44 (-0.26 to 1.14)	0.88 (0.32 to 1.44)
	Eta^2 = 0.012 (90% CI: 0.000-0.054), *P* = 0.511			Eta^2 = 0.006 (90% CI: 0.000-0.036), *P* = 0.766			Eta^2 = 0.013 (90% CI: 0.000-0.066), *P* = 0.642		
Overall appetite delta AUC (2 h mm)	-79.3 (-89.1 to -69.5)	-72.4 (-87.4 to -57.5)	-79.2 (-90.1 to -68.4)	-75.3 (-85.2 to -65.4)	-73.6 (-88.8 to -58.3)	-82.1 (-93.0 to -71.2)	-73.4 (-85.4 to -61.4)	-75.6 (-92.7 to -58.4)	-75.6 (-89.3 to -61.8)
	Eta^2 = 0.006 (90% CI: 0.000-0.036), *P* = 0.718			Eta^2 = 0.012 (90% CI: 0.000-0.056), *P* = 0.568			Eta^2 = 0.001 (90% CI: 0.000-0.021), *P* = 0.965		

Model I: Univariable model with FTO genotype as single fixed effect. Model II: Multivariable model with FTO genotype as a fixed effect and sex, age, fat mass and visceral adipose tissue as covariates. Model III: Multivariable model with FTO genotype as a fixed effect and sex, age, body mass index, peak oxygen uptake, resting metabolic rate, visceral adipose tissue, abdominal subcutaneous adipose tissue, liver fat, sitting time and moderate-to-vigorous physical activity as covariates.

Values are mean (95% confidence interval (CI)). Eta^2, 90% CI and *P* values are from the fixed effect of the FTO genotype group.

5.4.4 Sex-specific Pearson's correlation coefficients

5.4.4.1 Appetite-related outcomes and individual characteristics

No significant correlations were observed between fasting acylated ghrelin and age, BMI, fat mass, $\dot{V}O_2$ peak, resting metabolic rate, visceral fat, abdominal subcutaneous adipose tissue, liver fat, average sitting or average MVPA in men (r = -0.18 to 0.07, $P \geq 0.185$) or women (r = -0.19 to 0.06, $P \geq 0.175$). Similarly, no significant correlations were observed between fasting total PYY and any of the individual characteristics in men (r = -0.13 to 0.14, $P \geq 0.330$) or women (r = -0.14 to 0.10, $P \geq 0.323$). Pearson's correlation coefficients between individual characteristics and fasting insulin, glucose and leptin are presented in Table 5.4. In summary, fasting insulin was positively correlated with general and abdominal adiposity parameters in both sexes and with liver fat in men (r = 0.32 to 0.53, $P \leq 0.010$). Fasting insulin was negatively correlated with $\dot{V}O_2$ peak in both sexes and with MVPA in men (r = -0.35 to -0.47, $P \leq 0.004$). Fasting glucose was positively correlated with total and abdominal adiposity parameters in both sexes, with age and liver fat in men, and with resting metabolic rate in women (r = 0.28 to 0.44, $P \leq 0.017$). Fasting glucose was negatively correlated with $\dot{V}O_2$ peak in both sexes (r = -0.29 to -0.28, $P \leq 0.020$). Fasting leptin was positively correlated with general and abdominal adiposity parameters in both sexes, and with age and liver fat in men (r = 0.24 to 0.83, $P \leq 0.040$). Fasting leptin was negatively correlated with $\dot{V}O_2$ peak in both sexes and with MVPA in men (r = -0.35 to -0.64, $P \leq 0.006$). In men, fasting overall appetite was negatively associated with fat mass (r = -0.31, $P = 0.022$, 95% CI = -0.53 to -0.05) and abdominal subcutaneous adipose tissue (r = -0.30, $P = 0.032$, 95% CI = -0.53 to -0.02). No significant correlations between fasting overall appetite and individual characteristics were observed in women (r = -0.12 to 0.09, $P \geq 0.391$).

Delta AUC for acylated ghrelin was positively associated with sitting time (r = 0.29, $P = 0.048$, 95% CI = 0.00 to 0.53) and negatively associated with age (r = -0.32, $P = 0.017$, 95% CI = -0.54 to -0.06) in men. Insulin AUC was positively associated with visceral adipose tissue in men (r = 0.38, $P = 0.007$, 95% CI = 0.11 to 0.59) and women (r = 0.32, $P = 0.021$, 95% CI = 0.05 to 0.55), and with fat mass (r = 0.39, $P = 0.003$, 95% CI = 0.14 to 0.59), abdominal subcutaneous adipose tissue (r = 0.31, $P = 0.026$, 95% CI = 0.03 to 0.54) and liver fat (r = 0.47, $P = 0.001$, 95% CI = 0.21 to 0.66) in men. Insulin AUC was negatively associated with $\dot{V}O_2$ peak (r = -0.44, $P = 0.001$, 95% CI = -0.63 to -0.20) and MVPA (r = -0.38, $P = 0.007$, 95% CI

= -0.60 to -0.11) in men. None of the correlations between AUC for total PYY, glucose and overall appetite and individual characteristics were statistically significant (r = -0.23 to 0.24, P ≥ 0.061).

5.4.4.2 Perceived appetite and appetite-related blood parameters

Fasting overall appetite was negatively associated with fasting insulin (r = -0.32, *P* = 0.015, 95% CI = -0.54 to -0.06) and fasting leptin (r = -0.35, *P* = 0.008, 95% CI = -0.56 to -0.10) in men. Delta AUC for overall appetite was positively associated with insulin AUC (r = 0.35, *P* = 0.009, 95% CI = 0.10 to 0.56) in women. No other significant correlations between overall appetite and appetite-related blood parameters were evident in the fasted or postprandial state (r = -0.20 to 0.26, *P* ≥ 0.052).

Table 5.4 Sex-specific Pearson's correlation coefficients between fasting appetite-related blood markers and individual characteristics.

	Fasting insulin ($pmol \cdot L^{-1}$)	Fasting glucose ($mmol \cdot L^{-1}$)	Fasting leptin ($ng \cdot mL^{-1}$)
Age (years)	Men: r = -0.01, *P* = 0.457, 95% CI = -0.27 to 0.25 Women: r = -0.16, *P* = 0.123, 95% CI = -0.40 to 0.11	**Men: r = 0.34, *P* = 0.005, 95% CI = 0.08 to 0.55** Women: r = 0.08, *P* = 0.270, 95% CI = -0.19 to 0.33	**Men: r = 0.24, *P* = 0.040, 95% CI = -0.02 to 0.47** Women: r = -0.07, *P* = 0.298, 95% CI = -0.33 to 0.20
Body mass index ($kg \cdot m^{-2}$)	**Men: r = 0.39, *P* = 0.003*, 95% CI = 0.14 to 0.59** **Women: r = 0.53, *P* < 0.001*, 95% CI = 0.31 to 0.69**	**Men: r = 0.33, *P* = 0.013, 95% CI = 0.07 to 0.54** **Women: r = 0.35, *P* = 0.004*, 95% CI = 0.10 to 0.56**	**Men: r = 0.62, *P* < 0.001*, 95% CI = 0.43 to 0.76** **Women: r = 0.77, *P* < 0.001*, 95% CI = 0.64 to 0.86**
Fat mass (kg)	**Men: r = 0.49, *P* < 0.001*, 95% CI = 0.26 to 0.67** **Women: r = 0.32, *P* = 0.008, 95% CI = 0.06 to 0.54**	**Men: r = 0.44, *P* < 0.001*, 95% CI = 0.20 to 0.63** **Women: r = 0.28, *P* = 0.017, 95% CI = 0.02 to 0.50**	**Men: r = 0.83, *P* < 0.001*, 95% CI = 0.73 to 0.90** **Women: r = 0.75, *P* < 0.001*, 95% CI = 0.61 to 0.85**
$\dot{V}O_2$ peak ($mL \cdot kg \cdot min^{-1}$)	**Men: r = -0.47, *P* < 0.001*, 95% CI = -0.65 to -0.24** **Women: r = -0.35, *P* = 0.004*, 95% CI = -0.56 to -0.10**	**Men: r = -0.29, *P* = 0.015, 95% CI = -0.51 to -0.03** **Women: r = -0.28, *P* = 0.020, 95% CI = -0.50 to -0.02**	**Men: r = -0.64, *P* < 0.001*, 95% CI = -0.77 to -0.45** **Women: r = -0.58, *P* < 0.001*, 95% CI = -0.73 to -0.37**
Resting metabolic rate (kcal)	Men: r = -0.04, *P* = 0.381, 95% CI = -0.31 to 0.23 Women: r = 0.03, *P* = 0.402, 95% CI = -0.24 to 0.29	Men: r = -0.12, *P* = 0.205, 95% CI = -0.38 to 0.15 **Women: r = 0.35, *P* = 0.005, 95% CI = 0.09 to 0.56**	Men: r = 0.05, *P* = 0.369, 95% CI = -0.22 to 0.32 Women: r = 0.05, *P* = 0.359, 95% CI = -0.22 to 0.31
Visceral adipose tissue (L)	**Men: r = 0.41, *P* = 0.002*, 95% CI = 0.15 to 0.62** **Women: r = 0.33, *P* = 0.010, 95% CI = 0.06 to 0.55**	**Men: r = 0.42, *P* = 0.001*, 95% CI = 0.15 to 0.63** **Women: r = 0.36, *P* = 0.005, 95% CI = 0.09 to 0.58**	**Men: r = 0.65, *P* < 0.001*, 95% CI = 0.45 to 0.79** **Women: r = 0.62, *P* < 0.001*, 95% CI = 0.42 to 0.76**
Abdominal subcutaneous adipose tissue (L)	**Men: r = 0.43, *P* = 0.002*, 95% CI = 0.17 to 0.63** **Women: r = 0.44, *P* = 0.001*, 95% CI = 0.19 to 0.64**	**Men: r = 0.39, *P* = 0.005, 95% CI = 0.13 to 0.60** **Women: r = 0.34, *P* = 0.013, 95% CI = 0.07 to 0.56**	**Men: r = 0.79, *P* < 0.001*, 95% CI = 0.66 to 0.87** **Women: r = 0.79, *P* < 0.001*, 95% CI = 0.66 to 0.87**
Liver fat (%)	**Men: r = 0.49, *P* < 0.001*, 95% CI = 0.24 to 0.68** Women: r = 0.06, *P* = 0.338, 95% CI = -0.22 to 0.33	**Men: r = 0.33, *P* = 0.010, 95% CI = 0.05 to 0.56** Women: r = 0.07, *P* = 0.305, 95% CI = -0.21 to 0.34	**Men: r = 0.44, *P* = 0.001*, 95% CI = 0.18 to 0.64** Women: r = 0.18, *P* = 0.112, 95% CI = -0.11 to 0.44
Average sitting time ($min \cdot day^{-1}$)	Men: r = -0.06, *P* = 0.340, 95% CI = -0.34 to 0.23 Women: r = 0.12, *P* = 0.196, 95% CI = -0.17 to 0.39	Men: r = -0.12, *P* = 0.210, 95% CI = -0.39 to 0.17 Women: r = 0.13, *P* = 0.190, 95% CI = -0.16 to 0.40	Men: r = -0.12, *P* = 0.207, 95% CI = -0.39 to 0.17 Women: r = 0.05, *P* = 0.353, 95% CI = -0.23 to 0.33
Average MVPA time ($min \cdot day^{-1}$)	**Men: r = -0.44, *P* = 0.001*, 95% CI = -0.64 to -0.18** Women: r = -0.01, *P* = 0.493, 95% CI = -0.28 to 0.27	Men: r = -0.03, *P* = 0.420, 95% CI = -0.31 to 0.25 Women: r = 0.09, *P* = 0.274, 95% CI = -0.19 to 0.36	**Men: r = -0.35, *P* = 0.006, 95% CI = -0.57 to -0.08** Women: r = -0.10, *P* = 0.241, 95% CI = -0.36 to 0.18

AUC, area under the curve; FTO, fat mass and obesity-associated gene; MVPA, moderate-to-vigorous physical activity, PYY, peptide YY; $\dot{V}O_2$ peak, peak oxygen uptake.

* $P < 0.005$.

5.5 Discussion

The primary finding of this study is that the association between the FTO genotype, fasting and postprandial perceived appetite and appetite-related blood outcomes was weak in healthy men and women. Explained variance for FTO group on all outcomes was small (< 5%) according to the thresholds suggested by Cohen (1998). Even the upper 90% confidence limits of the explained variance were low for each outcome (< 15%). In the context of precision medicine, we maintain that explained variance would need to be much larger than our observed values for the FTO gene to be a useful predictor of appetite-related outcomes. We also found that fasting and postprandial acylated ghrelin and total PYY were not associated with general or abdominal adiposity, while leptin, glucose and insulin concentrations were consistently associated with adiposity variables. Our study is the first to employ an integrative approach to investigate associations between a variety of genetic, physiological and lifestyle characteristics with appetite-related outcomes. Previous research has provided limited evidence on the influence of specific individual characteristics on appetite-related blood parameters and appetite perceptions.

The FTO gene represents the most extensively-studied gene that has been associated with a higher risk of obesity (Frayling et al. 2007), yet evidence on the physiological mechanisms involved is limited. The study undertaken by Karra et al. (2013) supported the hypothesis that satiety control differs between FTO genotype groups. Specifically, the group with higher obesity risk (AA) presented attenuated suppression of acylated ghrelin and perceived hunger after consumption of a meal, which can naturally lead to increased energy intake and, consequently, higher body mass (Karra et al. 2013). However, our results do not support this hypothesis as we found weak associations between genotype group, acylated ghrelin concentrations and perceived appetite ratings. Differences between study samples can possibly explain discrepancies between findings, as Karra et al. (2013) recruited healthy young males, while our sample was composed of a heterogeneous group of males and females. Additionally, Karra et al. selectively sampled their participants in order to match groups for certain variables, whereas we adopted a multivariate-adjusted approach to our data analysis. Interestingly, recent studies have reported lower postprandial total ghrelin concentrations in AA compared to AT and TT individuals (Magno et al. 2018; Melhorn et al. 2018), and postprandial hunger ratings were either similar between genotype groups (Melhorn et al. 2018) or were lower in AA individuals (Magno et al. 2018). These findings were observed despite the AA individuals

exhibiting higher energy intake during an *ad libitum* buffet (Melhorn et al. 2018). Of note, the active part of ghrelin (acylated ghrelin) only represents approximately 10% of total ghrelin (Hosoda et al. 2000; Yoshimoto et al. 2002) and, therefore, the assessment of total ghrelin in these studies could potentially explain the variability in findings.

Our research group has recently conducted a replicated crossover study to examine individual appetite responses to meal intake in healthy men recruited according to their FTO rs9939609 genotype (AA or TT) (Goltz et al. 2019; Chapter 6). The findings from this study highlighted the existence of interindividual variability in perceived appetite and acylated ghrelin, total PYY, insulin and glucose responses to a standardised meal over and above any measurement errors and/or natural variance of the outcomes. However, the magnitude of postprandial appetite parameter responses after meal intake was not associated with the FTO genotype (Goltz et al. 2019; Chapter 6). In line with our findings, previous studies have reported no differences between FTO genotype groups for fasting glucose and insulin (Speakman et al. 2008), fasting leptin (Speakman et al. 2008; Karra et al. 2013; Melhorn et al. 2018), fasting and postprandial PYY_{3-36} (Karra et al. 2013) and fasting and postprandial glucagon-like peptide 1 (Melhorn et al. 2018). Beyond the subjective appetite and appetite-related blood outcomes assessed in this study, AA and TT individuals have been shown to exhibit divergent neural responsiveness to food cues within homeostatic and reward brain regions in both fasted and postprandial states (Karra et al. 2013). Specifically, AA individuals rated high-energy food images as more appealing than TT individuals, and positive associations between circulating acylated ghrelin and central neural system responsiveness to food cues were observed only in TT individuals (Karra et al. 2013). Additional studies are needed to elucidate the precise role that FTO plays in moderating appetite control and energy intake which include both central and peripheral factors implicated in appetite regulation.

Although evidence to date suggests a negligible impact of FTO genotype on energy expenditure, higher levels of physical activity seem to exert a protective effect on the obesity risk associated with FTO (Sonestedt et al. 2009; Speakman, 2015). On the contrary, diets with higher fat content can exacerbate the susceptibility to obesity linked to the FTO high-risk genotype (Sonestedt et al. 2009; Speakman, 2015). Our study included objectively assessed sitting time, MVPA and cardiorespiratory fitness as covariates in the statistical analyses. However, only 20% of our participants accumulated, on average, less than 30 min of MVPA per day, indicating that most participants in our sample had relatively high levels of physical

activity. Therefore, we cannot rule out the possibility of this hindering our ability to detect differences in appetite-related outcomes between the genotype groups (Speakman et al. 2008). Our study did not include any assessment of habitual dietary intake and, therefore, fat intake was not taken into consideration in our analyses. Nevertheless, it is well known that the currently available dietary intake assessment tools do not provide reliable data, and this currently represents a major challenge for those involved in nutrition-related research, clinical practice or policy development (Dhurandhar et al. 2015; Archer et al. 2018).

In contrast to previous studies (Alajmi et al. 2016; Douglas et al. 2017), we did not observe a statistically significant difference in fasting concentrations of acylated ghrelin between men and women. The reason for this disparity is unclear but it is worth noting that two female participants were identified as clear outliers within our sample, with fasting acylated ghrelin concentrations of 2,899 and 4,411 $pg \cdot mL^{-1}$. These extremely high concentrations of acylated ghrelin were observed consistently in all four samples collected for each participant, indicating these values represented physiological characteristics of these two individuals rather than merely one-off measurement errors. Further studies are needed to investigate potential causes and consequences of such extreme concentrations of acylated ghrelin, and care should be taken when interpreting group mean results, as group means can be greatly impacted by such outliers. Nevertheless, exclusion of the outliers did not influence any of the statistical models in this study and, therefore, data are presented with the outliers included. Higher concentrations of fasting glucose were observed in men than women in the current study, which may be indicative of a greater degree of insulin resistance resulting from the higher visceral adipose tissue and liver fat levels observed in men (Marchesini et al. 2001; Ibrahim, 2010). Higher levels of fasting leptin were observed in women, likely because of the higher fat mass values in relation to total body mass in women, compared to men (Marshall et al. 2000; Rosenbaum and Leibel, 2014).

After meal consumption, greater changes in acylated ghrelin and overall appetite were observed in women than men. It should be noted that all participants received an identical standardised meal and, as women had significantly lower body mass and fat-free mass, and consequently lower resting metabolic rate, it was expected that the postprandial suppression of appetite would be stronger in women. However, it is interesting to observe that, apart from acylated ghrelin, no other statistically significant differences were observed between men and women in any of the remaining postprandial appetite-related blood parameters. Previous evidence has demonstrated a stronger suppression of acylated ghrelin in women than men after acute exercise

and standardised meals (Douglas et al. 2017), but not after the consumption of a standardised liquid meal (Carroll et al. 2007).

Our exploratory analyses did not identify any statistically significant or meaningful association between adiposity parameters and fasting or postprandial concentrations of acylated ghrelin and total PYY. This is in contrast with findings from previous studies which demonstrated a lower postprandial suppression of total and acylated ghrelin (Le Roux et al. 2005; Carrol et al. 2007) and a blunted postprandial elevation in PYY (Le Roux et al. 2006) in individuals with obesity. However, as expected, fasting insulin, glucose and leptin and postprandial insulin were all positively associated with general and visceral adiposity, demonstrated by moderate to very large correlation coefficients, which is consistent with the well-established role of leptin in signalling adiposity levels (Rosenbaum and Leibel, 2014) and the impact of adiposity on insulin resistance (Ibrahim, 2010). Additionally, fat-free mass, which represents the largest determinant of resting metabolic rate, has been identified as a primary determinant of appetite and energy intake (Blundell et al. 2015b). However, our findings did not reveal any significant associations of appetite-related hormones or perceived appetite with resting metabolic rate.

While acute bouts of exercise have been shown consistently to transiently suppress appetite (King et al. 2017), chronic exercise and high levels of physical activity have been suggested to increase the overall drive to eat and, concomitantly, to increase the satiating effect of a standardised meal (King et al. 2009; Beaulieu et al. 2016). We did not identify any significant associations between habitual physical activity levels and fasting or postprandial acylated ghrelin, total PYY, glucose or perceived appetite. However, a negative association was observed between MVPA and fasting leptin and insulin, and postprandial insulin in men. It is well established that acute and chronic exercise augments insulin sensitivity (Borghouts and Keizer, 2000), and a recent meta-analysis showed that leptin concentrations can be reduced by exercise in individuals who are overweight even in the absence of dietary interventions or major weight loss (BMI reduction of > 2.5%) (Rostás et al. 2017). Postprandial acylated ghrelin was positively associated with sitting time in men, but this correlation was small in magnitude and would not be considered significant if the stricter threshold of $P < 0.005$ was applied in line with recent recommendations (Benjamin et al. 2017).

Perceived fasting overall appetite was negatively associated with total fat mass in men, supporting previous evidence suggesting the existence of negative feedback signals originating from fat mass in order to regulate appetite and maintain body weight (Weise et al. 2014;

Blundell et al. 2015a). However, no association was observed between postprandial perceived appetite and any adiposity parameter in our study. Interestingly, no statistically significant associations between fasting or postprandial perceived overall appetite and acylated ghrelin or total PYY were identified. Even though circulating concentrations of acylated ghrelin and PYY vary on a meal-to-meal basis, concomitantly with perceived appetite, the magnitude and direction of the changes in hormone concentrations are not always mirrored by changes in perceived appetite (Goltz et al. 2018; Chapter 4). In contrast, postprandial overall appetite AUC was positively associated with postprandial insulin AUC in women, which is consistent with previous findings showing that postprandial insulin concentrations are positively associated with postprandial satiety and negatively associated with postprandial hunger (Flint et al. 2007).

The strengths of our study include the use of an integrative approach and objective assessment methods to explore the associations of the FTO genotype with fasting and postprandial appetite-related hormones and perceived appetite, taking into consideration a variety of individual characteristics that have been previously suggested to influence appetite parameters. Furthermore, the recruitment of a highly heterogeneous sample for parameters such as age, adiposity and cardiorespiratory fitness levels adds strength to our analyses. Finally, the careful standardisation of diet and physical activity in the 24 h preceding the laboratory visit, as well as the inclusion of a cannula acclimatisation period, also contributed to the quality of the study outcome measurements obtained. However, it should be highlighted that our study employed an exploratory approach and the cross-sectional design makes it impossible to imply any causation in our results. In addition, our results may have been compromised by the reduced sample size and by the loss of power in some of the statistical models due to missing data.

In conclusion, the FTO genotype was not significantly associated with fasting and postprandial perceived appetite and appetite-related blood parameters in healthy men and women. Further research is needed to clarify the precise role of the FTO gene in moderating appetite control and energy intake. Specifically, well-controlled long-term studies are needed to improve understanding of the effect of the FTO genotype on appetite and energy intake during and after interventions targeting weight loss and/or prevention of weight gain. Understanding the complex interaction between genetics and other individual characteristics, physiological appetite parameters and perceived appetite is of crucial importance for planning targeted strategies for weight control.

CHAPTER 6

True interindividual variability exists in postprandial appetite responses in healthy men but is not moderated by the FTO genotype

6.1 Abstract

Background: After meal ingestion, a series of coordinated hormone responses occur concomitantly with changes in perceived appetite. It is not known whether interindividual variability in appetite exists in response to a meal. **Objectives:** This study aimed to 1) assess the reproducibility of appetite responses to a meal; 2) quantify individual differences in responses; and 3) explore any moderating influence of the fat mass and obesity-associated (FTO) gene. **Methods:** Using a replicated crossover design, 18 healthy men (mean ± SD: age = 28.5 ± 9.8 years; body mass index = 27.0 ± 5.0 $kg \cdot m^{-2}$) recruited according to FTO genotype (9 AA, 9 TT) completed two identical control and two identical standardised meal conditions (5025 kJ) in randomised sequences. Perceived appetite and plasma acylated ghrelin, total peptide YY (PYY), insulin and glucose concentrations were measured before and after interventions as primary outcomes. Interindividual differences were explored using Pearson's product-moment correlations between the first and second replicate of the control-adjusted meal response. Within-participant covariate-adjusted linear mixed models were used to quantify participant-by-condition and genotype-by-condition interactions. **Results:** The meal suppressed acylated ghrelin and appetite perceptions (standardised effect sizes (ES): 0.18 - 4.26) and elevated total PYY, insulin and glucose (ES: 1.96 - 21.60). For all variables, SD of change scores was greater in the meal versus control conditions. Moderate-to-large positive correlations were observed between the two replicates of control-adjusted meal responses for all variables (r = 0.44 - 0.86, $P \leq 0.070$). Significant participant-by-condition interactions were present for most variables ($P \leq 0.031$), while the interactions for acylated ghrelin and fullness were just above the threshold for statistical significance ($P \leq 0.056$). FTO genotype-by-condition interactions were not significant ($P \geq 0.19$) and treatment effect differences between genotype groups were small (ES ≤ 0.27) for all appetite parameters. **Conclusions:** Reproducibility of postprandial appetite responses is generally good. True interindividual variability is present beyond any random within-subject variation in healthy men but is not

moderated by the FTO genotype. These findings highlight the importance of exploring individual differences in appetite for the prevention and/or treatment of obesity.

6.2 Introduction

Interindividual variability in response to an intervention, including manipulations of dietary intake and exercise energy expenditure, has received considerable interest from the scientific community in recent years (Atkinson and Batterham, 2015; Hecksteden et al. 2015; Senn, 2016). The notion that individuals may differ in the magnitude of response to an identical stimulus has widespread implications and the potential to engender tailored strategies that optimise health outcomes for individuals is undoubtedly appealing (Betts and Gonzalez, 2016). Within this sphere, an increasing number of studies have been undertaken to quantify individual differences in appetite and energy intake responses to an intervention (Hopkins et al. 2014; King et al. 2017). Specifically, marked individual variability in *ad libitum* energy intake has been reported in response to a single bout of cycling (Hopkins et al. 2014); however, recent evidence from a large pooled data set suggests that such variability can be explained by normal day-to-day variation in most cases (King et al. 2017). This line of enquiry has direct implications for energy balance and weight control.

The consumption of a meal suppresses the gut hormone acylated ghrelin and perceived hunger, and increases peptide YY and perceived satisfaction (Neary and Batterham, 2009). Standardised meals, as opposed to *ad libitum* buffet meals, provide a fixed amount of pre-selected food items, and can be used to compare meal-related outcomes when the exact same stimulus is given. Previous studies have suggested that individuals exhibit reproducible *ad libitum* energy intake, as well as changes in subjective appetite perceptions, glucose and appetite-related hormones in response to a standardised meal provided on separate occasions (Flint et al. 2000; Nair et al. 2009; Gonzalez et al. 2012; Horner et al. 2014). Whilst such efforts to explore reproducibility and individual responses alongside the mean effects of an intervention should be encouraged, there are significant methodological and analytical challenges to adequately quantifying interindividual variability (Atkinson and Batterham, 2015). In the context of a crossover study, these challenges include replicating each intervention and control condition and partitioning true response heterogeneity from within-subjects random measurement variability with an appropriate statistical model (Atkinson and Batterham, 2015).

Current evidence is often limited by the absence of a control group or condition which has been highlighted as an imperative study design feature to account for random within-subject variability over time (Atkinson and Batterham, 2015; Senn, 2016; Williamson et al. 2017). A recent approach proposed to quantify individual differences in the intervention response involves quantifying the participant-by-response interaction from replicated intervention and control groups or conditions (Senn et al. 2011; Senn, 2016; Atkinson et al. 2018). We have recently adopted this framework to highlight the presence of individual variability in subjective and hormonal appetite responses to acute exercise (Goltz et al. 2018; Chapter 4); however, it is not known whether such variability in appetite responses exists in response to a standardised meal.

A further consideration, if true individual differences are present, involves identifying potential moderators that could explain the individual variability (Atkinson and Batterham, 2015). The FTO gene represents the most extensively studied gene associated with obesity, with individuals homozygous for the obesity risk A allele (AA) of FTO rs9939609 weighing, on average, 3 kg more and having a 1.7-fold higher obesity risk than those homozygous for the low-risk T allele (TT) (Frayling et al. 2007). Whilst the physiological mechanisms underlying this heightened risk are not fully understood, it has been demonstrated that AA individuals exhibit a blunted postprandial suppression of acylated ghrelin and hunger compared with adiposity-matched TT individuals (Karra et al. 2013). Given that interindividual variability in the responses of appetite to repeated meal intake is suspected, it is possible that groups with different risk variants of the FTO gene may moderate these responses.

Therefore, the aims of this study were (1) to investigate whether the perceived appetite and appetite-related hormone responses to a standardised meal are reproducible on repeated occasions; (2) to examine whether there is true individual variability in appetite responses to a standardised meal; and (3) to determine whether the FTO genotype moderates the magnitude of appetite responses to a standardised meal.

6.3 Methods

6.3.1 Ethical approval

All procedures included in this study were approved by Loughborough University Ethics Advisory Committee. All participants provided written informed consent before taking part in any aspect of the study.

6.3.2 Participants

Participants were selected from two previous study databases where participants were genotyped for the rs9939609 allele of the FTO gene. Genomic deoxyribonucleic acid (DNA) was extracted from the whole blood samples using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). The samples were genotyped using the Applied Biosystems TaqMan® (Roche Molecular Systems, Pleasanton, USA) genotyping assay and real-time polymerase chain reaction system. Eighteen healthy white European men were recruited for this study between January and April 2018 according to their FTO genotype: 9 homozygous minor allele (AA) and 9 homozygous major allele (TT). Participants were informed about the study purpose; however, their genotype was not disclosed until the end of the study in order to avoid any potential effect on the outcomes of interest. Participants were body mass stable (≤ 3 kg change in the previous 3 months), non-smokers, had no history of cardiovascular or metabolic disease, and were not dieting or taking any medications. Participants were habitual breakfast eaters and 'moderately active' according to the International Physical Activity Questionnaire (Craig et al. 2003), which was used to ensure homogeneity in physical activity levels across the study sample.

6.3.3 Anthropometry

Height was measured to the nearest 0.1 cm and body mass to the nearest 0.1 kg using an electronic measuring station (Seca, Hamburg, Germany). Body mass index (BMI) was calculated as body mass in kilograms divided by the square of height in meters. Skinfold thickness was measured at three sites (chest, abdomen and thigh) and body fat percentage was estimated using the equations of Jackson and Pollock (Jackson and Pollock, 1978) and Siri (Siri, 1961).

6.3.4 Experimental design

Using a replicated crossover experimental design (Senn, 2016), participants completed four visits in a randomised order, each separated by an interval of at least three days: two identical fasting control and two identical standardised meal conditions. The block randomization plan was obtained from www.randomization.com by the main investigator, who also enrolled participants and assigned participants to interventions. Participants completed a weighed food record in the 24 h preceding the first visit and were instructed to replicate this feeding pattern

before each subsequent visit. Participants refrained from alcohol, caffeine, and strenuous physical activity during the same period. A standardised meal was consumed in the evening before the laboratory visits consisting of a pizza (3054 kJ, 44% carbohydrate, 22% protein, 34% fat). Participants were instructed to consume the whole meal without any additional food or drink items except plain water, and compliance was confirmed from the food record completed prior to the first visit, and verbally on the remaining visits. After this meal, participants consumed no food or drink except plain water before arriving at the laboratory the next day.

6.3.5 Main conditions

Participants arrived at the laboratory at 09:00 after a 13 h overnight fast. A cannula (Becton Dickinson Venflon, Helsingborg, Sweden) was inserted into an antecubital vein 60 min before the collection of venous blood samples to eliminate any stress effects associated with cannula insertion (Chandarana et al. 2009). A fasting venous blood sample and rating of perceived appetite was taken at ~10:00 (0 h). Participants rested throughout all four conditions but were provided with a standardised breakfast meal after the fasting measurements during the two meal conditions. Breakfast was consumed within 15 min and consisted of croissants, butter, chocolate spread, cereal biscuits and milkshake which provided 5025 kJ energy (47% carbohydrate, 9% protein, 44% fat). Subsequent venous blood samples were taken at 0.5 and 1 h, and appetite perceptions were assessed at 1 h. Environmental temperature and humidity were monitored and kept constant throughout all main experimental conditions using a wireless weather station (Opes, London, UK).

6.3.6 Subjective appetite ratings

Subjective appetite ratings (hunger, satisfaction, fullness and prospective food consumption (PFC)) were assessed at 0 and 1 h using 100 mm visual analogue scales (Flint et al. 2000) as primary study outcomes. The scales were anchored by a descriptor at each end defining the extremes of the appetite perception being measured.

6.3.7 Blood sampling and biochemical analysis

Venous blood samples were collected in the semi-supine position for the measurement of plasma acylated ghrelin, total PYY, insulin and glucose concentrations as primary study outcomes. Plasma acylated ghrelin and total PYY concentrations were quantified from samples

at 0 and 1 h, and plasma insulin and glucose concentrations were measured at 0 and 0.5 h to capture the peak change in concentration after the meal.

Blood sample collection and processing methods were described in detail in Chapter 3. Duplicate haemoglobin and haematocrit measurements were taken at each blood sampling time point to calculate the acute change in plasma volume (Dill and Costill, 1974). Commercially available enzyme immunoassays were used to determine the plasma concentrations of acylated ghrelin (Bertin Technologies, Montigney le Bretonneux, France), total PYY (Millipore, Watford, UK) and insulin (Mercodia, Uppsala, Sweden). Plasma glucose concentrations were analysed by enzymatic, colorimetric methods using a benchtop analyser (Horiba Medical Pentra 400, Montpellier, France). All samples were analysed in duplicate and, in order to eliminate inter-assay variation, samples for each participant were analysed in the same run. The within-batch coefficient of variation values for acylated ghrelin, total PYY, insulin and glucose concentrations were 4.0%, 4.6%, 5.9% and 0.4%, respectively.

6.3.8 Statistical analyses

In our previous replicated crossover study (Goltz et al. 2018; Chapter 4), we detected statistically significant participant-by-treatment interactions with a sample size of 15 participants. Based on information from this study, we assumed a correlation between trials of 0.7. Using G*Power version 3.1.9.2 (University of Kiel, Kiel, Germany), it was estimated that a total sample size of 16 participants would provide 80% statistical power to detect a statistically significant interaction between our 2-level between-subjects factor of genotype and within-subjects factor of treatment effect when this interaction amounted to a standardised effect size of 0.2 (alpha = 0.05). The four measurements of each outcome associated with our replicated crossover design increases statistical power over a conventional 2-level crossover study for detection of this group-by-treatment interaction.

Between-genotype differences in participant characteristics were quantified using linear mixed models with group (AA *vs* TT) modelled as a fixed factor. The presence of interindividual differences in appetite-related blood parameters and perceived appetite responses to a standardised meal were examined according to three analytical approaches (Senn et al. 2011; Atkinson and Batterham, 2015; Senn, 2016). The three approaches, detailed recently by Goltz et al. (Goltz et al. 2018; Chapter 4), were as follows:

(i) The association between the first and second replicate of control-adjusted treatment effect was quantified for each outcome using Pearson's product-moment correlation coefficients (Senn, 2016). The first meal condition in any participant's sequence was paired to the first control condition in the same individual's sequence. Thresholds of 0.1, 0.3 and 0.5 were used to label correlation coefficients as small, moderate and large, respectively (Cohen, 1988). This correlation coefficient quantifies the consistency of meal effect across the replicated experimental conditions.

(ii) The following equation (Atkinson and Batterham, 2015) was used to provide an overall estimate of the true (control condition adjusted) between-subject differences in treatment response:

$$SD_{IR} = \sqrt{SD_M^2 - SD_C^2}$$

SD_{IR} represents the true interindividual variation in treatment effect. SD_M and SD_C are the standard deviations of the pre-to-post change scores for the meal and fasting control conditions (averaged over the two replicates using the relevant equation for pooling SDs (Higgins and Green, 2018)).

(iii) While the equation in (ii) estimates response variance adjusted for control condition change variance, the associated standard errors and confidence intervals (CI) are not appropriate for our within-subjects crossover study design, hence our adjunct approach of within-subjects general linear modelling. Using the MIXED procedure in SAS OnDemand for Academics, a within-participant linear mixed model was formulated to quantify any participant-by-condition interaction for each outcome. Condition and period (sequence), and their interaction effects, were modelled as fixed effects, and participant and participant-by-condition terms were modelled as random effects (refer to the SAS code supplied in Appendix J). Standard residual diagnostics were undertaken to assess the "influence diagnostics" of a potential set of observations on the adequacy and the stability of the modelled covariance parameter estimates (Oman, 1995; Schabenberger, 2004; West and Galecki, 2012).

The grand mean differences between conditions and associated 95% CI were quantified with a within-subjects linear mixed model run in version 23 of SPSS (IBM Corporation, New York, USA) without the participant-by-condition random effect, but with a covariate of baseline

values. The FTO genotype was included in this model as a fixed between-subjects effect, and the genotype-by-condition interaction was quantified.

Correction of hormone and glucose concentrations for acute changes in plasma volume had a negligible influence on our findings and, therefore, the unadjusted plasma concentrations are displayed for simplicity. In the absence of a robust and precise prognostic anchor for an important difference in our appetite-related outcomes, we calculated distribution-based standardised effect sizes (ES) (Cook et al. 2018). An ES of 0.2 denoted the minimum important mean difference for all outcomes, with an ES of 0.5 being moderate and an ES of 0.8 being large (Cohen, 1988). To calculate the minimal clinically important difference (MCID) for individual responses, the threshold of 0.2 for interpreting standardised mean changes (Cohen, 1988) was halved, i.e. 0.1, and multiplied by the baseline between-subject standard deviation (SD) (Atkinson and Batterham, 2015; Williamson et al. 2018). Pearson's product-moment correlation coefficients were quantified between the mean control-adjusted meal response for each of the appetite measures and body adiposity measurements. Correlation coefficients were also quantified between the pooled mean pre-to-post change in concentrations of plasma constituents and the pooled mean pre-to-post change in appetite perceptions across the four conditions.

Data are presented as mean ± SD. Mean differences or changes and correlation coefficients are presented along with respective 95% CI. Statistical significance was accepted as $P < 0.050$ and P values are expressed in exact terms apart for very low values, which are expressed as $P < 0.001$.

6.4 Results

6.4.1 Participant characteristics

Participant characteristics are presented in Table 6.1. All 95% CI for the difference between AAs and TTs overlapped zero ($P \geq 0.411$), although these 95% CIs were relatively wide. All standardised effects sizes were very small, except for the small-to-moderate effect sizes found for body fat percentage and fat-free mass.

Table 6.1. Participant characteristics.

	All (n = 18)	FTO homozygous minor allele (AA) (n = 9)	FTO homozygous major allele (TT) (n = 9)	AA vs TT mean difference (95% CI)	ES
Age (years)	28.5 ± 9.8	28.5 ± 9.6	28.4 ± 10.5	0.1 (-9.9, 10.2)	0.01
Height (m)	1.78 ± 0.06	1.78 ± 0.07	1.78 ± 0.05	-0.002 (-0.06, 0.06)	0.03
Body mass (kg)	85.5 ± 16.0	85.7 ± 14.2	85.3 ± 18.5	0.4 (-16.1, 16.9)	0.03
Body mass index ($kg \cdot m^{-2}$)	27.0 ± 5.0	27.1 ± 4.7	26.8 ± 5.6	0.2 (-4.9, 5.4)	0.05
Body fat percentage (%)	20.2 ± 9.1	18.9 ± 9.1	21.4 ± 9.5	-2.6 (-11.8, 6.7)	0.27
Fat-free mass (kg)	67.1 ± 7.8	68.7 ± 8.8	65.5 ± 6.8	3.1 (-4.7, 11.0)	0.40

Values are means ± SDs, n = 18 healthy men (9 AA, 9 TT).

ES, standardised effect size (mean difference); FTO, fat mass and obesity-associated gene.

6.4.2 Plasma hormone and metabolite concentrations

6.4.2.1 Acylated ghrelin

No significant correlation was observed between the two replicates of control-adjusted meal responses for acylated ghrelin (r = 0.22, 95% CI -0.27 to 0.62, P = 0.384, Figure 6.1A). In agreement with our post-estimation residuals diagnostics, two distinct outliers can be seen in Figure 6.1A, which were more than three times higher or lower than the sample SD. Although we explored several data transformations, these were not successful in improving the non-normal distribution of the ghrelin data. We could not identify any systematic protocol variation or measurement issues that would explain these two outliers. The removal of the two outliers in a sensitivity analysis improved the correlation coefficient of treatment effect between replicates to 0.86 (95% CI 0.64 to 0.95, P < 0.001). The SD of within-trial change was substantially greater for the meal than control conditions, which remained after removal of the two outliers (Table 6.2). After adjustment for period (sequence) influences, the estimated marginal mean acylated ghrelin concentration was 62 $pg \cdot mL^{-1}$ lower (95% CI -69 to -54 $pg \cdot mL^{-1}$, P < 0.001, ES = 0.18) in the meal versus control conditions. The P value for the participant-by-condition interaction was just above the threshold for statistical significance after the

removal of the two outliers (Table 6.2). The magnitude of change in individual replicated mean responses after the meal for acylated ghrelin ranged from -128 to -38 pg·mL^{-1}, with all participants demonstrating a meal-mediated suppression of ghrelin beyond the MCID (± 34.8 pg·mL^{-1}) (Figure 6.2A).

6.4.2.2 Total PYY

A large positive correlation of 0.50 (95% CI 0.04 to 0.78, P = 0.034) was observed between the two replicates of control-adjusted meal responses for total PYY (Figure 6.1B). The within-trial SD for total PYY was substantially greater for the meal than control conditions (Table 6.2). The period-adjusted mean total PYY concentration was 78 pg·mL^{-1} higher (95% CI 70 to 87 pg·mL^{-1}, P < 0.001, ES = 1.96) in the meal versus control conditions. A statistically significant participant-by-condition interaction was found (Table 6.2). The magnitude of change in individual replicated mean responses after the meal for total PYY ranged from 15 to 115 pg·mL^{-1}, with all participants demonstrating an increase beyond the MCID (± 3.99 pg·mL^{-1}) (Figure 6.2B).

6.4.2.3 Insulin

A large positive correlation of 0.64 (95% CI 0.25 to 0.85, P = 0.004) was observed between the two replicates of control-adjusted meal responses for insulin (Figure 6.1C). Following our residuals diagnostics, we undertook a sensitivity analysis where we removed one outlier, which was more than 4 times higher than the sample SD. This improved the correlation to 0.82 (95% CI 0.56 to 0.93, P < 0.001). The within-trial SD for insulin was substantially greater for the meal than control conditions (Table 6.2). The period-adjusted mean insulin concentration was 526 pmol·L^{-1} higher (95% CI 442 to 610 pmol·L^{-1}, P < 0.001, ES = 21.60) in the meal versus control conditions. The participant-by-condition interaction was statistically significant both with and without inclusion of the outlier (Table 6.2). The magnitude of change in individual replicated mean responses after the meal for insulin ranged from 123 to 1130 pmol·L^{-1}, with all participants demonstrating an increase beyond the MCID (± 2.43 pmol·L^{-1}) (Figure 6.2C).

6.4.2.4 Glucose

A moderate positive correlation of 0.44 (95% CI -0.03 to 0.75, P = 0.070) was observed between the two sets of control-adjusted meal responses for glucose (Figure 6.1D). The within-trial SD for glucose was substantially greater for the meal than control conditions (Table 6.2).

The period-adjusted mean glucose concentration was 1.30 mmol·L^{-1} higher (95% CI 1.14 to 1.46 mmol·L^{-1}, $P < 0.001$, ES = 3.61) in the meal versus control conditions. The participant-by-condition interaction was statistically significant (Table 6.2). The magnitude of change in individual replicated mean responses after the meal for glucose ranged from 0.52 to 2.39 mmol·L^{-1}, with all participants demonstrating an increase beyond the MCID (± 0.04 mmol·L^{-1}) (Figure 6.2D).

6.4.3 Subjective appetite ratings

Moderate-to-large positive correlations were observed between the two sets of control-adjusted meal responses for hunger (r = 0.59, 95% CI 0.17 to 0.83, $P = 0.010$), satisfaction (r = 0.74, 95% CI 0.42 to 0.90, $P < 0.001$), fullness (r = 0.41, 95% CI -0.07 to 0.73, $P = 0.091$) and PFC (r = 0.65, 95% CI 0.26 to 0.86, $P = 0.003$) (Figure 6.3). Removal of one outlier for fullness improved the correlation coefficient to 0.62 (95% CI 0.22 to 0.84, $P = 0.008$). The within-trial SD was substantially greater for the meal than control conditions for hunger, satisfaction, fullness and PFC (Table 6.2).

The participant-by-condition interaction was statistically significant for hunger, satisfaction and PFC, while it was just above the threshold for statistical significance for fullness (Table 6.2). Exclusion of the previously mentioned outlier for fullness did not affect the significance of the participant-by-condition interaction or the estimated individual differences SD and therefore data for fullness is presented with the outlier. The period-adjusted mean ratings of hunger and PFC were 49 mm (95% CI -53 to -44 mm, $P < 0.001$, ES = 2.16) and 43 mm (95% CI -48 to -38 mm, $P < 0.001$, ES = 4.26) lower in the meal versus control conditions, respectively. The period-adjusted mean ratings of satisfaction and fullness were 52 mm (95% CI 47 to 56 mm, $P < 0.001$, ES = 3.46) and 51 mm (95% CI 45 to 57 mm, $P < 0.001$, ES = 3.23) higher in the meal versus control conditions, respectively. The magnitude of change in individual replicated mean responses after the meal ranged from -97 to 14 mm for hunger, 11 to 88 mm for satisfaction, 13 to 89 mm for fullness and -96 to -4 mm for PFC. All participants demonstrated a response beyond the MCID for hunger (± 2.27 mm), satisfaction (± 1.49 mm), fullness (± 1.59 mm) and PFC (± 1.59 mm) (Figure 6.4).

6.4.4 Moderating effect of FTO genotype on individual variability

The FTO genotype-by-condition interaction was not statistically significant for acylated ghrelin ($P = 0.423$), total PYY ($P = 0.192$), insulin ($P = 0.540$), glucose ($P = 0.698$) or any of the perceived appetite ratings ($P \geq 0.474$). The differences in the mean treatment effects observed for all appetite parameters between genotype groups were small and not statistically significant (ES ≤ 0.27, $P \geq 0.174$).

6.4.5 Correlations between appetite outcomes and individual characteristics

Large positive correlations were observed between mean acylated ghrelin control-adjusted meal responses and body mass (r = 0.55, $P = 0.019$), BMI (r = 0.56, $P = 0.015$) and body fat percentage (r = 0.56, $P = 0.016$). Large positive correlations were also observed between hunger mean responses and BMI (r = 0.53, $P = 0.023$) and body fat percentage (r = 0.55, $P = 0.018$). None of the remaining appetite parameters mean responses were significantly correlated with the adiposity parameters assessed in this study (r = -0.46 to 0.41, $P \geq 0.055$). No significant correlations were observed between fat-free mass and any of the mean appetite parameter responses to the meal (r = -0.14 to 0.36; $P \geq 0.147$).

6.4.6 Correlations between changes in study outcome variables

A large positive correlation was observed between the pre-to-post change in acylated ghrelin and the change in both hunger and PFC, whereas a large negative correlation was observed between the pre-to-post change in acylated ghrelin and the change in both satisfaction and fullness. A large negative correlation was observed between the pre-to-post change in total PYY and the change in both hunger and PFC, whereas a large positive correlation was observed between the pre-to-post change in total PYY and the change in both satisfaction and fullness. A moderate negative correlation was observed between the pre-to-post change in insulin and the change in both hunger and PFC, whereas moderate-to-large positive correlations were observed between the pre-to-post change in insulin and the change in both satisfaction and fullness. A large negative correlation was observed between the pre-to-post change in glucose and the change in both hunger and PFC, whereas, a large positive correlation was observed between the pre-to-post change in glucose and the change in both satisfaction and fullness (Table 6.3).

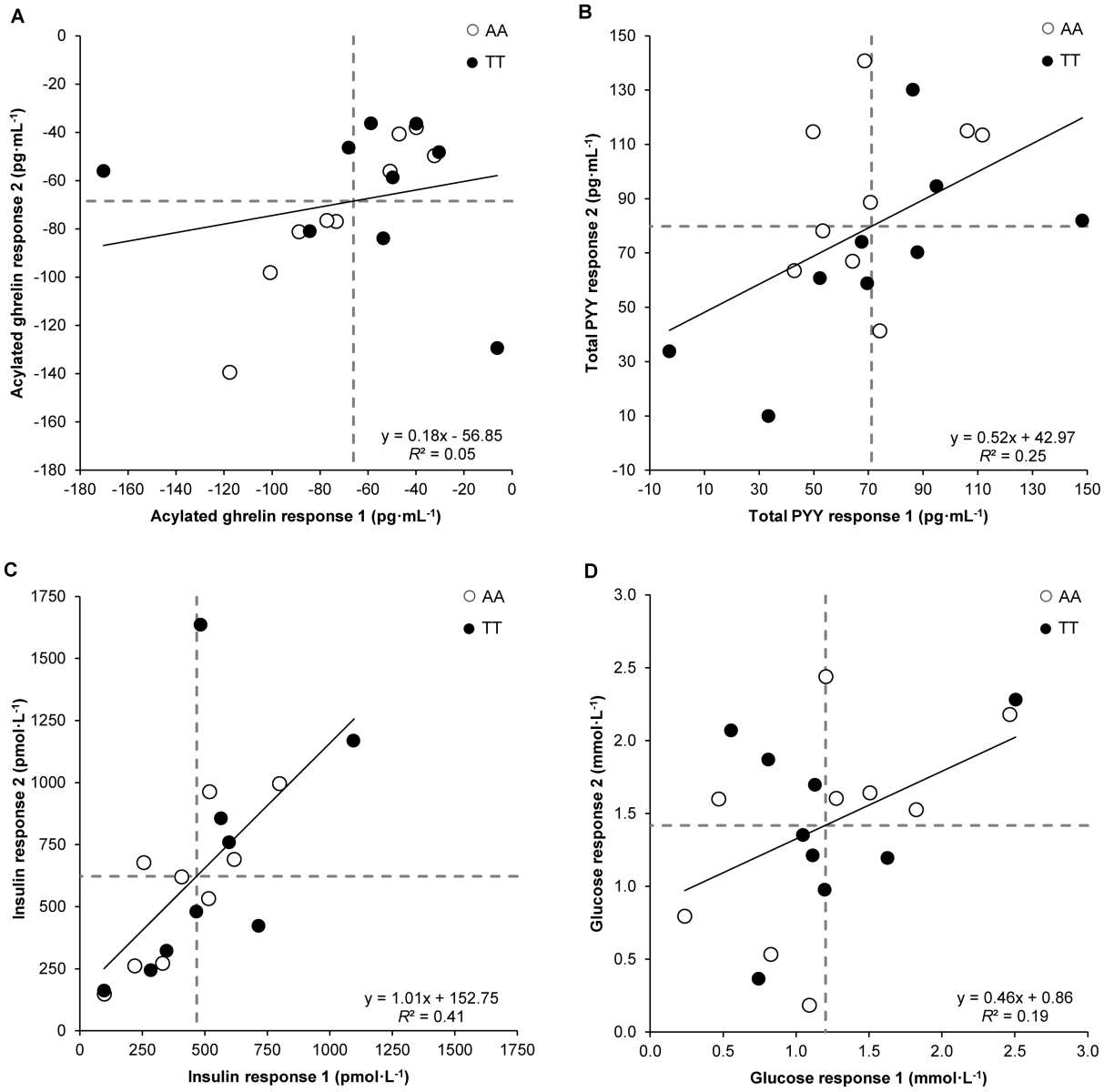


Figure 6.1 Correlation between meal (standardised meal providing 5025 kJ) and control (no intervention) pre-to-post change scores on the two occasions for (A) plasma acylated ghrelin, (B) plasma total PYY, (C) plasma insulin, and (D) plasma glucose in 18 healthy men genotyped for FTO rs9939609 (n = 9 AA, n = 9 TT). ‘‘Response 1’’ corresponds to the first pair of conditions (meal 1 minus control 1) and ‘‘response 2’’ to the second pair of conditions (meal 2 minus control 2). Dashed lines represent the mean responses.

FTO, fat mass and obesity-associated gene; PYY, peptide YY.

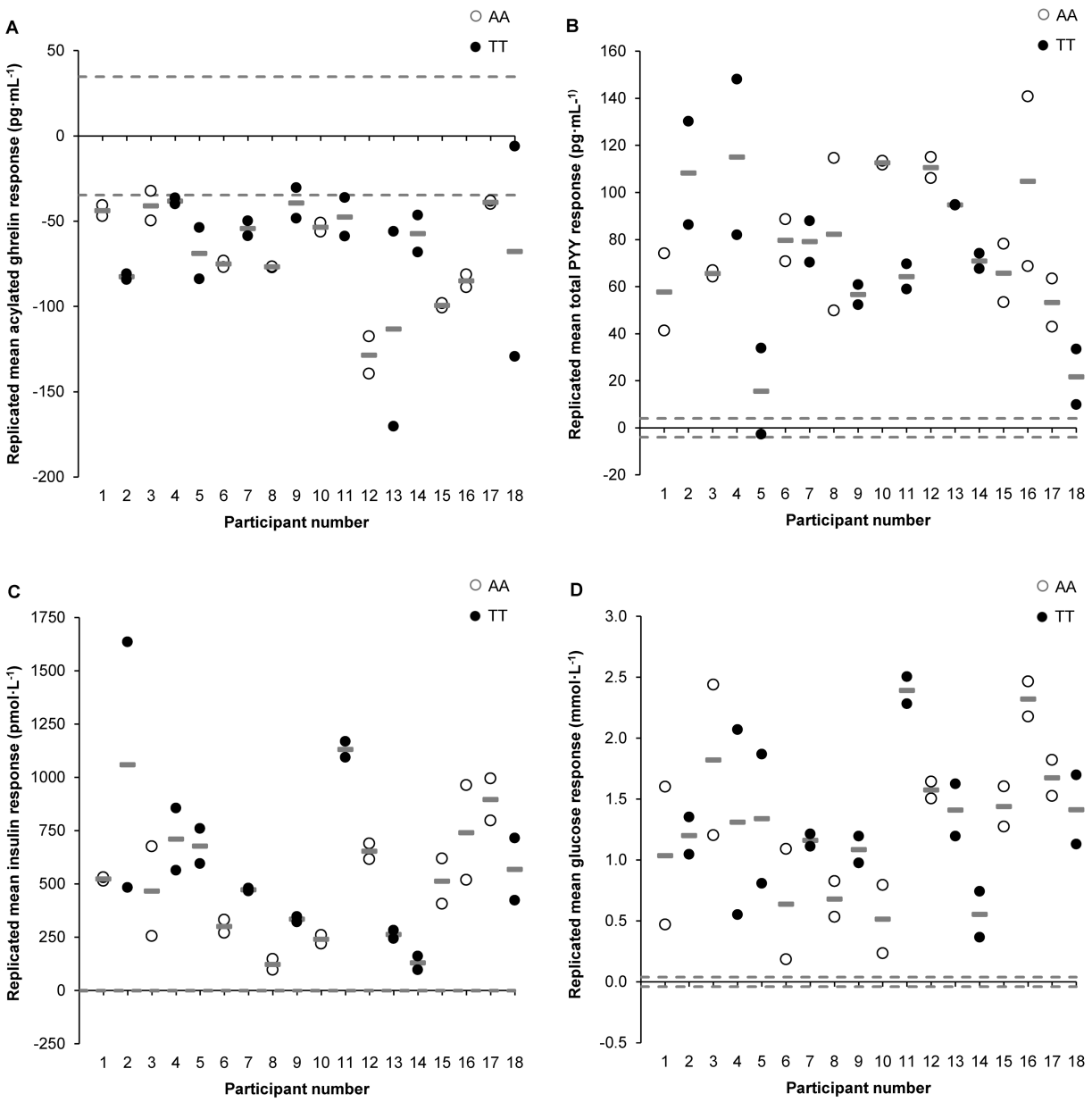


Figure 6.2 Individual changes in hormone and glucose concentrations between the meal (standardised meal providing 5025 kJ) and control (no intervention) conditions (meal minus control): (A) plasma acylated ghrelin, (B) plasma total PYY, (C) plasma insulin, and (D) plasma glucose in 18 healthy men genotyped for FTO rs9939609 (n = 9 AA, n = 9 TT). Pre-to-post change scores for ‘‘response 1’’ and ‘‘response 2’’ are indicated by white and black circles. Grey lines (—) represent each participant’s replicated mean response. Dashed lines indicate the standardised minimal clinically important difference calculated as 0.1 multiplied by the baseline between-subject SD.

FTO, fat mass and obesity-associated gene; PYY, peptide YY.

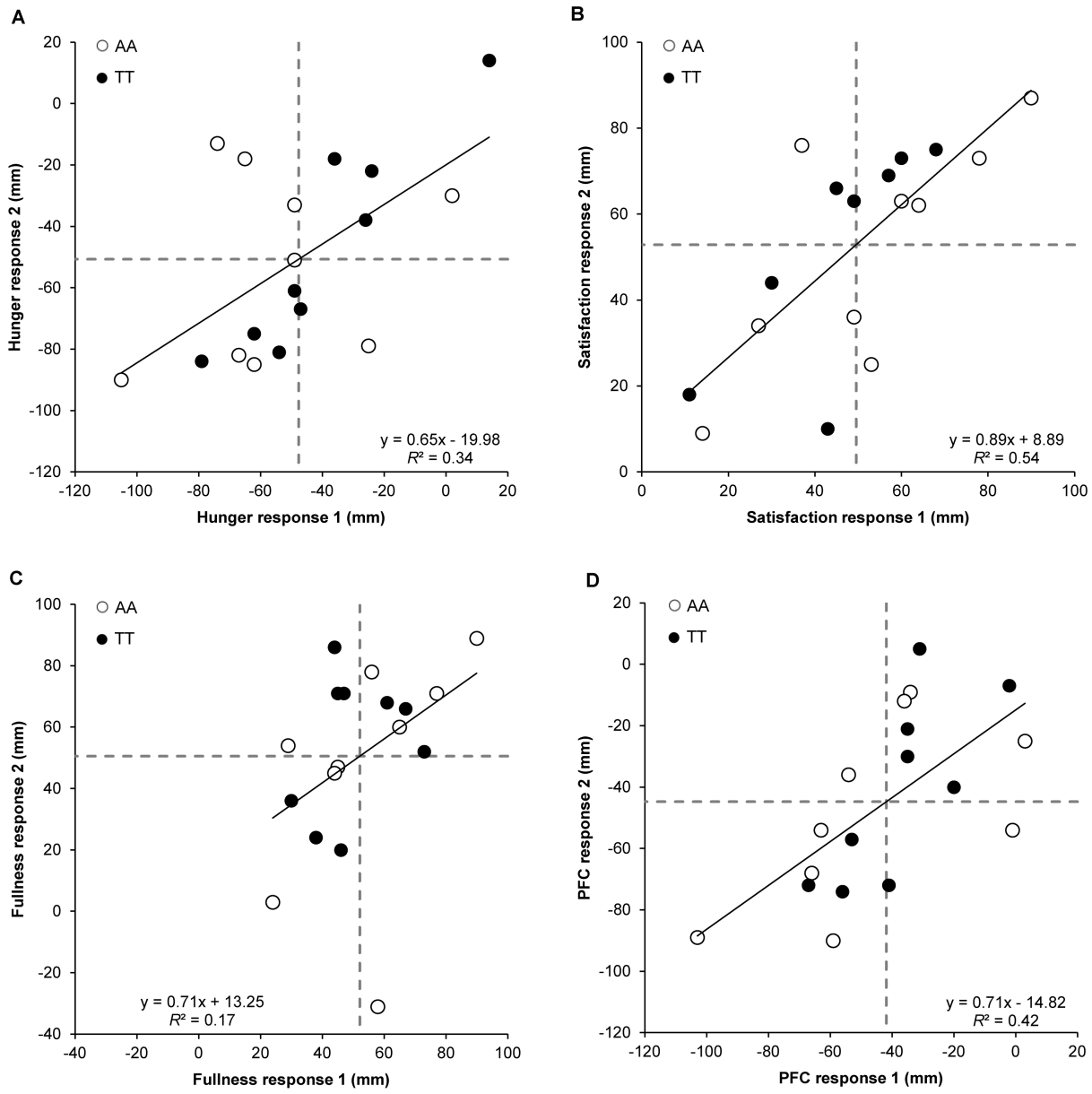


Figure 6.3 Correlation between meal (standardised meal providing 5025 kJ) and control (no intervention) pre-to-post change scores on the two occasions for (A) hunger, (B) satisfaction, (C) fullness, and (D) prospective food consumption in 18 healthy men genotyped for FTO rs9939609 (*n* = 9 AA, *n* = 9 TT). ‘‘Response 1’’ corresponds to the first pair of conditions (meal 1 minus control 1) and ‘‘response 2’’ to the second pair of conditions (meal 2 minus control 2). Dashed lines represent the mean responses.

FTO, fat mass and obesity-associated gene; PFC, prospective food consumption.

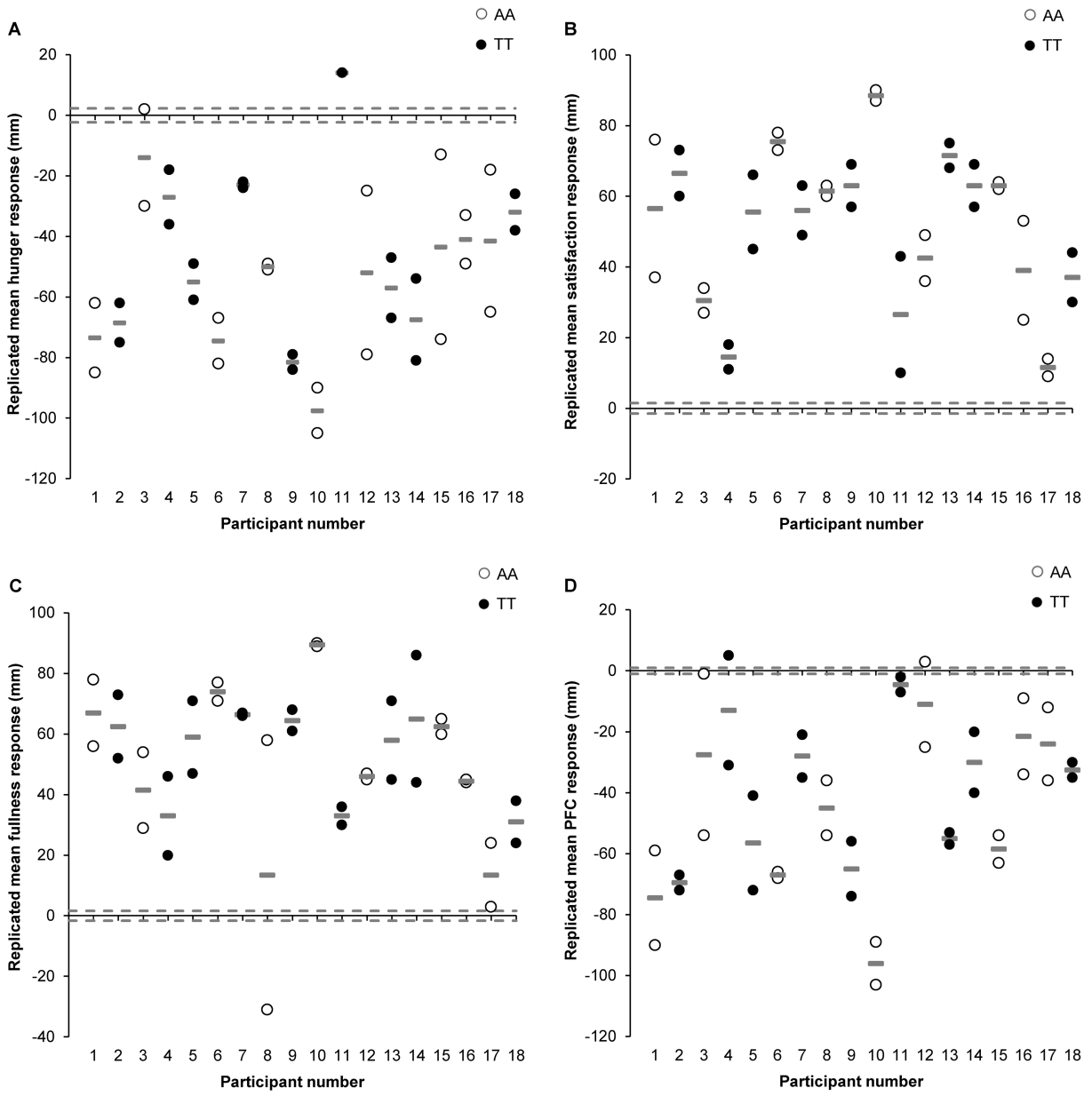


Figure 6.4 Individual changes in each appetite perception between the meal (standardised meal providing 5025 kJ) and control (no intervention) conditions (meal minus control): (A) hunger, (B) satisfaction, (C) fullness, and (D) prospective food consumption in 18 healthy men genotyped for FTO rs9939609 (n = 9 AA, n = 9 TT). Pre-to-post change scores for ‘‘response 1’’ and ‘‘response 2’’ are indicated by white and black circles. Grey lines (—) represent each participant’s replicated mean response. Dashed lines indicate the standardised minimal clinically important difference calculated as 0.1 multiplied by the baseline between-subject SD.

FTO, fat mass and obesity-associated gene; PFC, prospective food consumption.

Table 6.2 Means and standard deviations (SD) of the pre-to-post change scores for the meal (standardised meal providing 5025 kJ) and control (no intervention) conditions and the true individual differences SD.

Variable	Meal change Mean ± SD	Control change Mean ± SD	Estimate 1[a]	Estimate 2[b]	
			Individual differences SD	Individual differences SD (SE)	*P* value
Plasma acylated ghrelin ($pg \cdot mL^{-1}$)	-61.1 ± 36.2	6.2 ± 27.7	23.3	4.9 (16.1)	0.930
	-57.0 ± 20.4[c]	7.4 ± 12.5[c]	16.2[c]	18.0 (13.0)[c]	0.056[c]
Plasma total PYY ($pg \cdot mL^{-1}$)	61.6 ± 35.1	-13.9 ± 11.1	33.3	31.8 (20.6)	0.020
Plasma insulin ($pmol \cdot L^{-1}$)	545 ± 324	0.1 ± 11.5	324	502 (300)	0.005
	515 ± 287[c]	0.0 ± 11.8[c]	286[c]	349 (208)[c]	0.005[c]
Plasma glucose ($mmol \cdot L^{-1}$)	1.22 ± 0.62	-0.09 ± 0.17	0.60	0.58 (0.37)	0.012
Hunger (mm)	-40.4 ± 30.0	8.7 ± 15.2	25.9	22.7 (15.4)	0.031
Satisfaction (mm)	48.3 ± 24.0	-3.0 ± 9.4	22.0	19.5 (12.7)	0.018
Fullness (mm)	52.8 ± 20.2	1.4 ± 12.3	16.0	13.6 (9.8)	0.054
Prospective food consumption (mm)	-44.1 ± 27.8	2.2 ± 9.8	26.0	23.9 (15.3)	0.015

Data are presented for $n = 18$ healthy men.

[a] Estimate 1: Individual differences SD estimated using $SD_{IR} = \sqrt{SD_M^2 - SD_C^2}$ where SD_{IR} is the SD of the true individual response, and SD_M and SD_C are the SDs of the pre-to-post change scores for the meal and control conditions (averaged over both replicates), respectively (Atkinson and Batterham, 2015).

[b] Estimate 2: Period-adjusted individual differences SD estimated using a random effects statistical model (Senn et al. 2011). The SD was derived from the SAS model participant-by-condition interaction term (as a random effect) (refer to the SAS code supplied in Appendix J). The *P* value shown is also for this interaction term.

[c] After the removal of outliers.

SE, standard error; PYY, peptide YY.

Table 6.3 Pearson's correlation coefficients between the pooled mean pre-to-post change in plasma hormone and glucose concentrations and the pooled mean pre-to-post change in appetite perceptions across the two meal (standardised meal providing 5025 kJ) and two control (no intervention) conditions.

	Plasma total PYY ($pg \cdot mL^{-1}$)		Plasma insulin ($pmol \cdot L^{-1}$)		Plasma glucose ($mmol \cdot L^{-1}$)		Hunger (mm)		Satisfaction (mm)		Fullness (mm)		Prospective food consumption (mm)	
	r (95% CI)	*P* value	r (95% CI)	*P* value	r (95% CI)	*P* value	r (95% CI)	*P* value	r (95% CI)	*P* value	r (95% CI)	*P* value	r (95% CI)	*P* value
Plasma acylated ghrelin ($pg \cdot mL^{-1}$)	-0.66 (-0.86, -0.28)	0.003	-0.49 (-0.78, -0.03)	0.039	-0.61 (–0.84, -0.20)	0.007	0.53 (0.08, 0.80)	0.024	-0.68 (-0.87, -0.31)	0.002	-0.66 (-0.86, -0.28)	0.003	0.53 (0.08, 0.80)	0.024
Plasma total PYY ($pg \cdot mL^{-1}$)			0.67 (0.30, 0.87)	0.002	0.63 (0.23, 0.85)	0.005	-0.60 (-0.83, -0.19)	0.008	0.69 (0.33, 0.87)	0.002	0.71 (0.36, 0.88)	<0.001	-0.62 (-0.84, -0.22)	0.006
Plasma insulin ($pmol \cdot L^{-1}$)					0.85 (0.64, 0.94)	<0.001	-0.40 (-0.73, 0.08)	0.100	0.45 (-0.02, 0.76)	0.060	0.56 (0.13, 0.81)	0.016	-0.45 (-0.76, 0.02)	0.060
Plasma glucose ($mmol \cdot L^{-1}$)							-0.50 (-0.78, -0.04)	0.035	0.62 (0.22, 0.84)	0.006	0.64 (0.25, 0.85)	0.004	-0.53 (-0.80, -0.08)	0.024
Hunger (mm)									-0.82 (-0.93, -0.57)	<0.001	-0.80 (-0.92, -0.53)	<0.001	0.89 (0.72, 0.96)	<0.001
Satisfaction (mm)											0.90 (0.75, 0.96)	<0.001	-0.88 (-0.95, -0.70)	<0.001
Fullness (mm)													-0.86 (-0.95, -0.66)	<0.001

Statistical analyses conducted on $n = 18$ healthy men. PYY, peptide YY; 95% CI, 95% confidence interval.

6.5 Discussion

The main findings from our study are that control-adjusted appetite-related blood parameters and perceived appetite responses to a standardised meal are reproducible when measured on two separate occasions. True interindividual variability exists in the post-meal responses of all studied outcomes, but we did not detect any worthwhile or statistically significant moderating influence of the FTO genotype on the magnitude of post-meal responses.

As expected, meal intake after an overnight fast resulted in mean suppressions of acylated ghrelin, hunger and PFC, concomitantly with increases in total PYY, insulin, glucose, fullness and satisfaction. Correlation coefficients between the two replicates of control-condition-adjusted responses were positive, significant and large for total PYY, insulin, hunger, satisfaction and PFC. The correlation for acylated ghrelin was positive, but small and not significant. However, the exclusion of two outliers improved the correlation markedly and we could not identify any methodological factors that could explain the one-off large or small values. Correlation coefficients for glucose and fullness were positive and moderate, although not significant. Removal of one outlier for fullness improved the correlation. Overall, the postprandial changes in appetite parameters were similar between the two experiment replicates suggesting good reproducibility of appetite responses to meal intake.

Previous studies have also reported good reproducibility of *ad libitum* energy intake, cholecystokinin, glucose and insulin (Nair et al. 2009; Horner et al. 2014), and appetite perceptions after repeated fixed test meals (Flint et al. 2000; Gonzalez et al. 2012; Horner et al. 2014). Although Nair et al. observed good reproducibility of blood glucose area under the curve after a glucose preload on three occasions, the time taken for glucose to peak varied between visits (Nair et al. 2009). In our study, the pre-to-post change score was calculated between the fasting state and a single postprandial time point when the peak post-meal change was expected. It is possible that the relatively small correlation for pre-to-post changes in glucose on two occasions reflects inconsistency in the time taken for glucose to peak after a meal for some participants, rather than a lack of reproducibility of the response magnitude *per se*.

Recently, Gonzalez et al. observed poor reproducibility at the individual level in perceived appetite after the consumption of liquid meals (Gonzalez et al. 2017). Data were pooled from two previous studies comparing low and high energy liquid meals, but no control condition

was included. The inclusion of a condition where no intervention takes place is essential so that the natural oscillation in the outcomes can be quantified and, therefore, the "true" effect of the intervention can be assessed (Atkinson and Batterham, 2015; Senn, 2016). Our results indicate that, besides the good reproducibility of appetite responses to meal intake, the magnitude of change varied considerably between individuals, supported by the participant-by-condition interactions. No previous studies have examined the interindividual variability in appetite responses to a meal including control conditions in a replicated crossover design. Therefore, our study adds to the literature by using a novel and appropriate study design and statistical analysis approach (Atkinson and Batterham, 2015; Senn, 2016).

The SD of the pre-to-post change scores was substantially larger in the meal compared to control conditions, indicating that the individual differences could not be explained by random within-subject variation or measurement errors (Atkinson and Batterham, 2015). All participants exhibited perceived appetite and appetite-related blood parameter responses beyond our defined MCID, but a few participants were ‘‘small responders’’ whereas others were ‘‘very large responders’’ according to the degree of change in the appetite parameters after meal intake. Of note, there are no clinically relevant target differences established for appetite parameters and the MCID thresholds chosen were based on the statistical threshold of 0.1 SD for judging clinical importance of individual differences. Clinically relevant differences are most appropriately defined using "hard" anchors to changes in morbidity and/or mortality (Cook et al. 2018), but information is lacking on this at present. All participants exhibited the expected direction of meal-induced change in the various outcomes i.e. suppression of acylated ghrelin, hunger and PFC, and increase in total PYY, insulin, glucose, fullness and satisfaction, except for one participant who exhibited an increase in hunger after meal intake on both occasions.

The FTO genotype-by-condition interaction was not statistically significant for any of the appetite parameters. While statistical power can be lower for the detection of sub-group by treatment interactions versus the overall treatment effect, effect sizes were low for all FTO gene sub-group interaction terms. It is well established that the homozygous AA variant of the FTO genotype is associated with higher obesity risk (Frayling et al. 2007). Although the mechanisms are not fully elucidated, it has been suggested that AAs demonstrate attenuated postprandial suppression of acylated ghrelin and hunger (Karra et al. 2013). In contrast, a recent study did not identify differences in hunger or total ghrelin between FTO genotype groups in

individuals with overweight or obesity after standardised and buffet meals, even though AAs had higher energy intake at the buffet meal (Melhorn et al. 2018). Of note, the assessment of total ghrelin in this study could have influenced the results as the active part of the hormone only represents ~10% of total ghrelin (Hosoda et al. 2000; Yoshimoto et al. 2002). Longer term studies are needed to confirm whether differences in appetite-related outcomes are observed between FTO genotype groups which may culminate in continuous differences in energy intake, and consequently, body mass alterations.

Exploratory analyses of our data indicated that higher adiposity was associated with smaller changes in the mean postprandial acylated ghrelin and hunger responses, supporting previous evidence suggesting that individuals with obesity exhibit a reduced postprandial suppression of ghrelin (Le Roux et al. 2005). However, our study was not designed to answer this question and participant recruitment aimed to match the two FTO-genotype groups for age and adiposity. Besides adiposity levels, individual differences in rates of stomach distention and gastric emptying (Janssen et al. 2011), as well as differences in gut microbiota, could potentially explain the interindividual variability in postprandial appetite responses (van de Wouw et al. 2017). Indeed, direct associations between insulin and glucose responses to a glucose preload and rates of gastric emptying have been observed (Nair et al. 2009), and a growing body of evidence points to the important role of gut microbiota in nutrient sensing and appetite regulation (Lam et al. 2017; van de Wouw et al. 2017). Future research is required to determine moderators of appetite responses to meal intake that may explain the individual variability.

All the correlations between the changes in perceived appetite and appetite-related blood parameters were significant apart from the correlations between insulin and hunger, satisfaction and PFC. Although the exact pathways are unclear, insulin has been associated previously with short-term appetite control in healthy individuals, as increased postprandial insulin concentrations were associated with increased satiety and decreased hunger (Flint et al. 2007). Overall, our results provide evidence of very strong associations between perturbations in appetite-related blood parameters and perceived sensations of hunger, satisfaction, fullness and PFC. This supports previous evidence showing that changes in glucose, insulin, acylated ghrelin, PYY_{3-36} and glucagon-like peptide-1 concentrations occur synchronously with changes in perceived appetite after the consumption of test meals (Lemmens et al. 2011).

The strengths of our study include the novel study design and statistical approaches employed, which have been advocated to quantify interindividual variability in responses to an intervention appropriately (Atkinson and Batterham, 2015; Senn, 2016). Furthermore, the combination of circulating blood parameters with perceived appetite ratings known to respond episodically to meal intake represents a further strength. Care should be taken when generalizing the findings as alternative blood processing or analysis methods, as well as inclusion of females, older individuals and individuals with obesity, may result in different findings.

In conclusion, the reproducibility of appetite responses to standardised meals is generally good. True interindividual variability is present in appetite-related blood parameters and perceived appetite responses to meal intake beyond any random within-subject variation over time in healthy men, but the magnitude of change in postprandial appetite responses was not influenced by the FTO gene. Our study supports the existence of true interindividual variability in postprandial appetite changes between individuals, which should be considered in future research as well as for interpreting group mean results from intervention studies. Furthermore, these findings highlight the importance of exploring individual differences in appetite response in the context of the prevention and/or treatment of obesity. Further studies with longer-term interventions using appropriate study designs and statistical analyses are needed to identify potential moderators responsible for the individual variability in postprandial appetite responses and to confirm the exact clinical relevance of our findings.

Chapter 7

A pilot study to explore the association between brown adipose tissue activity, FTO genotype and appetite-related blood parameters in healthy males

7.1 Abstract

Background: The ability of brown adipose tissue (BAT) to increase energy expenditure and its potential impact on appetite regulation has stimulated great research interest. Limited evidence in rodents suggest a possible role of BAT on the obesity risk associated to the fat mass and obesity-associated gene (FTO), although this is yet to be investigated in humans. **Objective:** To explore any potential associations between BAT activity, FTO genotype and fasting and postprandial appetite-related blood parameters in healthy males. **Methods:** Eighteen healthy men recruited according to FTO rs9939609 genotype were divided into two groups matched for age and adiposity: 9 homozygous minor allele (AA) and 9 homozygous major allele (TT). Measurements of fasting and postprandial acylated ghrelin, total PYY, insulin and glucose were obtained during two fasting trials and two meal trials (as described in Chapter 6), and BAT activity was assessed after an overnight fast. A thermal imaging camera was used to measure the temperature of the skin overlying the supraclavicular (SCV) area during 10 min of acclimatisation and 10 min of a forearm cooling protocol at 15°C. The observed change in SCV temperature (delta) during the cooling protocol was used as indication of BAT activity. Within-participant linear mixed models were used to assess the effect of the cooling protocol and the FTO genotype on SCV temperature, as well as the genotype-by-condition interaction. Pearson's correlation coefficients were calculated between BAT activity and fasting and postprandial appetite-related blood parameters. **Results:** Positive delta SCV temperature values, indicative of BAT activation as a result of the cooling protocol, were observed in 8 participants. However, no significant effect of cooling on SCV temperature was identified at the group level ($P = 0.240$). Additionally, no significant effect of FTO genotype ($P = 0.861$) or genotype-by-condition interaction ($P = 0.916$) was detected on SCV temperature. Pearson's correlation coefficients were trivial to moderate and not statistically significant between BAT activity and fasting and postprandial appetite-related blood parameters (r = -0.38 to 0.30, $P \geq 0.115$). **Conclusions:** Results from this pilot study do not

support the existence of a significant association between BAT activity, FTO genotype and appetite-related blood parameters. The cooling protocol employed may not have been sufficient to activate BAT in all study participants. Additionally, larger study samples may be needed to elucidate the potential effect of FTO genotype on BAT in humans, and to investigate whether appetite-regulatory hormones are involved.

7.2 Introduction

BAT is a specialised and highly metabolically active form of adipose tissue containing uncoupling protein 1 (UCP-1) in the mitochondria, which uses glucose and free fatty acids to produce heat when activated (Cannon and Nedergaard, 2004; Nedergaard et al. 2007). For a long time, the main function of BAT was believed to be the maintenance of body temperature during infancy, although the identification of BAT depots mainly in the cervical and SCV areas of adults, together with its ability to increase energy expenditure and improve glucose and blood lipid profiles, has stimulated increasing interest on BAT as a potential target tissue for the treatment of obesity and associated metabolic complications (Chechi et al. 2014; Sidossis and Kajimura, 2015). The volume of BAT present in adults and its exact contribution to total energy expenditure remain unknown due to the heterogeneity in the conditions and assessment methods employed in studies published to date (Law et al. 2018a).

BAT activity is controlled by the action of norepinephrine, from the sympathetic nervous system, and its activation can be acute or chronic, resulting from continuous enhanced BAT recruitment to maintain body temperature (Law et al. 2018a). Acute activation can be a result of either meal ingestion or cold exposure, although the latter seems to exert a stronger effect (Orava et al. 2011). During extreme cold exposure, shivering is the main mechanism responsible for temperature homeostasis maintenance. However, BAT is the main source of heat production during less intense cold exposure i.e. above shivering threshold (Law et al. 2018a). Current evidence suggests that, besides the classical brown adipocytes, inducible brown adipocytes, often called beige adipocytes, are also found in adults, mainly within subcutaneous white adipose tissue (Sidossis and Kajimura, 2015). Beige adipocytes differ from brown adipocytes in developmental and anatomical characteristics, and the development of beige adipocytes within white adipose tissue, a process known as ‘browning’, seems to be induced by factors such as chronic cold exposure and exercise (Sidossis and Kajimura, 2015).

The assessment of BAT activity can be made directly, with biopsy samples, or indirectly, with imaging techniques. The location of BAT depots in adults make biopsies impractical due to the proximity to great vessels, and the current gold standard for measuring BAT activity in vivo, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake assessed by positron emission tomography – computed tomography scanning (PET-CT), has its application limited due to radiation exposure, high cost and long time needed for the assessment (Law et al. 2018a). Recently, the use of infrared thermography (IRT) has been suggested as a safe and non-invasive alternative to measure temperature changes on the skin overlying BAT depots, which occur as a result of thermogenesis together with increased blood flow during tissue activation. This method has shown good reproducibility and comparable results to the glucose uptake of activated BAT measured by PET-CT (Haq et al. 2017; Law et al. 2018a).

While the association between variants in the fat mass and obesity-associated gene (FTO) and obesity risk is well established, evidence on the mechanisms involved are still scarce and study results are often controversial. Interestingly, studies in FTO knockout mice have shown that FTO deficiency leads to enhanced browning in white adipose cells, resulting in increased energy expenditure (Tews et al. 2013; Ronkainen et al. 2016). Together with previous evidence showing that the volume and activity of BAT is inversely related to body mass index and body fat mass (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Vijgen et al. 2011), these findings led to speculations about the involvement of BAT on the increased obesity risk associated with FTO. It was hypothesized that individuals with the FTO high-risk allele present reduced expression of UCP-1 and thermogenesis in white adipose tissue, i.e. diminished browning of white adipocytes, leading to chronically reduced energy expenditure and, as a consequence, increased risk of fat mass accumulation (Tews et al. 2013). Whether classical brown adipocytes are equally involved remains to be elucidated.

Of note, previous evidence suggested that the mechanisms through which FTO increases obesity risk are associated with increased energy intake, rather than lower energy expenditure (Speakman et al. 2008). Recent studies have shown a potential link between BAT and hormones associated with appetite regulation and energy homeostasis, such as leptin, ghrelin and secretin (Chondronikola et al. 2017; Li et al. 2018), which may indicate a combined effect of BAT on both energy intake and energy expenditure. Whether the obesity risk associated with FTO is influenced by these dual mechanism is still to be determined. Therefore, the exact

involvement of BAT in the physiological mechanisms affected by the FTO gene in humans deserves further investigation.

Considering these findings, this pilot study aimed to explore any potential associations between BAT activity and fasting and postprandial appetite-related blood parameters in healthy males with the high-risk (AA) and low-risk (TT) variants of the FTO genotype.

7.3 Methods

7.3.1 Ethical approval

All procedures included in this study were approved by Loughborough University Ethics Advisory Committee and written informed consent was obtained from all study participants before any aspect of the study was conducted.

7.3.2 Participants

All participants from the study described in Chapter 6 were invited and agreed to participate in this pilot study. Eighteen healthy white European men were recruited according to the rs9939609 allele of the FTO gene and divided into two groups matched for age and adiposity: 9 homozygous minor allele (AA) and 9 homozygous major allele (TT). Inclusion criteria and anthropometric measurements are described in detail in Chapter 6. Participants' physical activity level was assessed with the International Physical Activity Questionnaire (Craig et al. 2003).

7.3.3 Experimental design

All participants attended four laboratory visits (two control and two meal conditions) where circulating levels of fasting and postprandial appetite-related blood parameters were assessed. These visits have been described in detail in Chapter 6. In summary, participants arrived at the laboratory at 9:00 after an overnight fast and a cannula (Venflon; Becton Dickinson, Helsingborg, Sweden) was inserted into an antecubital vein 60 min before the collection of venous blood samples. After the fasting venous blood sample was taken, participants rested throughout all four conditions but consumed a standardised breakfast meal (5025 kJ energy) during the two meal conditions. Subsequent venous blood samples were taken at 0.5 and 1 h.

After completing the four visits, participants attended one single laboratory visit for the assessment of BAT activity. Participants refrained from alcohol, caffeine and strenuous physical activity during the 24 h preceding the visit. Participants arrived at the laboratory between 8:00 and 9:00 after a 12 h overnight fast (no food or drink except plain water) and were asked to wear standardised cotton clothes (shorts and vest) before being positioned sitting on a chair facing a thermal imaging camera (FLIR Systems T620, West Malling, UK). The distance between the participant's chair and the camera tripod was standardised as 1 m. A cooling blanket connected to a cooling unit was placed around the participant's right forearm and six reflective markers were placed on the neck and shoulders region in order to determine the apices of the region of interest for posterior analyses of the images (Figure 7.1). Temperature wireless data loggers (Maxim iButton DS1219H-F50, Sunnyvale, USA) were placed at seven body sites (forehead, trunk, arm, hand, lower leg, thigh and foot), a heart rate monitor strap (Garmin 920XT, Olathe, USA) was positioned under the chest, and a blood pressure arm cuff (Omron HEM-FL31, Kyoto, Japan) was positioned on the participant's left arm. Environmental temperature and humidity were monitored and kept constant using a wireless weather station (Opes, London, UK).

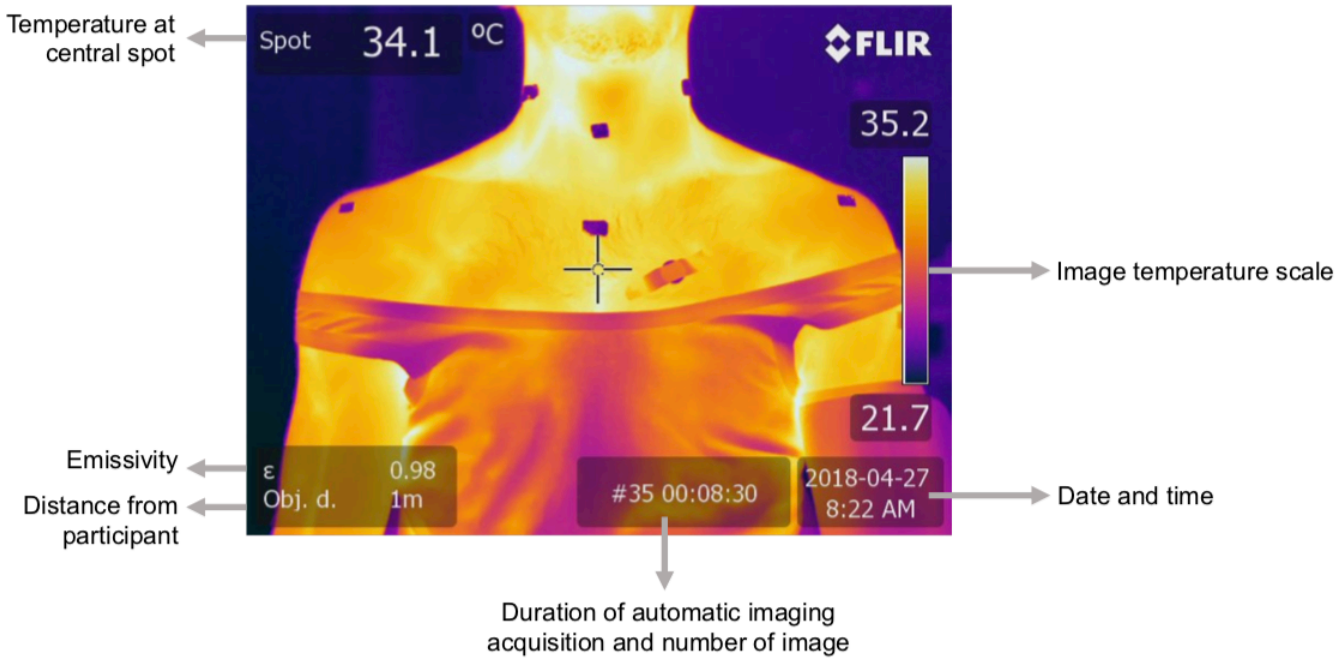


Figure 7.1 Thermal image with reflective markers determining the apices of the region of interest for the assessment of brown adipose tissue activity.

7.3.4 Brown adipose tissue activity

Participants were asked to stay as still as possible while thermal images were automatically captured every 15 seconds during 10 minutes of acclimatisation (with the cooling blanket switched off) and 10 minutes of forearm cooling at 15°C (stimulation period). This short cooling protocol has been previously shown to be sufficient for the stimulation of BAT activity (Haq et al. 2017; Law et al. 2018b). Skin temperature at seven body sites and heart rate were recorded every minute. Blood pressure and tympanic temperature (Braun ThermoScan PRO 4000, Kronberg, Germany) were recorded before and after the acclimatisation period, and after the stimulation period. Mean skin temperature was calculated using the data obtained from the seven data loggers and the following formula (Hardy and Oppel, 1938):

Mean skin temperature = (0.07 x temperature head) + (0.14 x temperature arm) + (0.05 x temperature hand) + (0.07 x temperature foot) + (0.13 x temperature leg) + (0.19 x temperature thigh) + (0.35 x temperature trunk)

The temperature of the skin overlying the SCV region was measured using infrared thermography and used as an indicator of BAT activity during cooling stimulation. The region of interest was defined as that bounded by the right sternocleidomastoid muscle, clavicle and lateral contour of the neck using Matlab 2017b software (The Mathworks Inc., Natick, USA). The hottest ten percent of points within the region of interest were identified and the median of the temperature in these points was calculated in each captured image (Figure 7.2).

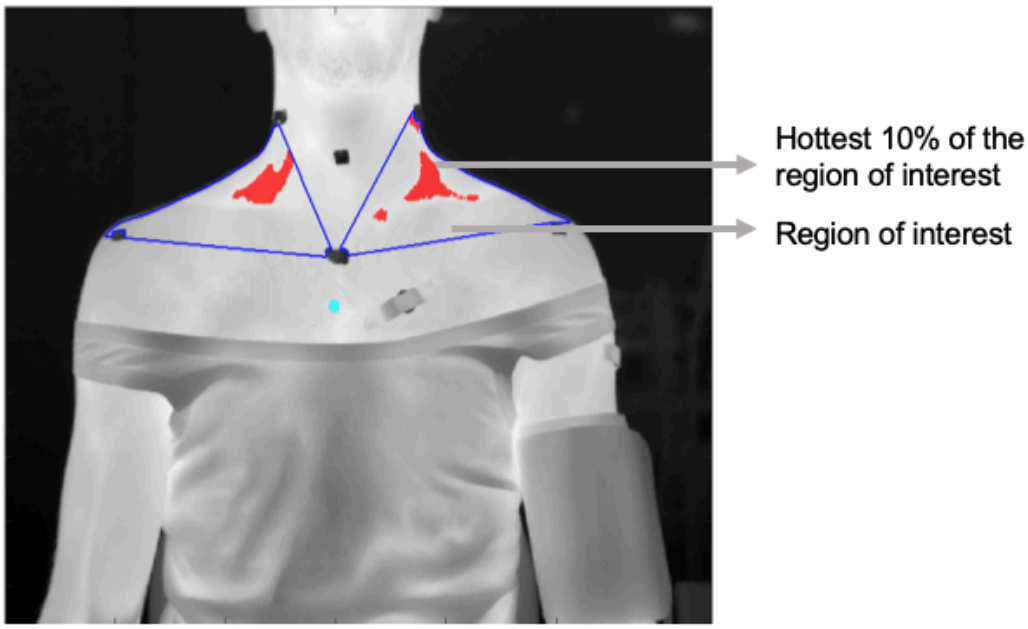


Figure 7.2 Thermal image with the region of interest for the assessment of brown adipose tissue activity marked by the blue lines and the hottest ten percent of points shown in red.

Baseline SCV temperature was defined as the mean temperature on the images acquired during the last minute of the acclimatisation period to ensure participants had achieved a steady state. The same criterium was used to determine baseline mean skin temperature, and the difference between baseline SCV temperature and baseline mean skin temperature was calculated in order to obtain the relative baseline SCV temperature, taking into account the natural difference between both measurements. A moving average for every minute of the stimulation period was applied to reduce the effect of natural variation in measurements, and the highest average value was selected as the stimulation SCV temperature, representing the peak BAT activity. The same criterium was used to determine the stimulation period mean skin temperature, and the difference between stimulation SCV temperature and stimulation mean skin temperature was calculated in order to obtain the relative stimulation SCV temperature, taking into consideration the changes in skin temperature due to factors other than BAT activation, e.g. any potential effect of ambient temperature. Finally, delta SCV temperature was calculated as the difference between relative stimulation SCV temperature and relative baseline SCV temperature in order to assess BAT activity, using each participant as their own control, where positive values indicated BAT activation as a result of the acute cold stimulus.

At the end of the session, a final thermal image of both participant's forearms was acquired in order to confirm the effectiveness of the cooling protocol (Figure 7.3).

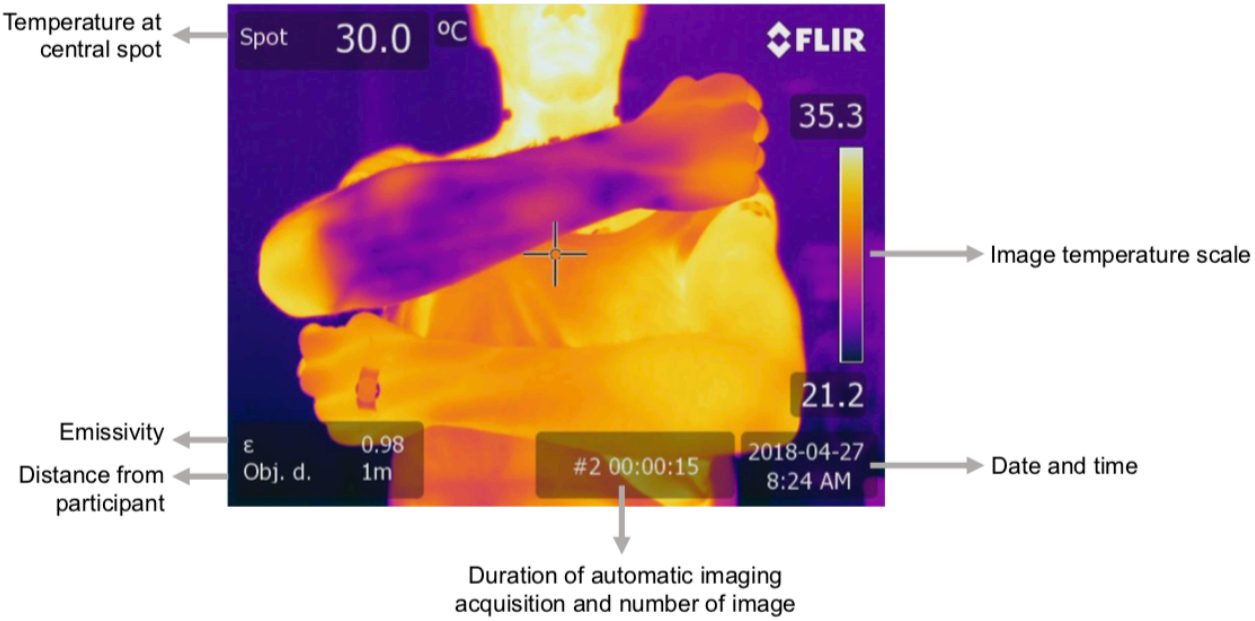


Figure 7.3 Thermal image comparing participant's right forearm after cooling period with cooling blanket set at 15°C for 10 minutes and left forearm used as control.

7.3.5 Fasting and postprandial appetite-related blood parameters

Acylated ghrelin, total PYY, insulin and glucose concentrations in the fasted state and after consumption of a standardised meal were obtained in four separate visits, explained in detail in Chapter 6. In summary, plasma acylated ghrelin and total PYY concentrations were quantified from samples at 0 and 1 h, and insulin and glucose concentrations were measured at 0 and 0.5 h to capture the peak change in concentration after the meal. The average between fasting concentrations obtained for each blood parameter in the four visits was calculated and used in the analyses. The average between two control-adjusted meal responses (i.e. change in blood concentration during meal condition – change in blood concentration during control condition) for each appetite-related blood parameter was calculated and used in the analyses.

7.3.6 Statistical analysis

Univariable general linear models with FTO genotype as a single fixed effect were used to

quantify differences between genotype groups for physiological parameters, mean skin temperature and SCV temperature between both FTO genotype groups. Within-participant linear mixed models were used to quantify the effect of the cooling protocol and the FTO genotype on SCV temperature, and the genotype-by-condition interaction was quantified. Pearson's correlation coefficients were calculated to investigate any potential associations between BAT activity, fasting and postprandial appetite-related blood parameters (acylated ghrelin, total PYY, insulin and glucose), and individual characteristics. Thresholds of 0.0, 0.1, 0.3 and 0.5 were used to define trivial, small, moderate and large correlation coefficients, respectively (Cohen, 1988). Data are presented as mean (SD). Statistical significance was accepted as $P < 0.050$. Data were analysed using SPSS version 23 (IBM Corporation, New York, USA).

7.4 Results

Positive delta SCV temperature values, indicative of BAT activation as a result of the cooling protocol, were observed in 8 participants. Individual delta SCV temperature values are presented in Figure 7.4.

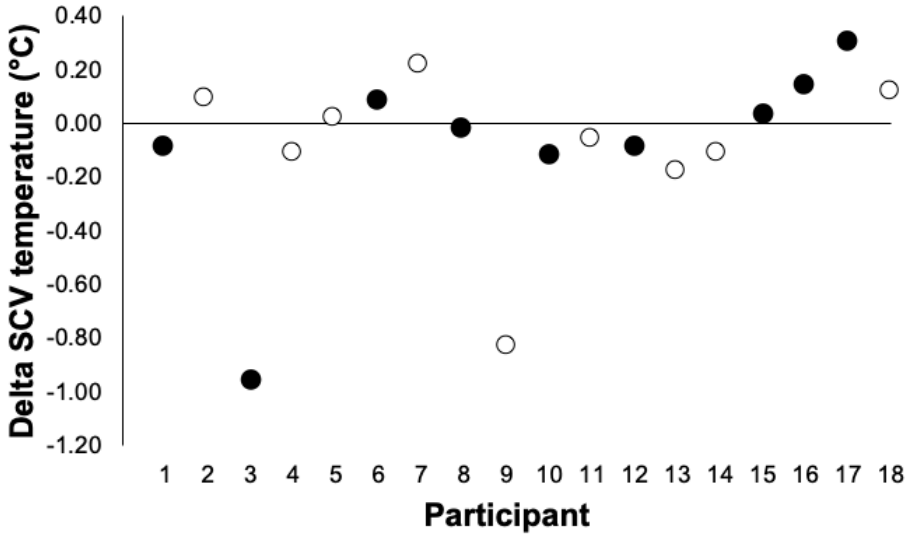


Figure 7.4 Individual delta values of supraclavicular (SCV) temperature relative to changes in mean skin temperature following a 10-min cooling stimulation of the forearm at 15°C. White circles indicate individuals homozygous for the minor allele (AA) and black circles indicate individuals homozygous for the major allele (TT) of FTO rs9939609.

Physiological parameters, mean skin temperature and SCV temperature measured at baseline and after cooling stimulation are presented in Table 7.1.

Table 7.1 Physiological parameters, mean skin temperature and supraclavicular temperature measured before and after cooling stimulation. Mean (SD).

	Baseline			Cooling stimulation		
	AA (n = 9)	TT (n = 9)	P value*	AA (n = 9)	TT (n = 9)	P value*
Heart rate (bpm)	66 (8)	73 (9)	0.087	66 (8)	74 (10)	0.059
Systolic blood pressure (mmHg)	130 (19)	122 (14)	0.320	127 (15)	121 (12)	0.412
Diastolic blood pressure (mmHg)	85 (13)	88 (16)	0.674	86 (11)	88 (12)	0.810
Tympanic temperature (°C)	36.4 (0.4)	36.3 (0.4)	0.716	36.4 (0.4)	36.5 (0.5)	0.682
SCV temperature (°C)	34.6 (0.4)	34.1 (0.8)	0.107	34.9 (0.4)	34.3 (0.7)	0.060
Mean skin temperature (°C)	31.3 (0.7)	30.8 (0.7)	0.148	31.6 (0.8)	31.1 (0.7)	0.178
Relative SCV temperature (°C)	3.3 (0.4)	3.3 (0.5)	0.874	3.2 (0.6)	3.2 (0.7)	0.859
Delta relative SCV temperature (°C)				-0.08 (0.35)	-0.10 (0.30)	0.905

* P values are from univariable general linear models with FTO genotype as a single fixed effect.

SCV, supraclavicular.

The effect of the cooling protocol was not statistically significant at the group level (P = 0.240) and no effect of FTO genotype was identified on SCV temperature (P = 0.861). Similarly, no statistically significant genotype-by-condition interaction could be identified (P = 0.916).

Pearson's correlation coefficients were trivial to moderate and not statistically significant between BAT activity and fasting acylated ghrelin (r = 0.13, $P = 0.611$), fasting total PYY (r = 0.20, $P = 0.414$), fasting insulin (r = 0.00, $P = 0.990$) or fasting glucose (r = -0.38, $P = 0.115$). Similarly, correlation coefficients were small to moderate and not statistically significant between BAT activity and control-adjusted meal responses for acylated ghrelin, total PYY, insulin and glucose (r = -0.26 to 0.30, $P \geq 0.221$). Exploratory analyses of the data showed that correlation coefficients were trivial to moderate and not statistically significant between BAT activity and body mass index (r = -0.25, $P = 0.311$), body fat mass (r = -0.31, $P = 0.213$), fat-free mass (r = 0.04, $P = 0.870$) and self-reported physical activity level (r = -0.12, $P = 0.622$).

7.5 Discussion

The main finding from this pilot study was that no significant association between FTO rs9939609 genotype and BAT activity, indicated by changes in SCV skin temperature, could be identified following a short cooling protocol. Even though BAT activation was identified in 8 out of eighteen study participants, the effect of the cooling protocol on SCV temperature was not significant at the group level. Further findings were that BAT activity was not associated with fasting or postprandial appetite-related blood parameters, body composition or self-reported physical activity level.

While previous studies have shown a clear link between FTO, beige adipocytes and energy expenditure in mice, to date, this association has not been explored in humans. In mice, the absence of the FTO protein has a protective effect against obesity due to increased energy expenditure resultant from an up-regulation of UCP-1 in adipocytes (Tews et al. 2013; Ronkainen et al. 2016). Similar findings were observed in isolated human adipocytes, leading to the hypothesis that lower browning of white adipose tissue is a contributing factor to the obesity risk associated to FTO gene (Tews et al. 2013). Whether the volume and activity of classical brown adipocytes are also affected by FTO is currently unknown, and we could not identify a significant difference in BAT activity measured by IRT after a short cooling stimulation protocol between FTO rs9939609 homozygous genotype groups in healthy males.

We speculate that larger study samples are needed to elucidate any potential effect of FTO genotype on BAT activity. In addition, previous studies in rodents indicated an inverse association between genetic susceptibility to obesity and browning propensity in white adipose tissue, indicated by UCP-1 expression (Xue et al. 2007; Kozak, 2011; Sidossis and Kajimura,

2015). However, this association may not be seen with classical BAT depots. Finally, in light of previous evidence suggesting FTO increases obesity risk through impaired appetite control and, as a result, increased energy intake (Speakman et al. 2008), it is possible that a difference in BAT activity following food intake, rather than cold exposure, exists between FTO genotype groups and exerts an impact on appetite regulation.

BAT is known to contribute to both acute meal thermogenesis and long-term diet-induced thermogenesis, however, the mediators and the magnitude of the associated energy expenditure remain to be elucidated. The mechanisms involved in BAT activity following meal consumption seem to differ from those responsible for cold-activated BAT activity (Orava et al. 2011; Peterson et al. 2016). It has been shown that BAT is stimulated by insulin (Orava et al. 2011; Trayhurn, 2017), and the meal-associated thermogenesis induces satiation via brain signalling (Li et al. 2018). Following observations that ghrelin inhibits norepinephrine release in BAT of rodents (Mano-Otagiri et al. 2009), BAT volume was recently associated with a greater suppression in acylated ghrelin concentration during cold exposure in humans (Chondronikola et al. 2017). In addition, BAT volume was associated with lower fasting leptin, gastric inhibitory peptide and glucagon concentrations during thermoneutrality, and leptin and glucagon concentrations were significantly reduced following five hours of cold exposure (Chondronikola et al. 2017). Whether such links between BAT and appetite-related hormone concentrations are seen during meal-related thermogenesis is still unclear. Interestingly, recent evidence identified secretin, a gut hormone released during meal consumption, as a non-sympathetic activator of BAT thermogenesis which induces satiation (Li et al. 2018). Therefore, further studies are needed to elucidate other potential mechanisms through which BAT may influence appetite regulation.

Even though concentrations of appetite-related hormones typically change on a meal-to-meal basis, and often reflect perceived appetite ratings, this is not observed in every study (Goltz et al. 2018; Chapter 4). This observation reflects the notion that appetite regulatory mechanisms are still not fully understood and other physiological factors influencing appetite are still to be identified. The potential effect of BAT on appetite regulation is undoubtedly appealing and could help to explain many inconsistencies seen in appetite research, as well as being a potential contributor to the well documented interindividual variability in appetite responses to a meal (Goltz et al. 2019; Chapter 6) and to exercise (Goltz et al. 2018; Chapter 4). We could not identify any significant associations between BAT activity and fasting or postprandial

acylated ghrelin, total PYY, insulin and glucose in our pilot study. This is in contrast with previous findings showing an inverse association between BAT activity and blood glucose (Lee et al. 2010). Indeed, BAT is believed to be a major organ in glucose utilization, playing a role in insulin sensitivity and glucose homeostasis (Lee et al. 2014; Sidossis and Kajimura, 2015; Trayhurn, 2017). Of note, circulating concentrations of appetite-related blood parameters were assessed in a separate visit from the cold-stimulation protocol, and BAT activity was not measured during or after meal intake in this pilot study. Future studies should investigate whether BAT is activated following meal consumption concomitantly and proportionally to observed changes in appetite-related blood parameter concentrations.

Previous studies have classified individuals as BAT-positive and BAT-negative depending on whether tissue activity could be identified following cold exposure (Yoneshiro et al. 2011b). We identified BAT activation following an acute cold stimulus in 8 out of eighteen study participants. The remaining participants showed greater temperature increases in the mean skin temperature compared to the change in the temperature of the skin overlaying BAT depots in the SCV area. This observation is similar to findings from a previous study using IRT measurements, and it is possibly a result of whole-body vasoconstriction induced by cold exposure, leading to increased skin temperature in body areas other than SCV (Boon et al. 2014; Peterson et al. 2017). Interestingly, the two individuals (participant 3 and participant 9 in Figure 7.4) presenting the lowest delta SCV temperature within our sample, -0.96 and -0.84, indicating that the change in mean skin temperature after cold stimulation was nearly 1°C larger than the change in temperature at the SCV area, were 48 and 50 years old, respectively, while the remaining study participants ranged from 18 to 37 years old. This observation is consistent with previous findings showing that the incidence of cold-activated BAT decreases with age (Yoneshiro et al. 2011b).

Besides age, adiposity has also been negatively associated with the volume and activity of BAT (Cypess et al. 2009; Lee et al. 2010; van Marken Lichtenbelt et al. 2009; Vijgen et al. 2011; Trayhurn, 2017). While cold stimulation was shown to increase energy expenditure and SCV temperature measured with IRT in lean subjects, this was not observed in obese subjects (El Hadi et al. 2016). This observation may be due to lower BAT activity in obese and/or resultant of the presence of a thicker layer of subcutaneous adipose tissue in the SCV area, which would provide higher thermal insulation to these subjects (El Hadi et al. 2016). The majority of our study participants had a BMI higher than 25 $kg \cdot m^{-2}$ and it is possible that higher fat mass

affected our ability to identify BAT activity in this study sample. However, no significant correlations between BAT activity and body mass index or body fat mass were observed. Additionally, a previous study showed that BAT activity assessed by IRT was not associated to neck adiposity in healthy men with BMI ranging from 19.3–32.3 kg/m^2 (Haq et al. 2017).

Exercise has been previously suggested as a contributing factor to white adipose tissue browning, leading to a chronic increase in energy expenditure (Sidossis and Kajimura, 2015; Aldiss et al. 2018). The impact of exercise on BAT is still not fully understood, although it is suggested that lactate and the myokines irisin, meteorin-like and interleukin-6, all upregulated by exercise, may stimulate white adipose tissue browning (Sidossis and Kajimura, 2015). Whether classical BAT can be affected by acute or chronic exercise and/or daily physical activity is still unclear and no association between BAT activity and self-reported physical activity levels could be identified in our pilot study. Our results support the findings of a recent cross-sectional study showing no association between objectively assessed physical activity levels and BAT volume and activity assessed by PET-CT in healthy sedentary young adults (Acosta et al. 2019). However, studies with larger and more heterogeneous samples are still needed in order to further investigate the impact of physical activity and/or exercise on BAT activity.

We cannot rule out the possibility that our short cooling protocol may not have been sufficient to activate BAT in all study participants, as no statistically significant effect of the cooling protocol could be seen at the group level. In that respect, we did not observe a decrease in mean skin temperature as reported in previous studies (Yoneshiro et al. 2011a; Boon et al. 2014), but rather a small increase in mean skin temperature, possibly indicating inefficiency of the cold stimulus. However, the decrease in the participants' forearm skin temperature was evident as shown by the IRT images obtained following 10 min of cooling. Previous studies have also reported increases in heart rate and blood pressure after cold stimulation, indicative of activation of the adrenergic system (Boon et al. 2014; El Hadi et al. 2016), although we could not identify any significant changes in heart rate or blood pressure during our cooling protocol. Utilizing individualised cooling protocols, which take into consideration the temperature in which each individual starts shivering, is an option to guarantee the delivery of an effective stimulus for each study subject. However, this is impractical when using IRT as the temperature needed for someone to start shivering can vary day-by-day, and achieving shivering on the day

of IRT measurements could affect the assessment of skin temperature at rest which should be used as a control for each individual (Law et al. 2018a).

Some limitations are inherent to the method we used to assess BAT activity. The efficacy of IRT can be limited by factors such as increased blood flow in the carotid arteries and the presence of high amounts of subcutaneous fat, although the extent of the impact of these factors is yet to be quantified (Law et al. 2018a). Furthermore, previous studies have shown that some subjects do not show any BAT activity, whereas others present very active BAT, even at thermoneutrality (Lee et al. 2010; Yoneshiro et al. 2011b; Jang et al. 2014). The latter may not increase thermogenesis by much when stimulated by cold and thus temperature changes measured by IRT would not be a reliable assessment of BAT activity in such cases (Law et al. 2018a). The inability of IRT to measure or estimate BAT volume is a further limitation, as the impact of BAT on energy homeostasis and body weight balance could be more impacted by the quantity rather than by the level of activation observed in a small pre-determined area. However, SCV skin temperature has been previously shown to be correlated with BAT volume and activity measured by PET-CT (Boon et al. 2014; Law et al. 2018a). Furthermore, the identification of BAT with PET-CT has shown poor reproducibility (Lee et al. 2010). Of note, besides glucose, free fatty acids and triglycerides are also utilized by BAT, and therefore glucose uptake measured in ^{18}F-FDG PET may not always be a reliable measure of BAT activity (Haq et al. 2017). Nevertheless, measurements of BAT with IRT have shown highly reproducible results (Haq et al. 2017), with a probability greater than 80% in predicting BAT (Jang et al. 2014).

In conclusion, we could not identify any significant association between FTO genotype, BAT activity and appetite-related blood parameters in our pilot study with a small sample of healthy men. We highlight the need of further studies investigating the potential link between FTO and BAT in larger and more heterogeneous samples. The effect of FTO genotype on BAT activity associated to meal-induced thermogenesis, and the associated effect on appetite, should be assessed where possible. As most evidence on BAT activity is derived from studies in mice, the understanding of BAT physiology in humans is still in its infancy and it is still unclear whether energy expenditure and/or the impact on energy intake derived from BAT make a significant contribution to energy homeostasis in human adults (Trayhurn, 2017). BAT might be a good candidate for the treatment of obesity and associated complications not only due to its ability to increase energy expenditure, but also because of its involvement in glucose

homeostasis and appetite regulation (Sidossis and Kajimura, 2015; Li et al. 2018). Identifying the physiological mechanisms involved in the activation of BAT is of great importance to the understanding of whether BAT can be a potent therapeutic option for preventing and/or treating obesity (Trayhurn, 2017).

CHAPTER 8

General discussion

8.1 Introduction

While the prevalence of overweight and obesity continues to rise worldwide, the success of currently available interventions to prevent weight gain and/or promote weight loss remains very limited. The focus on interindividual variability in responses to such interventions has increased considerably in recent years, as personalised medicine strategies arise as a valuable and potentially more effective alternative to 'one-size-fits-all' interventions. A few studies have suggested individuals differ in their appetite and energy intake responses to an exact same intervention (Finlayson et al. 2009; King et al. 2009; Hopkins et al. 2014), indicating that tailored individual approaches may be needed for the achievement of satisfactory results. However, important methodological limitations are present in these studies, such as the lack of a control condition, no replication of conditions, and/or no appropriate statistical model employed in order to differentiate true response heterogeneity from within-subjects random variability. Therefore, the affirmation of the existence of interindividual variability in responses to an intervention must be interpreted taking these limitations into consideration.

The studies within this thesis were designed to extend the knowledge on interindividual variability in perceived appetite and appetite-related blood parameter responses to acute exercise and to a standardised meal, as well as on potential factors influencing such variability. The purpose of this chapter is to discuss the main outcomes of the experimental studies presented here and to highlight directions for future research needed to build on this area of knowledge. Table 8.1 provides a summary of the characteristics of the protocols, subjects and variables assessed in each experimental study.

Table 8.1 Summary of the experimental studies presented in this thesis.

Chapter	*n*	Sex	Mean age (years)	Mean BMI ($kg \cdot m^{-2}$)	Study design	Conditions	Measurements
4	15	M	23	24.8	Replicated crossover	Control: 60-min resting Exercise: 60-min treadmill running at 70% peak $\dot{V}O_2$	Perceived appetite, acylated ghrelin and total peptide YY
5	112	M/F	34	25.2	Cross-sectional	Meal test	Resting metabolic rate, visceral adipose tissue, abdominal subcutaneous adipose tissue, liver fat, perceived appetite, acylated ghrelin, total peptide YY, leptin, insulin, glucose, moderate-to-vigorous physical activity, sitting time and FTO genotype
6	18	M	28	27.0	Replicated crossover	Control: 60-min resting Meal: 5025 kJ consumed within 15 min and 45-min resting	Perceived appetite, acylated ghrelin, total peptide YY, insulin, glucose and FTO genotype
7	18	M	28	27.0	Cross-sectional	Cooling protocol	Brown adipose tissue activity, and FTO genotype

F, females; FTO, fat mass and obesity-associated gene; M, males; $\dot{V}O_2$, oxygen uptake.

8.2 Interindividual variability in appetite responses to exercise

One aim of the experimental studies presented within this thesis was to examine the reproducibility and interindividual variability in perceived appetite and appetite-related hormone responses to acute exercise. The study presented in Chapter 4 extends the existing knowledge by being the first study to use a replicated crossover design to assess the reproducibility and quantify the interindividual variability in appetite responses to exercise. The statistical analyses employed have been indicated to appropriately quantify interindividual variability in responses to an intervention (Atkinson and Batterham, 2015; Senn, 2016). Our methodological approach was able to detect good reproducibility and true interindividual variability in appetite responses to exercise, over and beyond any random variability, in a small sample of 15 healthy men. The true interindividual variability was demonstrated by a significant participant-by-condition interaction for acylated ghrelin, total PYY and perceived appetite, together with substantially greater SD of change scores in the exercise condition, compared to the control condition, for all the outcomes.

Our study confirms previous findings indicating an immediate suppression of appetite produced by exercise performed at moderate intensity, previously termed 'exercise-induced anorexia' (Stensel, 2010; Schubert et al. 2014), as shown by the suppression in acylated ghrelin and perceived appetite, and the increase in peptide YY observed after 60 min of treadmill running. While no previous studies have assessed the reproducibility of the changes in appetite-related hormones after acute exercise, previous studies have shown poor reproducibility of energy intake after acute exercise (Brown et al. 2012; Unick et al. 2015). Interindividual variability has been suggested to exist in perceived appetite and energy intake responses to acute exercise in healthy subjects and those who are overweight; however, these studies only included one control and one exercise condition (Finlayson et al. 2009; Hopkins et al. 2014). Without the repetition of the study conditions, it is impossible to determine whether the variability in the observed responses is a result of interindividual variability *per se* or is only reflecting the random variability and measurements errors naturally occurring in any study.

Our findings are in agreement with previous evidence suggesting exercise-induced changes in perceived appetite do not always reflect the changes observed in appetite-related hormones (Deighton et al. 2013; Sim et al. 2014; Martins et al. 2015). While the changes in acylated ghrelin and total PYY were consistent with the changes in perceived appetite in most participants, this was not true for the whole study sample and further investigation is needed to

understand what other physiological and/or psychological factors may be influencing perceived appetite, in conjunction with the two hormones measured in our study. Future studies should include measurements of other hormones known to influence appetite, as well as psychological measurements such as the influence of hedonic processes and cognitive/behavioural cues. Our study did not explore potential factors which could explain the interindividual variability in appetite responses to exercise, and future studies should aim to determine what individual characteristics influence the magnitude of appetite suppression after a session of exercise.

8.3 Interindividual variability in appetite responses to a standardised meal

The second aim of this thesis was to assess the reproducibility and interindividual variability in perceived appetite and appetite-related blood parameter responses to the consumption of a standardised meal. Using a replicated crossover design and novel statistical analyses, the study presented in Chapter 6 adds to the literature by showing good reproducibility of appetite responses to a meal and the existence of true interindividual variability, over and above any random variability and measurement errors, in a small sample of 18 healthy men.

Both studies presented in Chapters 5 and 6 confirm previous findings by showing a suppression of perceived appetite and acylated ghrelin, concomitantly with increases in total PYY, insulin and glucose immediately after meal consumption (Flint et al. 2007; MacLean et al. 2017). Additionally, the findings presented in Chapter 6 are also in agreement with previous studies showing good reproducibility of glucose and insulin (Nair et al. 2009), as well as perceived appetite (Flint et al. 2000; Gonzalez et al. 2012; Horner et al. 2015) responses to a meal. One recent study suggested reproducibility of perceived appetite responses to liquid meals is poor at the individual level (Gonzalez et al. 2017); however, no control trial where no intervention takes place was included in this study. Therefore, the presence of random variability and measurement errors could not be quantified and could have affected the results, possibly explaining the discrepancy between findings. Our study is the first to show true interindividual variability in appetite responses to a meal using a replicated crossover design and robust statistical analyses, evidenced by a significant participant-by-condition interaction and substantially greater SD of change scores in the meal condition, compared to the control condition, for acylated ghrelin, total PYY, insulin, glucose and perceived appetite.

Exploratory analyses of the results presented in Chapter 6 supported previous findings by showing significant correlations between the changes in perceived appetite and appetite-related blood parameters after consumption of a meal (Lemmens et al. 2011). Additionally, higher adiposity was correlated with smaller postprandial suppression of acylated ghrelin and hunger, supporting previous findings showing attenuated postprandial changes in acylated ghrelin in individuals with obesity (Le Roux et al. 2005). However, the results presented in Chapter 5 showed no association between fasting and postprandial acylated ghrelin and total PYY with general or abdominal adiposity, while leptin, glucose and insulin concentrations were consistently associated with adiposity variables in a heterogeneous sample of healthy men and women. Results from the pilot study presented in Chapter 7 did not support an association between brown adipose tissue activity and fasting and postprandial perceived appetite and appetite-related blood parameters, in contrast with previous suggestions that brown adipose tissue volume and activity may be associated with appetite-related hormones (Chondronikola et al. 2017; Li et al. 2018). Therefore, the conflicting results highlighted here provide evidence of the need of further investigation in order to determine the exact role of adiposity in the regulation of appetite.

8.4 Effect of the FTO gene on appetite regulation

The third aim of the studies presented in this thesis was to explore the influence of genetic, physiological and behavioural characteristics on fasting and postprandial appetite-related outcomes. In light of the consistently reported effect of the FTO gene on obesity risk (Frayling et al. 2007; Hess and Brüning, 2014), the study presented in Chapter 5 employed an integrative approach where the association between FTO genotype, perceived appetite and appetite-related blood parameter responses to a meal was assessed in a heterogeneous sample of healthy men and women, taking into consideration the effect of a variety of physiological and lifestyle characteristics. Our findings showed no differences between the three FTO genotype groups in fasting and postprandial perceived appetite and appetite-related blood parameters, assessed using multivariable general linear models with and without the inclusion of sex, age, body mass index, peak oxygen uptake, resting metabolic rate, visceral adipose tissue, abdominal subcutaneous adipose tissue, liver fat, sitting time and moderate-to-vigorous physical activity as covariates. Additionally, the study presented in Chapter 6 found little moderating influence of the FTO genotype on the magnitude of postprandial appetite responses in healthy males when comparing age and adiposity-matched AA and TT individuals.

The findings from Chapters 5 and 6 do not support previous evidence suggesting individuals with the higher obesity risk FTO genotype (AA) show smaller postprandial suppression of acylated ghrelin and perceived hunger compared to TT subjects after consumption of a standardised meal (Karra et al. 2013). Differences in study samples can potentially explain the discrepancies between findings. A heterogeneous sample of men and women was recruited in the study presented in Chapter 5, while Karra et al. recruited young males with AA or TT genotype, matching both groups for age and adiposity. However, in the study presented in Chapter 6, participants had similar characteristics to the study sample selected by Karra et al. Our results support recent findings where postprandial hunger was similar between FTO genotype groups, despite the higher energy intake during an *ad libitum* buffet observed in AA individuals (Melhorn et al. 2018). Furthermore, the findings presented in Chapters 5 and 6 are in agreement with previous studies showing no differences between FTO genotype groups for fasting glucose and insulin (Speakman et al. 2008), fasting leptin (Speakman et al. 2008; Karra et al. 2013; Melhorn et al. 2018), fasting and postprandial PYY_{3-36} (Karra et al. 2013) and fasting and postprandial glucagon-like peptide 1 (Melhorn et al. 2018). Additional studies with longer-term interventions are needed to elucidate the precise role that FTO plays in moderating appetite control and energy intake.

Besides potentially influencing perceived appetite and appetite-related blood parameters, the FTO gene has also been suggested to play a role in brown adipose tissue metabolism (Tews et al. 2013; Ronkainen et al. 2016). While the association between FTO genotype and brown adipose tissue activity has not been previously explored in humans, evidence from studies in mice and in isolated human adipocytes led to the hypothesis that lower browning of white adipose tissue is a contributing factor to the obesity risk associated to the FTO gene (Tews et al. 2013; Ronkainen et al. 2016). However, the results presented in Chapter 7 do not support the existence of a significant association between FTO genotype and brown adipose tissue activity measured by infrared thermography during a short cooling protocol. Our pilot study assessed the change in temperature in the neck area, where classical brown adipose tissue is usually found in adults; however, we were not able to assess any outcomes related specifically to the browning of white adipose tissue, and future studies should attempt to do so in order to determine whether FTO genotype has a similar effect in humans as previously observed in mice.

8.5 Practical implications

It is hoped that the findings presented in Chapters 4 and 6 will bring attention to the need for well-planned study designs and statistical analyses when the quantification of interindividual variability in appetite responses to an intervention is intended. These studies provide important novel evidence of true interindividual variability in appetite responses to acute exercise and eating, which have great value for interpreting study results and also for planning future research. The findings from these studies also provide sound evidence to justify the investigation of individual characteristics which may explain the observed interindividual variability. Furthermore, these findings highlight the importance of exploring individual differences in appetite regulation in the context of the prevention and/or treatment of obesity. Specifically, it is speculated that individuals who show a smaller suppression in perceived appetite and smaller changes in appetite-related blood parameters, such as acylated ghrelin and total PYY, after acute exercise are less likely to show successful results in losing weight through exercise interventions. On the other hand, individuals with greater suppressions in perceived appetite and more expressive changes in acylated ghrelin and total PYY may show more promising results in weight loss interventions including exercise. The stronger appetite suppression after exercising can potentially counteract any increase in appetite resultant of significant weight loss. This hypothesis needs to be tested in future studies and, if proven to be true, the assessment of appetite responses to acute exercise could be used as a predictor for the success of exercise weight loss interventions, which can be easily employed in both research and clinical settings.

Similar applications are possible for dietary interventions. While the study presented in Chapter 6 used a mixed macronutrient meal with high energy content in order to evaluate the effect of the FTO genotype on postprandial meal responses, the same study design and statistical analyses could be employed to test different meal compositions which may elicit similar findings. For example, if true interindividual variability is observed in appetite responses to a high-protein meal, the hypothesis of these acute responses being useful predictors of the success of weight loss interventions through high-protein diets should be tested in future studies. This is valid for any other research areas such as the effect of exercise or meal composition on blood lipids or blood pressure, for example.

The findings presented in Chapters 5 and 6 challenge previous research findings and hypotheses in which FTO genotype was suggested to influence appetite regulation via gut

hormones and highlight the need for additional studies in order to understand the mechanisms involved in the increased obesity risk associated with FTO genotype. Additionally, the study presented in Chapter 5 provides a comprehensive analysis of the association of perceived appetite, appetite-related blood markers and a variety of physiological and lifestyle factors previously indicated to influence appetite regulation, which can be helpful in future research planning. In Chapter 7, a very novel topic was explored in a small pilot study and it is hoped that these preliminary findings can help in designing future studies exploring the association between FTO genotype, brown adipose tissue and appetite regulation.

8.6 Limitations and future directions

Some important limitations have been identified within each experimental chapter of this thesis and some common limitations are highlighted here, together with directions for future research. While the controlled laboratory settings and detailed standardisation protocol followed by all participants preceding laboratory visits in all the studies add credibility to the data presented, it is not known whether these findings reflect what would be observed during individuals' daily lives. Additionally, all studies included healthy young (18 to 50 years old) subjects and the results reported cannot be generalized to other populations such as individuals who are obese, older or present any medical conditions.

The results presented in Chapters 4 and 6 may be restricted to the exact conditions employed in the studies, meaning that different exercise modes, intensities and session durations, as well as different meal compositions could elicit differences in reproducibility and interindividual variability. Of note, exercise was performed after an overnight fast in the study presented in Chapter 4, in order to isolate the effect of exercise *per se* on appetite parameters. However, future studies should investigate whether the findings are similar when exercise is performed in the postprandial state, which may be more representative of the condition in which most people exercise in their daily lives. Therefore, further research is needed to assess the reproducibility and interindividual variability of appetite responses to exercise and meal intake employing different interventions. The need for longer-term intervention studies using a replicated crossover design and appropriate statistical analyses is especially relevant in order to provide valuable evidence on the clinical meaning of the findings reported here, as well as to improve the understanding of the effect of the FTO genotype on appetite and energy intake during and after interventions targeting weight loss and/or prevention of weight gain. It is,

however, acknowledged that the complex logistics, as well as the time and resources needed to plan and perform such studies, represent great challenges.

When the reproducibility and true interindividual variability of responses to an intervention are confirmed, other sources of variability beyond the ones explored in the studies presented in this thesis should be investigated in future studies, such as differences in the gut microbiota and gastric distension, as well as hedonic and psychological factors. Furthermore, the studies presented in Chapters 4 and 6 explored minimal clinically important differences in appetite parameters using a statistical approach as there are no clinically relevant target differences established for appetite parameters. Future studies should aim to determine minimal clinically important differences in appetite parameters that can be applied in research and clinical practice.

The studies presented in Chapters 5 and 7 employed an exploratory approach with cross-sectional design, and therefore, no causation relationship can be implied in the results. Specifically, in Chapter 7, the limitations highlighted by the pilot study should be considered when planning future research. Future studies should first test the efficacy of the brown adipose tissue stimulation protocol within the study sample, as well as determining the reproducibility and interindividual variability of brown adipose tissue measurements, in order to investigate potential factors influencing such variability, if it exists.

The publication of more studies investigating interindividual variability in appetite responses to varied interventions, and potential contributing factors, may stimulate the development of more efficient weight management strategies by determining whether an intervention is likely to be beneficial, ineffective or detrimental for different individuals. Particular attention should be given to the assessment of whether appetite responses to acute interventions, such as a single exercise session or the consumption of a specific meal, can be useful predictors of weight loss interventions. Future studies should first ensure true interindividual variability is present in acute responses to a specific intervention, and then test whether such responses show a clear link with individual results from weight loss interventions. This information will help to identify individuals who may achieve more favourable appetite responses through alternative exercise and/or nutritional interventions.

8.7 Conclusion

The experimental studies presented in this thesis provide new evidence showing generally good reproducibility and the existence of true interindividual variability in perceived appetite and appetite-related blood parameter responses to acute exercise and to a standardised meal in healthy men. Additionally, the results indicate that the effect of the FTO genotype was weak and not statistically significant or worthwhile in influencing fasting and postprandial perceived appetite and appetite-related blood parameters, as well as brown adipose tissue activity. This work contributes to the literature by highlighting, for the first time, true interindividual variability in appetite regulation using gold standard study design and statistical analyses. It is hoped that the evidence presented here helps in directing and developing future research studies aiming to build on existing knowledge by conducting longer-term interventions with larger samples in order to clarify the clinical relevance of the findings.

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APPENDIX A

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

Taking Part	**Please initial box**
The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.	☐
I have read and understood the information sheet and this consent form. I understand that taking part in the project will include being photographed using a thermal imaging camera.	☐
I have had an opportunity to ask questions about my participation.	☐
I understand that I am under no obligation to take part in the study, have the right to withdraw from this study at any stage for any reason, and will not be required to explain my reasons for withdrawing.	☐
I agree to take part in this study.	☐
Use of Information	
I understand that all the personal information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others or for audit by regulatory authorities.	☐
Bodily Samples (please choose one of the options below)	
I agree that the bodily samples taken during this study can be stored until November/2022 for future research in the same research theme as this project.	☐
OR	
I agree that the bodily samples taken during this study can **only be** used for this study and will be disposed of upon completion of the research [June 2018].	☐

_____	_____	_____	
Name of participant	[printed]	Signature	Date
_____	_____	_____	
Researcher	[printed]	Signature	Date

APPENDIX B

Name/Number .....…….

Date of Birth .....…….

BMI .....…….

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness 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 or injury 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/chest pains ……	Yes		No	
(i)	Problems with muscles, 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)	Problems with blood pressure	Yes		No	

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

.....

4. Smoking, physical activity and family history

(a)	Are you a current or recent (within the last six months) smoker?	Yes		No	
(b)	Are you physically active (30 minutes of moderate intensity, physical activity on at least 3 days each week for at least 3 months)?	Yes		No	
(c)	Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?	Yes		No	

5. Allergy Information

(a)	Are you allergic to any food products?	Yes		No	

(b) Are you allergic to any medicines? Yes ☐ No ☐

(c) Are you allergic to plasters? Yes ☐ No ☐

(d) Are you allergic to latex? Yes ☐ No ☐

If YES to any of the above, please provide additional information on the allergy

……

6. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details.

……

7. Have you recently given blood or been involved with research involving blood samples?

Yes ☐ No ☐

If yes, please provide details.

……

8. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name

……

Telephone Number

……

Work ☐ Home ☐ Mobile ☐

Relationship to Participant

……

9. Height: …… cm

10. Weight: …… kg

APPENDIX C

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

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

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → **Skip to question 3**

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → **Skip to question 5**

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → **Skip to question 7**

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

APPENDIX D

Food Preferences

Please circle the number which best describes your liking of the following foods. Focus on how much you like the foods/drinks rather than how frequently you consume them:

White bread

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Brown bread

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Cheddar cheese

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Ham

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Chicken

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Margarine

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Butter

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Vanilla milkshake

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Strawberry milkshake

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Chocolate milkshake

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Milk

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Kit Kat chocolate

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Snickers chocolate

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Strawberry yoghurt

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Chocolate chip muffin

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Double chocolate muffin

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Chocolate chip cookies

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Banana

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Cheese and tomato pizza

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

Pepperoni pizza

(Dislike extremely) 1 2 3 4 5 6 7 8 9 10 (Like extremely)

□ Unsure

APPENDIX E

Three Factor Eating Questionnaire

Part 1: please answer true/false

1. **When I smell a sizzling steak or see a juicy piece of meat, I find it very difficult to keep from eating, even if I have just finished a meal**

   True □ False □

2. **I usually eat too much at social occasions, like parties and picnics**

   True □ False □

3. **I am usually so hungry that I eat more than three times a day**

   True □ False □

4. **When I have eaten my quota of calories, I am usually good about not eating any more**

   True □ False □

5. **Dieting is too hard for me because I just get too hungry**

   True □ False □

6. **I deliberately take small helpings as a means of controlling my weight**

   True □ False □

7. **Sometimes things just taste so good that I keep on eating even when I am no longer hungry**

   True □ False □

8. **Since I am often hungry, I sometimes wish that while I am eating, an expert would tell me that I have had enough or that I can have something more to eat**

   True □ False □

9. **When I am anxious, I find myself eating**

   True □ False □

10. **Life is too short to worry about dieting**

    True □ False □

11. Since my weight goes up and down, I have been on weight reducing diets more than once

True □ False □

12. I often feel so hungry that I just have to eat something

True □ False □

13. When I am with someone who is overeating, I usually overeat too

True □ False □

14. I have a pretty good idea of the number of calories in common food

True □ False □

15. Sometimes when I start eating, I just can't seem to stop

True □ False □

16. It is not difficult for me to leave something on my plate

True □ False □

17. At certain times of the day, I get hungry because I have gotten used to eating then

True □ False □

18. While on a diet, if I eat food that is not allowed, I consciously eat less for a period of time to make up for it

True □ False □

19. Being with someone who is eating often makes me hungry enough to eat also

True □ False □

20. When I feel blue, I often overeat

True □ False □

21. I enjoy eating too much to spoil it by counting calories or watching my weight

True □ False □

22. When I see a real delicacy I often get so hungry that I have to eat it right away

True □ False □

23. I often stop eating when I am not really full as a conscious means of limiting what I eat

True □ False □

24. I get so hungry that my stomach often feels like a bottomless pit

True □ False □

25. My weight has hardly changed at all in the last ten years

True □ False □

26. I am always hungry so it is hard for me to stop eating before I finish all the food on my plate

True □ False □

27. When I feel lonely, I console myself by eating

True □ False □

28. I consciously hold back at meals in order not to gain weight

True □ False □

29. I sometimes get very hungry late in the evening or at night

True □ False □

30. I eat anything I want, anytime I want

True □ False □

31. Without even thinking about it, I take a long time to eat

True □ False □

32. I count calories as a conscious means of controlling my weight

True □ False □

33. I do not eat some foods because they make me fat

True □ False □

34. I am always hungry enough to eat at any time

True □ False □

35. I pay a great deal of attention to changes in my figure

True □ False □

36. While on a diet, if I eat a food that is not allowed, I often then splurge and eat other high calorie foods

True □ False □

Part 2:

37. How often are you dieting in a conscious effort to control your weight?

1 (rarely) 2(sometimes) 3(Usually) 4(always)

38. Would a weight fluctuation of 5 lbs affect the way you live your life?

1(not at all) 2(slightly) 3(moderately) 4(very much)

39. How often do you feel hungry?

1(only at meal times) 2(sometimes between meals) 3(often between meals) 4(almost always)

40. Do your feelings of guilt about overeating help you to control your food intake?

1(never) 2(rarely) 3(often) 4(always)

41. How difficult would it be for you to stop eating half way through dinner and not eat again for four hours?

1(easy) 2(slightly difficult) 3(moderately difficult) 4(very difficult)

42. How conscious are you of what you are eating?

1(not at all) 2(slightly) 3(moderately) 4(extremely)

43. How frequently do you avoid 'stocking up' on tempting foods?

1 (almost never) 2(seldom) 3(usually) 4(almost always)

44. How likely are you to shop for low calorie foods?

1(unlikely) 2(slightly unlikely) 3(moderately likely) 4(very likely)

45. Do you eat sensibly in front of others and splurge alone?

1(never) 2(rarely) 3(often) 4(always)

46. How likely are you to consciously eat slowly in order to cut down on how much you eat?

1(unlikely) 2(slightly likely) 3(moderately likely) 4(very likely)

47. How frequently do you skip desert because you are no longer hungry?

1(almost never) 2(seldom) 3(at least once a week) 4(almost every day)

48. How likely are you to consciously eat less than you want?

1(unlikely) 2(slightly likely) 3(moderately likely) 4(very likely)

49. Do you go on eating binges though you are not hungry?

1(never) 2(rarely) 3(sometimes) 4(at least once a week)

50. On a scale of 0-5, where 0 means no restraint in eating (eating whatever you want, whenever you want it) and 5 means total restraint (constantly limiting food intake and never 'giving in'), what number would you give yourself?

0

Eat whatever you want, whenever you want it

1

Usually eat whatever you want, whenever you want it

2

Often eat whatever you want, whenever you want it

3

Often limit food intake, but often 'give in'

4

Usually limit food intake, rarely 'give in'

5

Constantly limiting food intake, never 'give in'

51. To what extent does this statement describe your eating behaviour? 'I start dieting in the morning, but because of a number of things that happen during the day, by evening I have given up and eat what I want, promising myself to start dieting again tomorrow.'

1(not like me) 2(little like me) 3(pretty good description of me) 4(describes me perfectly)

APPENDIX F

Rating of Perceived Exertion

Rating	Perceived Exertion
6	
7	Very, very light
8	
9	Very light
10	
11	Fairly light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Very, very hard
20	Maximum

APPENDIX G

CONFIDENTIAL

FOOD RECORD DIARY

The most important contribution you can make as a participant in this study is to accurately record your dietary intake and replicate it precisely between trials.

Please record everything you eat and drink the **day before your first main trial**. You will need to consume identical amounts of the same food and drink prior to the following three main trials. Instructions and examples are given inside. **You will be provided with your evening meal the day before and are instructed to eat this from 7:00 pm – 8:00 pm. Please consume only water following the evening meal and arrive to the lab fasted on the morning of the trial.** Please also refrain from any structured physical activity and alcohol consumption the day before each main trial. We understand that some physical activity may not be preventable, and therefore we ask that you record any physical activity undertaken the day before your first main trial and repeat this prior to the following three main trials.

Information will be treated in confidence.

If you have any problems, please contact: Miss Fernanda Reistenbach Goltz: F.Reistenbach-Goltz@lboro.ac.uk

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INSTRUCTION FOR USING THE FOOD DIARY

For solid foods, the food should be placed on the scale on a plate or container. The plate or container must be weighed empty first and the scales can then be zeroed. Each item of food can then be added to the plate and weighed individually, returning the scales to zero between each item.

e.g.	Plate	150g zero scale
	Bread	80g zero scale
	Ham	50g zero scale
	Lettuce	30g zero scale

For drinks, a cup or glass must first be weighed and then the scale can be returned to zero and the drink added. Please remember to record separately the different components of your drink, for example, weigh the squash concentrate, zero the scale, and weigh the water.

Do not forget to weigh and record second helpings and between meal snacks.

Any leftovers (eg. apple cores) should also be weighted and recorded in the leftovers column.

Eating Out – Most people eat foods away from home each day; please do not forget to record these. Take your diary and scales with you where ever it is possible. If this is too inconvenient just record the types of food eaten with an estimated weight, although please say when a weight has been estimated.

Most snack foods will have the weight of the food on the packet so they do no need weighing if you eat the whole packet yourself.

Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

e.g. Cheese – is insufficient information.

Cheese, cheddar (Shape reduced fat) – is sufficient information.

Record each item on a separate line and include the time of day in the first column of each line.

e.g. 10:30 am McVities Digestive Biscuits (2) 50g

The space provided at the foot of each page for general comments is for you to give any further information about your diet and your activity for that day.

e.g. Slow walk to work, morning 10 minutes. Missed lunch due to stomach pains

DAY 1

Date: / /

Please use a separate line for each item eaten; write in weight of plate; leave a line between different 'plate' entries.

A	B		C	D	E	F	Office Use
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned -cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away			(gms)	(gms)	(gms)

GENERAL COMMENTS and ACTIVITY UNDERTAKEN:

APPENDIX H

Visual Analogue Scale

Time:

Please indicate how hungry you are now by circling a relevant number

Not Hungry Fairly Hungry Hungry Very Hungry

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Place a mark on the horizontal lines below after considering the following questions:

How hungry do you feel?

I am not hungry at all — I have never been more hungry

How satisfied do you feel?

I am completely empty — I cannot eat another bite

How full do you feel?

Not at all full — Totally full

How much do you think you can eat?

Nothing at all — A lot

How nauseous do you feel?

Not at all nauseous — Very nauseous

Temperature (°C)	Humidity (%)

APPENDIX I

SAS OnDemand for Academics code used in Chapter 4

SAS PROC MIXED CODES for mixed linear model with participant and the participant-by-condition interaction terms as random effects. This code is based on that reported by Senn et al. (2011) with the addition of a "dummy variable" (XVARE) designed to represent the true interindividual response variance for the exercise condition (adjusted for the within-subjects random variance in the control condition (Senn et al. 2011)). The resulting estimate of the participant-by- condition interaction term consequently approximates to the true individual response SD calculated using the equation reported by Atkinson & Batterham (2015) and Hopkins (2015).

"Loughborough" is the data file. "period" are the coding data for which condition was undertaken in which order (codes of 1-4). "treat" is the condition codes, exercise (1) or control (0). "Subject" are the codes for each participant (1-15). "Score" is the stacked column of data (ghrelin, etc.,). XVARE is coded in the same way as "treat" but added to the model as a random effects covariate.

```
proc mixed data=Loughborough method=reml covtest;
class period treat subject;
model score=period treat XVARE/ddfm=kr solution CL;
random subject subject*XVARE/solution;
parms/nobound;
run.
```

ADJUSTED FOR BASELINE VALUES

```
proc mixed data=Loughborough method=reml covtest;
class period treat subject;
model score=period treat XVARE baseline/ddfm=kr solution CL;
random subject subject*XVARE/solution;
parms/nobound;
run.
```

APPENDIX J

SAS OnDemand for Academics code used in Chapter 6

The following code was used in SAS OnDemand for Academics in order to quantify any participant-by-condition interaction for each study outcome:

```
proc mixed data=Loughborough covtest ic cl alpha=0.05 nobound plots=residualpanel;

class period condition subject;

model Score=period condition period*condition/ddfm=kr outp=pred cl alpha=0.05 cl vciry residual solution;

random subject subject*XVART;

lsmeans Condition Period Condition*Period/diff cl alpha=0.05;

lsmestimate Condition*Period "Meal versus Control" 1 -1 -1 1/ cl alpha=0.05;

run.
```