EFFECTS OF PROTEIN ON GASTROINTESTINAL FUNCTION AND APPETITE REGULATION

A thesis submitted by

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for the degree of

Doctor of Philosophy

Discipline of Medicine

School of Medicine

University of Adelaide

November 2015
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Abstract

The prevalence of obesity and associated diseases, including type-2 diabetes mellitus, continues to increase at an alarming rate. The available therapies have largely ignored the key role of the gastrointestinal tract in determining appetite and blood glucose regulation in responses to ingested nutrients. A detailed understanding of these gastrointestinal mechanisms is critical in aiding development of new and effective interventions for obesity.

The research presented in this thesis focuses on the complex gastrointestinal mechanisms involved in the regulation of glycaemia, appetite and energy intake in response to protein in lean and obese individuals. In particular, this research explores the gastrointestinal motor and hormonal responses to nutrients involved in energy intake regulation and blood glucose control in both healthy lean and obese individuals. Using the novel, non-invasive technique of 3-dimensional ultrasound, the study described in chapter 5 reports that, in lean individuals, the rate of gastric emptying of drinks containing 30g and 70g of protein was comparable (kcal/min; 30g: 2.6±0.2, 70g: 2.9±0.3), and within the ranges previously observed for fat and carbohydrate (1-4 kcal/min). This was reflected by similar releases of cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), glucose-dependent inhibitory polypeptide (GIP), insulin and glucagon, for ~45 min following the drinks. Beyond 45 min, the 70g load resulted in more sustained hormone release, reflecting greater total calories and thus prolonged delivery of nutrient to the small intestine. Energy intake was comparable between the two loads, suggesting that a threshold amount of protein may exist, beyond which no additional appetite-suppressive benefit occurs.

In the studies described in chapters 6-8, intraduodenal infusions, combined with high-resolution manometry, were used to evaluate the effects of nutrients in the small intestine on
antropyloroduodenal motility and gastrointestinal hormone release. Nutrients were infused directly into the duodenum at standardised rates, reflecting the normal range of gastric emptying; intraduodenal infusion bypasses orosensory and gastric influences, isolating the effects of nutrient to the small intestine.

The first of these studies reported that intraduodenal protein has load-dependent effects on antropyloroduodenal motility, ghrelin, CCK, GLP-1, peptide tyrosine tyrosine (PYY), insulin and glucagon, glycaemia, and energy intake at a subsequent meal in lean individuals. The second study reported that load-dependent effects of protein on antropyloroduodenal motility and CCK, GLP-1, GIP, insulin and glucagon release are also apparent in obese individuals, suggesting that small intestinal sensitivity to protein remains intact in obesity. The final study demonstrated, in lean individuals, that intraduodenal lipid modulates gastrointestinal motor responses and CCK and GLP-1 concentrations more potently than an equicaloric protein load. In contrast, protein had more pronounced effects on insulin and glucagon release. Despite these differences, protein and lipid suppressed energy intake comparably, suggesting that different mechanisms may underlie the suppression of energy intake by these nutrients.

These data provide novel insights into the roles that gastrointestinal motor and hormone responses to dietary protein play in the regulation of blood glucose, appetite and energy intake in lean and obese individuals. These observations provide potential mechanistic explanations for the effects of high-protein diets on glycaemic control, and appetite. Importantly, they provide a basis for future development of nutrition-based interventions for the treatment of obesity.
Declaration of Originality

I, Amy Hutchison, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Amy Hutchison

November 2015
Publications Arising From This Thesis

The data presented in this thesis have formed the basis of the publications listed below:


Other Publications


**Dedication**

Ko te manu e kai ana i te miro, nōna te ngahere.
Ko te manu e kai ana i te mātauranga, nōna te ao.

*The bird that partakes of the miro berry reigns in the forest.
The bird that partakes of the power of knowledge has access to the world.*

This thesis is dedicated to
my husband, Rob,
for always inspiring and challenging me,
and my whānau,
for your unwavering love and support.
Acknowledgements

The studies reported in this thesis were performed at the Discipline of Medicine, Royal Adelaide Hospital. While conducting this research, I was financially supported by a Faculty of Health Sciences PhD Scholarship.

First and foremost, I would like to thank my primary supervisor Professor Christine Feinle-Bisset. I am grateful for your continual support, encouragement and the attention to detail that you have instilled into me. To my secondary supervisor Dr Natalie Luscombe-Marsh, thank you for your positivity, perspective, support and friendship. Thank you both for your time and patience in bringing this thesis, and the manuscripts, to fruition, through countless, countless drafts, to-ing and fro-ing, and I am sure a mountain of paper. I am so lucky to have been afforded the opportunities you have both offered me, and the mentorship you have provided.

Special thanks must also go to all of those who have helped me over the years in the conduct of this research, as well as surviving a PhD. Alex Saies and Asimina Kallas, thank you both for all of your help in the earlier studies. Penelope Fitzgerald, thank you for your invaluable help and assistance, especially with the last studies, and for giving up so many of your weekends to assist. Thank you also, along with Briohny Johnston and Caroline Giezenaar, for all of your friendship and laughs, and the coffee breaks. A special thank you to Diana Piscitelli (Dr Di) for the many hours we spent together in your office going over at ultrasound images. I have learned a lot from you, and will be eternally grateful for both the academic, and life, knowledge that I have gained from you.

To all other staff and students in the Discipline of Medicine, thank you for being so supportive throughout my candidature, and for the friendship and welcome distractions you offered. Thank you to Judith Wishart and Scott Standfield for the hormone analysis and Kylie Lange for help with the statistical analysis. A special thank you to all the individuals who volunteered their time to participate in the studies presented in this thesis.

Thank to you all of my friends and family for your love and encouragement, especially those that have helped make our home Adelaide. Finally, the biggest thank you of all must go to Rob, my husband, for your unconditional love, support and encouragement over the years.
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD</td>
<td>Antropyloroduodenal motility</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficients of Variance</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbance assay</td>
</tr>
<tr>
<td>FFM</td>
<td>Fat free mass</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>ID</td>
<td>Intraduodenal</td>
</tr>
<tr>
<td>IPPW</td>
<td>Isolated pyloric pressure wave</td>
</tr>
<tr>
<td>LCD</td>
<td>Low calorie diet</td>
</tr>
<tr>
<td>MI</td>
<td>Motility index</td>
</tr>
<tr>
<td>MMC</td>
<td>Migrating motor complex</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PWs</td>
<td>Pressure waves</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide tyrosine tyrosine</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>TAA</td>
<td>Total Free Amino Acids</td>
</tr>
<tr>
<td>TFEQ</td>
<td>Three Factor Eating Questionnaire</td>
</tr>
<tr>
<td>TMPD</td>
<td>Transmucosal potential difference</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual Analog Scale Questionnaire</td>
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Chapter 1: Thesis Overview

Both in Australia, and worldwide, the prevalence of overweight and obesity, as well as obesity-related diseases, continues to rise. In the most simplistic sense, weight gain results from an imbalance between energy intake and energy expenditure. To date, considerable effort has been invested in developing surgical and pharmaceutical interventions, however, these are associated with numerous side-effects and, particularly in the case of pharmaceuticals, generally have limited long-term efficacy. As such, lifestyle interventions, including caloric restriction, increased physical activity, or a combination of these, remain the cornerstone of obesity treatment. Moreover, the current obesity epidemic, and the difficulty associated with maintaining weight loss, has generated considerable interest in understanding the regulation of eating behaviour in humans.

Maintaining energy balance requires the brain to detect the status of energy stores and to match energy intake with expenditure. The gut and brain are intimately linked, with signals arising from the gastrointestinal (GI) tract conveyed to the brain, and playing a role in the regulation of appetite and energy intake. The brain initiates responses to eating before any food has even been ingested. For example, visual, olfactory and gustatory stimuli initiate the release of endocrine and exocrine signals in the gut, and modulate GI motility, in anticipation of a meal. Following meal ingestion, a cascade of neural and hormonal responses is activated within, and from, the GI system, to impact responses in the central nervous system. These signals determine how nutrients are utilised, where they are stored, and the amount of food we eat at a meal, and subsequent meals.
After swallowing, the arrival of food in the stomach stimulates mechanoreceptors located in the gastric wall on vagal afferent nerve endings, which initiates a coordinated sequence of physiological changes, including gastric relaxation to accommodate the meal, and propulsion further down the GI tract to ensure digestion and absorption. As food moves from the stomach into the small intestine, peristaltic motor activity propels the nutrients along the small intestine, allowing interaction of nutrients with the mucosa to activate chemoreceptors. The latter sense changes in the GI environment, including nutrient composition, osmolarity and pH, and releases GI hormones in response to these stimuli. These hormones activate receptors located on vagal afferents, and signal to the brain via the vagus nerve. The hormonal signals are then integrated by the brain centres that control food intake, most importantly the medulla and arcuate nucleus (ARC) of the hypothalamus. They are then relayed either directly or via nerve fibres that project to other hypothalamic areas, including the paraventricular nuclei and lateral hypothalamic area. Together, the activation of mechanoreceptors and chemoreceptors induce negative feedback to slow gastric emptying, which in turn prolongs gastric distension, and regulates the rate at which nutrients enter the small intestine. The postprandial glycaemic response is also modulated by changes in gastric emptying and GI hormone release, along with the release of insulin and glucagon. In these ways, GI signals play a fundamental role in appetite regulation as well as blood glucose control. Critically, these signals have the potential to be influenced by the characteristics of a food and/or the diet, with the aim of altering energy intake.

The magnitude and pattern of the signals arising from the GI tract is dependent on the type of nutrient ingested. For example, it is well established that fat infused directly into the small intestine has potent effects on GI motility, the release of anorexigenic hormones, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide tyrosine tyrosine (PYY),
and the suppression of the release of ghrelin. Intraduodenal carbohydrate (as glucose) has also been shown to modulate GI motor and hormone function, albeit less potently than lipid. In contrast, ingestion of carbohydrate, or protein, stimulates the release of insulin and glucagon more than fat. However, the GI motor and hormone responses to protein have not been comprehensively characterised.

Dietary protein has been shown to have effects on acute suppression of appetite and energy intake, as well as specific metabolic effects, including regulating blood glucose responses to a meal. Moreover, high-protein diets have been shown to induce weight loss, while maintaining fat-free mass, improving glycaemic control and blood lipids under both calorie-restricted and ad libitum conditions, for up to 2 years. It has been proposed that both the suppression of appetite, and subsequent reductions in energy intake, are the primary mechanism through which high-protein diets exert their superior weight loss effects. However, despite the known role of the GI tract in modulating energy intake, whether GI mechanisms underlie the appetite-suppressive effects of protein, remains to be established.

The research presented in this thesis addresses two overarching aims: i) to characterise the GI motor and hormonal responses to protein involved in the regulation of energy intake in healthy individuals; ii) to establish whether obese individuals are sensitive to the load of protein, and to explore the comparative effects of protein on GI function, blood glucose control, appetite and energy intake in obese, compared with lean individuals. In addition, the data presented in this thesis also considers the relationship between oral ingestion of protein and GI function, including gastric emptying, and the implications of the amount of protein administered both orally and intraduodenally on these outcomes. As the prevalence of obesity worldwide continues to increase, expanding our knowledge of the physiological
factors involved in appetite regulation will contribute to a better understanding of how these mechanisms might be targeted using nutrition-based strategies to promote satiety, and improve the outcomes of treatment in obesity.
Chapter 2: Human Obesity And Strategies To Achieve And Maintain A Healthy Body Weight

2.1 Introduction

The prevalence of overweight and obesity has increased over the past twenty years at an alarming rate. In Australia, self-reported height and weight, collected during the 2007-08 National Nutrition Survey, and using the World Health Organisation (WHO) classifications for body mass index (BMI) (calculated as weight in kg/height in metres$^2$) (Table 2.1), indicated that, of Australians aged over 18 years, 37% were overweight and 25% were obese (Australian Bureau of Statistics 2009).

The current high rates of obesity and their future projections are of considerable relevance. Obesity represents a risk factor for a number of non-communicable diseases, including, but not limited to, metabolic syndrome, non-alcoholic fatty liver disease and type 2 diabetes mellitus (T2DM) (Moayyedi 2008; Colagiuri et al. 2010; National Health and Medical Research Council 2013). In addition, obesity is associated with significant financial costs, and a loss of productivity due to poor health (National Health and Medical Research Council 2013). A recent systematic review reported that, when compared with healthy weight individuals, approximately 70% of obese individuals have at least one established comorbidity, which results in an average increase in medical costs of ~ 30% (Withrow and Alter 2011). Perhaps most important to note is that almost all obesity-related conditions are preventable, and most are at least partially reversible through weight loss. Hence, the cost of obesity can be substantially reduced through lifestyle modifications that promote a healthy weight (National Health and Medical Research Council 2013).
While the maintenance of a healthy body weight is achieved by matching energy intake and energy expenditure, obesity, in the most simplistic sense, occurs when energy intake exceeds energy expenditure, over prolonged periods of time. Perturbations in energy balance, and the resultant weight loss (i.e. negative energy balance) or weight gain (i.e. positive energy balance), are influenced directly, and indirectly, by a number of factors. These factors include intrinsic influences such as genetic programming and extrinsic, socio-environmental influences. While genetic factors may increase an individual’s susceptibility for becoming overweight, current epidemiological trends suggest that obesity primarily results from a combination of extrinsic lifestyle and environmental factors, such as changes in the food supply (i.e. increases in the abundance of palatable foods (Drewnowski and Specter 2004)), increasing portion sizes influenced by culture and social cues, and the rapid decline in physical activity due to the increasing prevalence of energy-saving technology (National Health and Medical Research Council 2013).

Although the precise contribution of each of these inter-related factors to the development of a positive energy balance and weight gain remains unclear, caloric restriction, in combination with an increase in physical activity via a multi-modal exercise program, remains the cornerstone of obesity management (Furst et al. 2005; Valette et al. 2012). In more chronically obese individuals, these interventions may support prescribed pharmacological agents or bariatric surgery to achieve sufficient weight loss. Regardless of the nature of the lifestyle therapy, or combination of therapies employed, maintaining weight lost for more than a few years remains a key issue. Even those who undergo bariatric surgery, which generally induces the largest weight loss, can experience weight regain after up to 10 years. Whilst the mechanisms underlying this weight gain probably involve a complex interplay of intrinsic and extrinsic factors, they have not been fully elucidated.
A key factor that has been implicated in driving weight regain following weight loss is the regulation of appetite and energy intake by signals arising from the GI tract (Sumithran et al. 2011). A large body of evidence exists to suggest that signals in the body, particularly those arising from the GI tract in response to foods that are consumed, play a critical role in determining energy balance. When food is ingested, neural and hormonal signals from the GI tract determine how the nutrients are utilised, where they are stored, the amount of food we eat at a meal, and also at subsequent meals. The development of obesity may, at least in part, reflect a decreased sensitivity to these GI signals, whereby they are insufficient to maintain energy intake at a level appropriate for weight maintenance. Critically, the magnitude of these signals is dependent on the type of nutrient ingested, so that fat, carbohydrate and protein have differential effects on appetite and energy intake. In particular, protein has been shown to have unique effects on suppressing appetite and energy intake, as well as specific metabolic effects, including regulating blood glucose.

Despite the known role of the GI tract in regulating energy intake (reviewed in Chapter 4), and the reported effects of protein on appetite (reviewed in Chapter 3), the GI mechanisms underlying these effects are poorly characterised. Hence, the main aim of this PhD thesis is to understand how nutrients, particularly dietary protein, modulate the GI mechanisms that are known to be involved in the regulation of energy intake, and how these mechanisms may be implicated in the modulation of body weight. This chapter will outline the definition and prevalence of obesity and its associated economic cost and health consequences. It will also highlight the evidence regarding the efficacy of a number of strategies for obesity treatment, including lifestyle modifications, bariatric surgery, and pharmacological interventions.
2.2 Definition, prevalence and health consequences of obesity

2.2.1 Definition of obesity

The WHO defines obesity as “abnormal or excessive fat accumulation that may impair health” (World Health Organisation 2015). BMI remains the most common method for determining overweight and obesity in individuals within the wider population (World Health Organisation 2015). As indicated in Table 2.1, BMI classifications provide an indication of the risk of morbidity and mortality to an individual that result as BMI increases. BMI is also useful as a measure of the risks of overweight and obesity, at a population level, for secondary disease, morbidity and mortality (World Health Organisation 2015).

In addition to BMI, WHO also suggests the use of proxy indicators for health, specifically waist circumference and waist-to-hip ratio, to assess the risk of obesity-related complications within the broader community. These measures account for the relative distribution of excess adipose tissue in the body, and the associated risk for cardiovascular disease, glucose intolerance, hyperinsulinemia, T2DM and other obesity related diseases. They also reduce the likelihood of an individual being misclassified as overweight or obese, for example, athletes with a high proportion of lean muscle mass. It is important to note that these specific BMI classifications apply primarily to Caucasian populations, due to differences in natural body composition between ethnicities. For example, in Asian populations, individuals have been shown to have a tendency for a comparatively higher body fat percentage for a given BMI (Kagawa et al. 2006). In Maori, Polynesian and Torres Strait Islander populations, the opposite is true, in that equivalent levels of risk for disease occur at higher BMIs than in Caucasians (Rush et al. 1997; Swinburn et al. 1999). Conversely, these populations are predisposed to a greater risk of diabetes, despite an equivalent level of body fat, compared with Caucasians (McAuley et al. 2002). Thus, assessment of disease risk for non-Caucasian
ethnicities using these BMI classifications should be interpreted with some caution, since it may not reflect the same body fatness in different individuals (He et al. 2001). Similarly to BMI, thresholds for waist circumference measures in European populations may differ from those of other ethnicities.

Table 2.1: Proxy indicators for health risk in Adult Caucasian populations

<table>
<thead>
<tr>
<th>Classification</th>
<th>Waist circumference (cm)</th>
<th>Waist: Hip ratio</th>
<th>Risk of co-morbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Women</td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18.5</td>
<td>Underweight</td>
<td>&lt;80</td>
<td>&lt;94</td>
</tr>
<tr>
<td>18.5-24.9</td>
<td>Normal range</td>
<td>≥80</td>
<td>≥94</td>
</tr>
<tr>
<td>25-29.9</td>
<td>Overweight (pre-obese)</td>
<td>≥88</td>
<td>≥102</td>
</tr>
<tr>
<td>≥30</td>
<td>Obese</td>
<td>≥88</td>
<td>≥102</td>
</tr>
<tr>
<td>30.0-34.9</td>
<td>Class I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35-39.9</td>
<td>Class II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥40</td>
<td>Class III</td>
<td></td>
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</table>

2.2.2 Prevalence of obesity

In 2014, over 1.9 billion adults were overweight (~39 % of the worldwide population), and of these over 600 million (~13 %) were obese (World Health Organisation 2015). Of particular concern, projections based on previous population-based data from the National Health Survey (Haby et al. 2012) and AusDiab studies (Walls et al. 2012) suggest that, if recent patterns of overweight and obesity rates continue, up to 75 % of Australians could be overweight or obese by the year 2025 (Haby et al. 2012; Walls et al. 2012). Moreover, projections based on international figures suggest that by 2030, up to 58 % of the world’s population could be overweight or obese (Appel et al. 2005). These projections, as well as
global figures, highlight the urgency to develop robust strategies to both halt and reverse the current rates of weight gain, and thereby reduce the enormous social and economic burdens.

2.2.3 Obesity-related risk factors

Excess adiposity associated with obesity is a risk factor for an array of secondary health complications, including sleep apnoea, joint pain and osteoarthritis, hypertension, hyperlipidaemia, hyperinsulinemia, cardiovascular disease, T2DM, metabolic syndrome (characterised by insulin resistance, dyslipidaemia, hypertension and increased waist circumference), gall bladder disease, non-alcoholic fatty liver disease and some cancers (Thompson et al. 1999; Guh et al. 2009). Overweight and obesity may also have implications for psychological health, including secondary eating disorders, body dysmorphia, low self-esteem and depression (Black et al. 1992; Smith et al. 1998; de Zwaan 2001). Generally, the risk for developing these non-communicable diseases increases with increasing BMI (World Health Organisation 2015).

2.3 Strategies for the treatment of obesity

Weight loss is an essential component in the reduction of risk associated with obesity-related co-morbidities. While reducing body weight to a level that places the individual within the normal BMI range (i.e. 18-24.9 kg/m² for adults aged 18-65) is the most desirable outcome, reductions in body weight of as little as 10 % can reduce hypertension (Neter et al. 2003), and improve cholesterol (Wing et al. 2011) glycaemic control (Look Ahead Research Group et al. 2007) and psychological outcomes (Yanovski et al. 1993; Blaine et al. 2007), in overweight and obese individuals. To achieve significant and enduring weight loss, considerable research effort has concentrated on developing a number of interventions, including i) lifestyle changes using varying degrees/ types of caloric restriction, alone or in
combination with increased physical activity; ii) surgical interventions including gastric bypass surgery, gastric banding and sleeve gastrectomy which alter the volume and absorptive capacities of sectors of the GI tract; and iii) pharmacological treatments that enhance metabolism, suppress appetite or inhibit nutrient absorption (or some combination of these), with varying degrees of success (Figure 2.1).

As mentioned earlier, lifestyle changes are the first avenue in treating obesity. Current Australian obesity guidelines suggest that intensive interventions (i.e. Very low calorie diets [VLCD], pharmacological interventions, bariatric surgery) may be used as an adjunct to lifestyle interventions in individuals with a BMI ≥ 30 kg/m², or with a BMI ≥ 27 kg/m² with a co-existing health condition, whom have previously been unsuccessful in reducing weight or mitigating weight gain using lifestyle interventions (National Health and Medical Research Council 2013).

Figure 2.1: Model of energy balance in the treatment of obesity, and effects of therapeutic interventions. Changes in weight are resultant of inducing a negative energy balance (i.e. total energy intake > total energy expenditure). Therapeutic interventions target reducing energy intake (red arrows) and increasing activity (green arrows) through increasing physical activity, the thermic effect of food (TEF) and basal metabolic rate (BMR). Reproduced from Bray (2008).
2.3.1 Lifestyle modifications

Weight loss through lifestyle modification remains the safest and most accessible method in reducing both the co-morbidities of obesity, and excess body weight. While exercise alone may induce a small energy deficit via an increase in fat oxidation, this deficit is often insufficient to maintain weight loss. Instead, it is the sustained negative energy balance through energy restriction that is the most critical factor in inducing weight loss (Jakicic et al. 2001; Layman et al. 2005). Importantly, since physical activity offers additional advantages of maintaining metabolically active lean tissue and improving cardiovascular and metabolic health outcomes, it is widely recommended that individuals use a combination of both caloric restriction and increased physical activity (as both aerobic and resistance exercise) to gain maximum improvements in health from the lifestyle modifications they undertake (Furst et al. 2005; National Health and Medical Research Council 2013).

Weight loss diets

A number of dietary approaches have been advocated for the treatment of obesity, including very low calorie (VLCD), low calorie (LCD), low-fat/ high-carbohydrate, low-carbohydrate and high-protein, diets, with varying degrees of efficacy. For example, VLCDs (i.e. 400-800 kcal/day) produce greater short-term weight loss (i.e. up to 15 kg over 12 weeks) (Mathus-Vliegen and Balance Study 2005), whereas LCDs (800-1200kcal/day) (Johnston et al. 2004; Noakes et al. 2005) produce a comparatively modest weight loss (i.e. up to ~ 7kg over 12 weeks), but are comparatively easier to sustain, for at least 6-12 months (Johnston et al. 2004; Noakes et al. 2005). Moderate energy restriction (i.e. a reduction of 500-1000 kcal per day) has also been shown to induce clinically significant weight loss. Often, weight loss up to, and beyond 12 months is similar between VLCD and more moderate calorie-controlled diets, while almost all weight-loss diets are associated with a weight loss plateau (Curioni and
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Lourenco 2005). Regardless of the degree of caloric restriction, weight loss of between 5 and 10% of initial body weight has been shown to improve fasting plasma glucose and insulin, fasting lipids and blood pressure, independent of physical activity (McLaughlin et al. 2001; Reaven et al. 2001; Luscombe-Marsh et al. 2005; Noakes et al. 2005).

The macronutrient composition of the diet has been manipulated extensively in a number of studies to establish the most effective weight loss diet, which also produces significant changes in health outcomes. Considerable evidence has indicated that higher fat intakes contribute to increased body weight, as well as an increased risk of cardiovascular disease, while diets low in fat and high in complex-carbohydrates were thought to be protective against weight gain (Astrup and Raben 1995). Diets prescribing low-fat and high-carbohydrate intakes have been popular since the 1970’s when fat was first implicated as a factor in increasing body weight and cardiovascular disease. In contrast, the Atkins diet, formulated in 1972, prescribes large amounts of protein and fat in the diet, while restricting carbohydrates to < 20 g per day, based on the notion that hyperinsulinemia and insulin resistance are the primary causes of obesity.

More recently, high-protein diets, with more moderate amounts of fat and carbohydrate have come to the fore in light of the increasing rates of obesity. High-protein diets have been shown, in both normal and overweight populations, to have superior effects in maintaining satiety, suppressing appetite and improving a number of metabolic outcomes. Importantly, there is considerable evidence to suggest that energy-restricted, high-protein diets are an effective nutrient-based therapy for managing appetite, reducing weight and improving glycaemic control in the obese, at least over 6-12 months (Wycherley et al. 2012b). High-protein diets also favour retention of fat-free mass, which contributes considerably to energy
expenditure and metabolism. Finally, high-protein diets have been shown to reduce energy intake, and induce weight loss under *ad libitum* conditions, suggesting that high-protein diets have superior appetite suppressive effects that allow a negative energy balance to be induced and maintained (Weigle *et al.* 2005).

Given the effects of dietary protein on appetite, glycaemia and metabolism, diets with an emphasis on high-protein foods may be advantageous in the treatment of obesity. While the effects of dietary protein and high-protein diets on energy expenditure and metabolism have been widely examined (Barrows and Snook 1987; Westerterp *et al.* 1999; Mikkelsen *et al.* 2000; Luscombe *et al.* 2003b), these mechanisms do not appear to fully explain the effects of high-protein diets on satiation, weight loss and reduction in metabolic risk. In light of this, additional factors, such as GI mechanisms, are likely to underpin the metabolic effects of protein. High protein diets, their effects on metabolic outcomes and use in weight management are reviewed in Chapter 3.

2.3.2 Surgical interventions

Bariatric surgery is arguably the most successful treatment for individuals with severe and complex obesity; however, it is associated with higher risks of both acute and longer-term complications than both pharmacological treatments and lifestyle interventions. Whilst surgical interventions have been shown to have a relatively high long-term success rate (i.e. up to 20% weight loss for 10 years), in Australia bariatric surgery is currently only indicated in the very obese (BMI ≥ 40 kg/m² or ≥ 35 kg/m² with co-morbidities), or in individuals for whom other interventions are contraindicated, or have been unsuccessful (Sjostrom *et al.* 2004; National Health and Medical Research Council 2013). Established surgical procedures
currently utilised for treatment of obesity are Roux-en-Y gastric bypass (RYGB), gastric banding, and sleeve gastrectomy.

**Roux-en-Y gastric bypass**

The RYGB procedure involves creating a small-volume gastric pouch and producing a ~80-100 cm diversion, termed the alimentary limb, for food to bypass the duodenum and upper jejunum (Figure 2.2B), thus diverting nutrients directly to the more distal parts of the small intestine. The biliopancreatic limb (~60-80 cm in length), consists of the resected portion of the stomach, duodenum and jejunum, and joins the alimentary limb to transport bile and pancreatic secretions to the distal small intestine (Simons et al. 1999). RYGB induces weight loss primarily through the diminished size of the gastric pouch, which restricts intake, and the bypassing of nutrients to beyond the proximal small intestine (Elder and Wolfe 2007). In addition, the increased exposure of the distal small intestine to nutrients, resulting from the surgical redirection modulates the release of GI hormones, including CCK, PYY, and GLP-1, as well as leptin from adipose tissue, which ultimately result in reduced energy intake (Rubino et al. 2004; Beckman et al. 2010; Dirksen et al. 2013).

RYGB surgery has been shown to achieve significant long-term weight loss (up to 76 % of excess body weight loss at one year) (Cottam et al. 2006; Rosenthal et al. 2006) and has been shown to resolve type 2 diabetes in up to 85 % of patients almost immediately after surgery, even in the absence of any significant weight loss (Saliba et al. 2009; Jacobsen et al. 2012). This rapid reversal has been attributed to enhanced GLP-1, glucose-dependent insulinotropic peptide (GIP), PYY and insulin responses (Peterli et al. 2009). Risks of RYGB include micronutrient deficiencies, including ferritin, vitamin B$_{12}$, vitamin D and red blood cell folate deficiencies (Toh et al. 2009), gastric pouch enlargement, marginal ulceration, and dilation of
the gastrojejunostomy (Griffith et al. 2012). Of critical importance, RYGB may be complicated in some individuals by dumping syndrome, whereby food enters the small intestine too quickly, and which may cause nausea, cramping and diarrhoea, and presents a risk of inappropriate glycaemic responses in patients with T2DM (Clegg et al. 2002).

Adjustable gastric banding

Adjustable gastric banding (AGB) has been performed since ~1983, and involves the placement of an adjustable silicone cuff around the stomach, approximately 2 cm below the gastro-oesophageal junction at a 55° angle to the midline (Mehanna et al. 2006) (Figure 2.2C). A thin tube connects with an injection port located at the skin, allowing injection of saline for the adjustment of the tightness of the band. AGB modulates a complex interplay of mechanisms to reduce weight, including activation of peripheral satiety signals and is mediated, at least in part, by vagal mechanisms (Dixon et al. 2005).

The placement of the band distal to the gastro-oesophageal junction impacts the types of foods able to be consumed, and elicits behavioural adaptations, such as more restrained eating patterns and consumption of softer, less solid foods. In addition, the location of the band impacts intraluminal pressures of the oesophagus. These intraluminal pressures delay transit of food, particularly semi-solid food, into the portion of the stomach below the band (Burton and Brown 2011). Moreover, since only small portions can cross the AGB, the remaining portion of the bolus is refluxed into the oesophagus, which triggers additional peristalsis. This delayed transit results in a slowed eating pace, as patients must wait for the bolus to clear the AGB before consuming more food (Burton et al. 2010). Despite these changes, AGB has little impact on overall gastric emptying rate, at least in the regions of the stomach below the band (de Jong et al. 2009). Moreover, fasting concentrations of
anorexigenic GI hormones, including PYY and GLP-1, do not appear to be altered by AGB (Korner et al. 2006).

In a randomised, blinded, cross over trial, patients were allocated to have their bands inflated to an optimal level, or to have the fluid partially, or completely removed. When the bands were optimally adjusted, subjects rated satiety higher, and hunger lower, before and after a standardised meal, when compared with sub-optimal band adjustment (Dixon et al. 2005). A recent publication of a 15-year-total follow-up of ~3200 patients who underwent adjustable gastric banding procedures reported that 714 patients completed at least 10 years follow-up, and after 10-years, excess weight lost by those patients averaged 47%.

Complications associated with gastric banding include perforation of the stomach wall, slippage, pouch dilatation, oesophageal dysmotility and reflux, and erosion of the stomach wall by the band (Mehanna et al. 2006). In some patients, insufficient weight loss, or weight regain after band deflation, symptomatic proximal gastric pouch dilatation, intragastric band migration and psychological band intolerance necessitate conversion of the AGB to RYGB (Mognol et al. 2004).

*Sleeve gastrectomy*

Sleeve gastrectomy involves resection of the greater curvature and fundus of the stomach, leaving the remaining stomach fashioned into an elongated tube with limited accommodative space (~60-80 mL) (**Figure 2.2D**). Sleeve gastrectomy offers almost immediate weight loss, maintains GI continuity (i.e. does not bypass regions of the GI tract) thus preventing dumping syndrome, and does not involve implantation of foreign material (i.e. the silicone band as in AGB).
Sleeve gastrectomy employs a combination of both restrictive and neurohormonal mechanisms to induce weight loss. The reduced volume of the post-operative “stomach”, particularly the removal of the accommodative region, the fundus, restricts the amount of food able to be consumed as a single meal (Melissas et al. 2007). Removing the fundus also decreases release of the anorexigenic hormone ghrelin, and thus reduces baseline levels of hunger (Langer et al. 2005; Padwal et al. 2011). Sleeve gastrectomy has also been shown to result in accelerated gastric emptying of both liquid and solid portions of a meal (Melissas et al. 2008), probably as a result of the absence of accommodative relaxation, and altered contractile activity in the proximal stomach (Melissas et al. 2008). This increase in gastric emptying has been hypothesised to result in greater amounts of undigested food reaching the small intestine, stimulating increased release of GLP-1, GIP and PYY postprandially (Ramon et al. 2012).

Long-term weight loss following sleeve gastrectomy is generally between 60 – 85 % of excess weight lost. The sleeve gastrectomy procedure is advantageous in that, similarly to RYGB, glucose homeostasis and type 2 diabetes are promptly corrected in up to 67 % of patients (Gill et al. 2010). It is likely that enhanced GI hormone release, particularly GLP-1 and GIP, underpins these effects (Romero et al. 2012). Importantly, the potent stimulation of GI hormone release and the recovery of normal glucose homeostasis following both sleeve gastrectomy and RYGB highlight the critical importance of GI mechanisms in both blood glucose control and energy balance.

Risks associated with sleeve gastrectomy surgery include possibilities of staple-line leaks or bleeds, abscesses and, in the longer term, stricture (narrowing of the gastric lumen due to scar tissue), gastro-oesophageal reflux and nutritional deficiencies (Sarkhosh et al. 2013). The
procedure is also irreversible since a majority of the stomach is resected, and has a higher operative risk compared to other bariatric procedures (Noah et al. 2013).

**Figure 2.2:** Graphical depiction of commonly used bariatric surgery techniques. (A): Normal GI anatomy; (B): Roux-en-Y Gastric Bypass (RYGB); (C): Adjustable Gastric Banding (AGB); (D): Sleeve Gastrectomy. (adapted from Cummings (2012).
2.3.3 Pharmacological interventions

Pharmacotherapies represent a third avenue of intervention for overweight and obesity, and may be used in conjunction with lifestyle therapies, and surgery, to enhance weight loss outcomes. To correct the imbalance of energy intake with energy expenditure associated with weight gain, pharmacotherapies have been developed that increase energy expenditure, reduce energy intake, or target both aspects of the energy balance equation. Typically, energy expenditure in an individual remains relatively stable from day to day, while energy intake can vary greatly due to social, behavioural and hedonic factors. Previously utilised drugs targeting increased energy expenditure include thyroid hormone, amphetamines, and dinitrophenol to stimulate basal metabolic rate and reduce body weight. These therapies are no longer approved for use in treating obesity, due to a considerable number of side effects, including addiction (Adan 2013). Accordingly, targeting mechanisms regulating appetite and energy intake remains the primary role of current pharmacotherapies.

In general, pharmacotherapies, taken over a period of 1–2 years, have demonstrated a significant, albeit modest (<5 kg), decrease in weight, when compared with placebo, with attrition rates of up to 43 % in some clinical trials (Padwal et al. 2004). In the majority of cases, weight loss following pharmacological intervention is not sustained once the drug is withdrawn, with many individuals regaining some, if not all plus more, of the weight that was lost. Weight loss drugs are classified by their effects, including fat absorption inhibitors and appetite suppressants with, and without, stimulant effects on the central nervous system. Currently, the only drugs approved for the treatment of obesity in Australia are phentermine (Duromine™, Metermine™), a central nervous system stimulant, and Orlistat (Xenical®), a fat absorption inhibitor. Off-label usage of drugs that have known secondary effects on
weight loss is also becoming increasingly popular as a mechanism for inducing weight loss where diet and lifestyle interventions alone have failed.

**Fat absorption inhibitors**

Orlistat acts to reduce fat digestion and absorption within the small intestine, by inactivating gastric and pancreatic lipases, thereby inhibiting hydrolysis and absorption of dietary triacylglycerols by around 30% (Hauptman *et al.* 1992; Jandacek and Woods 2004). Orlistat is approved for longer-term treatment of obesity, and has been shown, in a meta-analysis of 11 placebo-controlled 12-month trials, to reduce weight by around 3% in ~6000 patients (Padwal *et al.* 2004). Whilst this weight loss is relatively modest, Orlistat has also been shown to have beneficial effects on blood pressure, cholesterol and blood glucose in type 2 diabetics (Padwal and Majumdar 2007). Compliance of patients with the regimen can be limited due to adverse GI effects (i.e. diarrhoea, flatulence, bloating, oily stools, abdominal pain, and dyspepsia), which are greater following high-fat foods, due to the amount of unabsorbed fat passing through the GI tract, and being excreted from the body (Jandacek and Woods 2004). Prolonged use can also result in deficiency in the fat soluble vitamins A, D, E and K.

**Appetite suppressants**

Over the last 40 years, a vast number of anorexiant have been developed for obesity treatment, which target various neurotransmitters and receptors, including dopamine, serotonin and noradrenalin (Haddock *et al.* 2002). Such medications include phentermine, sibutramine (Reductil®, Meridia®), a monoamine reuptake inhibitor primarily affecting noradrenaline, dopamine and serotonin, rimonabant (Acomplia®), an endocannabinoid receptor inhibitor, and lorcaserin (Belviq®), a selective serotonin receptor inhibitor.
Phentermine is currently the only available prescription appetite suppressant in Australia. Phentermine is a sympathomimetic amine that stimulates increased hypothalamic release of norepinephrine, with no detectable effect on serotonin (Rothman and Baumann 2009). Phentermine has been shown to have favourable effects on lipid profiles (i.e. lowering of total cholesterol and low-density lipoprotein [LDL-] cholesterol) and glycaemic control, thereby reducing the risk of diabetes (Kim et al. 2006). Phentermine is also associated with a number of side effects including palpitations, tachycardia, increased blood pressure, tremor, excessive sweating, anxiety, irritability, restlessness, dizziness, insomnia, euphoria, dysphoria, headache, dryness of the mouth and GI complaints (i.e. diarrhoea and constipation) (Snow et al. 2005; Kang et al. 2010). Phentermine is also being studied in combination with other drugs to treat obesity, and was approved by the Food and Drug Authority (FDA) as a combination drug (Qsymia®) with topiramate (an anti-epileptic) in the United States in 2012.

A number of previously approved weight loss drugs have been withdrawn from use in many countries, due to their limited efficacy (i.e. a placebo-corrected weight loss of <4.0 kg) and considerable associated adverse effects. For example, ‘Fen-Phen’, a combination of the drugs fenfluramine and phentermine was shown to induce an average placebo-corrected weight loss of ~10 kg over 32 weeks (~300 g/week weight loss) when used in conjunction with lifestyle modifications (Weintraub et al. 1992). The drug combination was withdrawn from the market in 1997, after it was reported to cause valvular heart disease and pulmonary hypertension (Connolly et al. 1997). Phenylpropanolamine (Acutrim®), a potent selective adrenaline, noradrenaline and dopamine mimetic, was found to be associated with an increased risk of haemorrhagic stroke in women (Kernan et al. 2000), and is no longer available as a weight loss treatment; however it is present in small doses in some cough medicines. Rimonabant, which suppresses appetite by inhibiting cannabinoid receptors, and
also increases thermogenesis, was withdrawn from the market after it was associated with depression and suicidal ideation (Padwal and Majumdar 2007). More recently, sibutramine, a centrally acting serotonin-norepinephrine and dopamine reuptake inhibitor, which induced weight loss of ~ 5 % per year, was withdrawn from the market due to an increased risk of cardiovascular events and strokes (James et al. 2010).

**Pharmacological treatments with secondary effects on weight loss**

There is considerable evidence to suggest that the mechanisms underlying food intake are tightly linked to those that also regulate mood (McElroy et al. 2004). For example, as serotonin is highly involved in the regulation of energy intake, and since many anti-psychotics target the serotonin system, it is conceivable that these drugs may have secondary effects on appetite and body weight (Jandacek and Woods 2004). In addition, drugs normally prescribed to treat neurological or mental disorders (i.e. epilepsy or depression), or T2DM have been noted for their effects on appetite or energy intake, and are increasingly being prescribed “off-label” to specifically achieve weight loss. Such drugs, including topiramate (an anti-epileptic), tesofensin and fluoxetine (anti-depressant medications) are used either as monotherapies, or in combination with, for example, phentermine, to suppress appetite (Sjodin et al. 2010; Allison et al. 2012). In the case of drugs targeting the treatment of T2DM, GLP-1 agonists, such as liraglutide and exenatide, dipeptidyl peptidase IV (DPP-IV, an enzyme that deactivates GLP-1) inhibitors, and metformin appear to have secondary effects on weight loss. Liraglutide (3.0 mg) has recently been approved for use in the USA, specifically for weight loss. Long-term efficacy of these drugs as a monotherapy for obesity, rather than diabetes, requires further substantiation.
2.4 Concluding remarks

The available evidence suggests that current therapies used for obesity vary in their efficacy, side effects and associated risks. Bariatric surgery represents an effective intervention to induce significant weight loss that is mostly sustained in the long term. Moreover, the changes in GI hormone release, and subsequent improvements in glycaemic control following RYGB and sleeve gastrectomy highlight the importance of GI neural and hormonal signals in determining energy intake. Unfortunately, bariatric surgery is only indicated in individuals with a high BMI, where previous lifestyle interventions have failed, and is associated with a number of risks. Pharmacological interventions are also able to induce modest weight loss; however the options for these are limited, and are also associated with a number of undesirable side effects. Conversely, lifestyle modifications have no qualification criteria, and in most individuals (excluding the very obese) present minimal risk. Hence, they remain the first avenue of treatment for obesity. In particular, dietary modification has beneficial effects for weight loss, glycaemic control and plasma lipids, however the magnitude of these improvements is dependent on the macronutrient profile of the diet. Some evidence suggests protein in the diet may offer metabolic advantages, as well as suppressing energy intake. High-protein diets, the importance of protein in the diet, physiological advantages of protein and the mechanisms underlying these, and the optimal amounts (i.e. grams) and percentage of energy intake of protein are reviewed in Chapter 3.
Chapter 3: Diets High In Protein As A Strategy For The Management of Obesity

3.1 Introduction

In light of the increasing prevalence of obesity, and the costs and risks associated with bariatric surgery and pharmaceutical treatments, much attention has fallen on dietary interventions to improve both body composition and metabolic health. While the most important factor in inducing weight loss is a negative energy balance, current literature underscores the controversy regarding the optimal macronutrient composition of weight loss diets, and whether an emphasis on protein, fat or carbohydrate in the diet is most effective in both reducing body weight and improving health (Gardner et al. 2007; Sacks et al. 2009). For example, for many years, low-fat diets with a moderate protein, and high carbohydrate (i.e. < 20 % fat, 20 % protein, > 60 % carbohydrate) content were advocated to promote weight loss, through lowered energy density, and increased fibre in the diet. Later, diets with low (i.e. < 100 g/day), or very low (i.e. < 20g/day), carbohydrate, and moderate to high (i.e. up to 40 %) fat and protein contents became of interest.

The WHO recommends a protein intake of 10-15 % of total daily energy, or a minimum of 0.83 g/kg body weight for healthy adults in energy balance (Joint WHO FAO UNU Expert Consultation 2007). Considerable evidence, however, suggests that the optimal intake for health indicators, including body composition, and maintaining satiety, may be in the range of 0.8 -1.6 g/kg body weight (Layman et al. 2003; Layman et al. 2009), while a protein intake of up to 2 g/kg body weight per day may be consumed without adverse effects (Poortmans and Dellarieux 2000; Bilsborough and Mann 2006). A large body of evidence suggests that increasing the protein, and reducing the carbohydrate, content of a meal, can reduce hunger
(Weigle et al. 2005; Veldhorst et al. 2008) and energy intake (Weigle et al. 2005), and improve plasma lipid profiles (Brinkworth et al. 2004a), postprandial glycaemic control (Farnsworth et al. 2003; Layman 2003), insulin resistance (Brinkworth et al. 2004a) and promote thermogenesis and energy expenditure (Lasker et al. 2008). In addition, increased protein in the diet may reduce the catabolism of fat-free mass and blunt the decrease in resting energy expenditure that usually occur during weight loss (Krieger et al. 2006; Leidy et al. 2007). It has been proposed that both the acute, and long-term, suppression of appetite, and subsequent reductions in energy intake, are the primary mechanism through which high-protein diets exert their superior weight loss effects (Weigle et al. 2005; Paddon-Jones et al. 2008). This chapter will discuss the effects of protein, consumed either as a single meal, or as part of a diet over a period of days to weeks, on appetite, energy intake and weight loss, as well as the metabolic advantages of an increased amount of protein in the diet. Potential risks and adverse effects of higher-protein diets will also be discussed.

### 3.2 Satiety and appetite responses to protein

Human eating behaviour is complex, and influenced by a number of factors. Typically, humans eat in episodes, i.e., distinct meals and snacks (de Graaf et al. 2004). The drive to eat determines individual eating episodes, and is influenced by, for example, environmental cues (i.e. time of day), sensory hedonic, emotional (including boredom) and social cues (de Graaf et al. 2004). The ingestion of a meal initiates the “satiety cascade”, which describes the physiological processes involved in modulating food intake (Figure 3.1). The satiety cascade is characterised by two distinct phases, satiation, and satiety, which are mediated by sensory, cognitive, post-ingestive and post-absorptive factors (Blundell et al. 1996). With respect to a meal, humans tend to eat until they are comfortably full (termed satiation), upon which the meal is usually terminated (de Graaf et al. 2004). The degree of satiation is determined by
the measured meal size (as volume, weight or caloric value of the meal). Immediately after a meal, the drive to eat is at its lowest, and satiation at its highest. Satiety refers to the inhibition of hunger and appetite between meals, and determines both the length of time between meals (inter-meal interval) and meal frequency. Both satiation and satiety, and the reciprocal suppression of appetite are modulated and maintained by sensory, cognitive, pre-ingestive (i.e. hormonal), and post-absorptive (i.e. the presence of absorbed nutrient in the blood) processes, as described in the “satiety cascade” (Figure 3.1) (Blundell et al. 1993).

![Figure 3.1: Satiety cascade (recreated from Blundell, 1996).](image)

The ingestion of a meal generates absorptive signals from the GI tract. Pre-absorptive signals are generated as the nutrient is ingested and digested. With respect to protein, pre-absorptive signals include the release of GI hormones (CCK, GLP-1, GIP, PYY, ghrelin, neuropeptide Y), as well as regulatory peptides implicated in energy balance (leptin, orexin, insulin), and these signals reach the brain via vagal afferent signalling. Collectively, these vagal signals

### 3.2.1 Pre-absorptive satiety signals in response to protein

The ingestion of a meal generates absorptive signals from the GI tract. Pre-absorptive signals are generated as the nutrient is ingested and digested. With respect to protein, pre-absorptive signals include the release of GI hormones (CCK, GLP-1, GIP, PYY, ghrelin, neuropeptide Y), as well as regulatory peptides implicated in energy balance (leptin, orexin, insulin), and these signals reach the brain via vagal afferent signalling. Collectively, these vagal signals
converge on the brainstem, where afferent vagal fibres terminate. In addition, ghrelin and PYY also directly stimulate the ARC of the hypothalamus and the area postrema of the brainstem, where the blood-brain barrier is incomplete (Journel et al. 2012). The hypothalamus plays a role in regulating appetite and food intake; the area postrema, tractus solitarius and ARC are involved in the regulation of satiety, while the mesolimbic reward system, including the nucleus accumbens, which is also stimulated by the presence of protein-digestive products in the bloodstream, appears to decrease the motivation to eat (Tome et al. 2009).

3.2.2 Post–absorptive satiety signals in response to protein

During the absorptive phase of digestion, individual amino acids are sensed in the small intestine and hepatoporal regions, and have been shown to have both excitatory and inhibitory effects on hepatic vagal afferent fibres (Chaudhari et al. 2000; Uneyama et al. 2006). Non-vagal, post-absorptive signals may also play a role in regulating the responses to dietary protein, since an intact vagal nerve is not required for protein to elicit effects on food intake (L’Heureux-Bouron et al. 2003). In rodents, elevations of plasma amino acid concentrations, as a result of protein ingestion, are directly detected by the ARC and the area postrema, and PYY-detecting neurons may play a critical role in initiating these effects (Tome et al. 2009).

In humans, as early as 1956, Melinkoff et al observed that a rise in serum amino acid concentrations correlated with a reduction in appetite (Mellinkoff et al. 1956). Melinkoff termed this the “aminostatic hypothesis” and postulated that a centre in the brain may sense changes in amino acid concentrations in the blood, and that a threshold concentration of amino acids signalled the termination of eating (Mellinkoff et al. 1956). Following this,
ingestion of protein has been shown to alter plasma amino acid concentrations (Fernstrom et al. 1979), and when mixed amino acids were consumed as a preload 30 min prior to a meal, food intake was reduced by ~ 22 % (Butler et al. 1981). Whether these effects are due solely to direct stimulation of specific areas of the brain by various amino acids is unclear, and it is likely that both direct stimulation of regions of the brain by plasma amino acids, and indirect vagal afferent signals generated from GI hormone release are implicated (Tome 2004). Additional post-absorptive mechanisms, including increased thermogenesis (Tome 2004), glucose signalling and homeostasis, via amino-acid induced gluconeogenesis, may also influence the satiating effects of protein (Tome 2004; Potier et al. 2009). The maintenance of satiety through these mechanisms is thought to enhance the effects of both energy-restricted diets for weight loss, and the maintenance of ideal body composition following weight loss. While it is known that both pre-, peri- and post-absorptive satiety signals play a role in determining the appetite responses to nutrient, the contributions of these signals to the superior satiating effects of protein remain unclear.

3.2.3 Protein-specific appetite – the protein leverage hypothesis

There is a large body of evidence to show that a number of animal species, including rodents, non-human primates, pigs, birds, fish and insects, will self-select diets to maintain protein intake within an optimal physiological range (Anderson et al. 1990). The protein leverage hypothesis, postulated by Simpson and Raubenheimer (2005), has suggested an integral role of “protein-specific appetite” in determining energy intake, whereby a species will aim to consume a target amount of protein within the diet. When protein sources are limited, they may increase total energy intake beyond that required, to ensure this protein threshold is reached. This protein-specific appetite is thought to exist to ensure adequate protein is consumed within the diet to meet physiological demand (Gosby et al. 2014).
In humans, only a handful of studies to date have directly tested the protein leverage hypothesis (Gosby et al. 2011; Griffioen-Roose et al. 2012; Martens et al. 2013a; Martens et al. 2013b; Martens et al. 2014). Gosby et al, using a 4-day dietary manipulation, showed that subjects consuming a low-protein (10% total daily energy [TDE]) as compared to a normal-protein (15% TDE) diet, increased energy intake by around 12% (Gosby et al. 2011). However, this increase in energy intake was insufficient to maintain protein intake at a constant. Griffioen-Roose et al reported that subjects consuming a low-protein diet (0.5g/kg body weight/day) for 14 days, followed by 2.5 days of ad libitum feeding, increased their protein intake by ~13% during the ad libitum feeding period, however, total energy intake remained unchanged (Griffioen-Roose et al. 2012).

In contrast, in two separate studies, Martens et al reported that individuals under-ate relative to energy balance, following a diet containing 30% protein, compared with diets containing normal (15%) and low (5%) amounts of protein respectively, however, in response to the low-protein diet they did not find evidence for a protein leveraging effect on energy intake (Martens et al. 2013a; Martens et al. 2013b). Collectively, while current examinations of the protein leverage hypothesis reinforce the effects of dietary protein on regulating appetite, no single study has provided evidence supporting protein leverage in both directions, and hence it remains unclear whether protein intake is regulated in humans for the purposes of protein balance.

### 3.3 Effects of high-protein meals on appetite and subsequent food intake

A macronutrient satiating hierarchy is well established. Laboratory studies utilising pure nutrient infusions have demonstrated that, kJ for kJ, regardless of the route of administration (i.e. oral vs. intravenous), protein is more satiating than fat, and fat more satiating than carbohydrate, at least in rodents (Walls and Koopmans 1992). A small number of human
studies that have simultaneously compared the effects of the three macronutrients on appetite and energy intake have also confirmed this hierarchy. For example, when compared with carbohydrate or fat preloads, a protein preload elicited greater subjective “satiety” ratings and resulted in fewer kJ being consumed ad libitum over the rest of the day (Rolls et al. 1988; Poppitt et al. 1998). This section will review the evidence for effects of high-protein meals on appetite and energy intake, as well as the importance of protein source, and quantity in a meal, for effects on satiety.

### 3.3.1 Effects of high-protein meals on appetite and energy intake

Protein exhibits a dose-dependent effect on satiety, across a range of concentrations offered acutely to weight-stable subjects in a single meal (Hill 1986; Barkeling et al. 1990; Westerterp-Plantenga et al. 2006; Smeets et al. 2008; Veldhorst et al. 2008) as well as an increased satiety over 24 hours (Westerterp-Plantenga et al. 1999; Westerterp-Plantenga et al. 2004; Lejeune et al. 2006). An overview of the results from a number of studies assessing the acute effects of high-protein meals on appetite energy intake is presented in Table 3.1.

In acute studies, mixed-nutrient meals containing up to 68 % protein have been shown to induce greater postprandial satiety than meals containing moderate amounts of protein (19 %) as quantified using visual analogue scales (VAS) (Stubbs et al. 1996; Crovetti et al. 1998), and to reduce the amount of energy consumed at a subsequent ad libitum meal (Porrini et al. 1995; Poppitt et al. 1998). For example, Latner and Schwartz (1999) reported that, following isocaloric (450 kcal), sensory-matched (taste, texture) liquid meals that were either high-protein (71.5 %), high-carbohydrate (99 %), or a balanced-protein/carbohydrate meal (50 % / 50 %), subjects consumed less energy at an ad libitum dinner following the high-protein and mixed-nutrient meals. In both lean and obese subjects, Batterham et al reported that a high-
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Protein mixed-nutrient meal (~65 % protein, 17 % carbohydrate, 17 % fat) suppressed hunger more than a high-fat (65 % fat, 17 % protein, 17 % carbohydrate) or high-carbohydrate (65 % carbohydrate, 17 % protein, 17 % fat) meal (Batterham et al. 2006). While all of these studies report potent effects on appetite in response to protein, the meals used contained very high amounts of protein, which are not sustainable to consume longer term, and over multiple meals.

Using protein loads that were more representative of the average protein load for a meal, a number of researchers also report potent effects on appetite and energy intake (Porrini et al. 1997; Veldhorst et al. 2009c; Veldhorst et al. 2009b; Brennan et al. 2012; Douglas et al. 2013; Ortinau et al. 2014). For example, Brennan et al administered a pasta, mince and tomato sauce meal with a vanilla yoghurt dessert, covertly manipulated to alter the macronutrient content, to lean and obese males. The meals were high- (45 %) or adequate-protein (30 %), high-carbohydrate/low-protein (10 % protein, 60 % carbohydrate), or high-fat (55 % fat, 15 % protein) (Brennan et al. 2012). The authors reported that, in lean subjects, the high-protein and high-fat meals reduced subsequent energy intake compared with the adequate protein meal by ~ 14 % and 9 % respectively. In obese participants, the high-protein meal reduced energy intake compared with the high-carbohydrate/low-protein (-23 %) and high-fat meals (-18 %), while the adequate protein meal also reduced energy intake compared with the high-carbohydrate meal (-10 %) (Figure 3.2) (Brennan et al. 2012).
Figure 3.2: Energy intake (kJ) in lean and obese individuals following high-protein (HP, % energy from protein/fat/carbohydrate/ 45/25/30), adequate-protein (AP, 30/30/40) high-carbohydrate/low-protein (HC/LP 10/30/60) or high-fat (HF 15/55/30) test meals. Lean: * P<0.05 vs. HC/LP, ^ P<0.05 vs. AP; Obese: * P<0.05 vs. HC/LP, # P<0.05 vs HF (Brennan et al. 2012).

In contrast, a number of researchers have found no significant differences in the effects of high-protein versus high-carbohydrate or high-fat meals on satiety and energy intake (Rolls et al. 1988; Johnson and Vickers 1993; Raben et al. 2003). For example, Raben et al gave 20 lean, healthy subjects isocaloric meals (595 kcal for females, 714 kcal for males) that were high in either protein (crisp bread with cheese, yogurt with muesli, boiled egg, and skim milk; 31.8 % protein, 37.2 % carbohydrate, 31.1 % fat), carbohydrate (corn flakes with skim milk, white bread with butter, cheese, jam, and honey; 12.2 % protein, 65.4 % carbohydrate, 23.7 % fat), fat (yogurt mixed with double cream and grated apple, honeydew melon, rye bread with butter, cream cheese, and whole milk; 11.6 % protein, 23.9 % carbohydrate, 64.6 % fat), or alcohol (rye and whole-grain bread with butter and cheese, yogurt with muesli, honeydew melon, and orange juice with vodka; 12.1 % protein, 42.9 % carbohydrate, 24.3 % fat, 23 % alcohol), and reported no significant differences in hunger, satiety or fullness or energy intake between the macronutrient enriched meals (Raben et al. 2003). Of considerable importance, it should be noted that the foods used in the above described studies were whole foods, which
varied greatly in taste, texture and appearance, and thus it is not clear to what extent the type of food, and associated hedonic influences, or the macronutrient content of the food, affected satiety and subsequent energy intake.

Overall, the collective evidence presented here suggests that meals containing ≥ 25 % protein may modulate appetite sensations and decrease subsequent energy intake, in both lean and obese individuals. More recent evidence suggests that the absolute amount of protein (i.e. grams), may be more important than the proportion (i.e. %) of a meal, which may explain why some groups, but not others, reported effects of protein-containing meals on appetite and subsequent energy intake. In addition, differences in methodology, for example the use of whole foods versus liquid meals, the palatability of the test meals offered, the sources of protein administered, the timing of subsequent meals, the study population (i.e. lean vs. obese, mixed or single gender) and often small sample sizes make it difficult to determine the optimum percentage contribution, or dosage of protein, to induce a significant effect on satiety and energy intake at a later meal. The potential importance of the amount of protein is discussed in the following section.

3.3.2 The importance of protein quantity and timing in appetite regulation

Recent data have highlighted the importance of ensuring an absolute quantity of protein is consumed at each individual meal during the day, not only as a critical factor for protection of lean tissues during weight loss, but to induce satiety and suppress appetite (Schoeller and Buchholz 2005; Weigle et al. 2005). To stimulate muscle protein synthesis, adults probably require a minimum of 15 g of essential amino acids, and a total of 30 g of protein in a single meal (Rasmussen et al. 2000; Paddon-Jones and Rasmussen 2009). Based on 7-day food diary data of ~870 individuals, De Castro et al reported that current dietary patterns generally result in protein intakes of ~10 g protein at the breakfast meal, ~20 g at the lunch meal, and
~60 g at the evening meal. With respect to appetite control, in this pattern, the ingestion of the majority of daily protein intake at the evening meal, i.e. before sleep, limits the satiating benefits of the meal (de Castro 2004). In contrast, spreading the total daily intake of protein evenly across the day, may promote more stable appetite control. Thus, consuming ~30 g of protein at each meal may improve the satiety response to that meal, and, in particular, consuming this amount at the breakfast meal, when the body is in a catabolic state after an overnight fast, may have the greatest impact on total daily energy intake (Rolls et al. 1988; Layman 2009). This information has specific implications for the short-term (between-meal) regulation of appetite, as well as energy intake at subsequent meals.

There is also some evidence, albeit limited, that there is a ceiling effect for protein, whereby additional protein consumption beyond a threshold does not further suppress energy intake. For example, Belza et al have observed that *ad libitum* energy intake was not significantly different in response to equicaloric normal (24 g), medium-high (44 g), and high- (80 g) protein mixed-meals, despite protein-load-dependent suppression of hunger and prospective consumption, and stimulation of satiety and fullness (Belza et al. 2013). In addition, Akhavan et al reported that 20 g, but not 10 g, of whey protein, when consumed 30 min before a buffet meal, resulted in suppression of energy intake compared with a non-nutritive control. However, they also found no additional suppression of energy intake following 30 g, as compared with 20 g (Akhavan et al. 2010). While the data are limited, the above studies suggest that a protein ceiling may exist, and while increased amounts of protein may suppress appetite, approximately 30 g of protein appears to represent the maximum threshold amount of protein to significantly suppress energy intake, beyond which additional protein intake has no further effects. This requires further investigation.
By comparison, little is known regarding the minimum amount of protein required to elicit effects on appetite and energy intake. For example, Douglas et al report that 160 kcal of Greek yoghurt containing 24 g of protein increased fullness, and reduced hunger and delayed the request of dinner compared with a low (5 g) or moderate (14 g) protein yoghurt snack, or no snack at all (Douglas et al. 2013). Interestingly however, they report that the energy content of the snacks was not fully compensated for at an ad libitum dinner. In overweight women, Poppitt et al showed that a 500 mL liquid drink containing 1, 2 or 4 % w/w whey protein decreased hunger, and suppressed it for longer compared with a water control (Poppitt et al. 2011). Moreover, fullness and satisfaction were increased in response to the 2 and 4 % drinks. All of the protein drinks reduced energy intake at a subsequent ad libitum meal in a dose-dependent manner, however the energy content of all of the drinks was not entirely compensated for. This suggests that, while modest amounts of protein may alter short term satiety, the effects are insufficient to induce a significant reduction in energy intake at a subsequent meal. Evidence for the minimum protein dose however is scant, and further research is required.

3.3.3 Effects of protein source on appetite

Within the diet, there is a wide variety of foods that provide protein, and different sources of protein have different effects on satiety. For example, almost all animal-derived proteins have been shown to maintain post-absorptive satiety for longer than plant-derived proteins (Mikkelsen et al. 2000; Anderson et al. 2004). In particular, whey protein (a derivative of milk) has been shown to suppress short-term energy intake, increase subjective satiety and stimulate satiety signalling mechanisms more than other protein sources, including casein (Hall et al. 2003), egg albumin (Anderson et al. 2004) soy protein (Anderson et al. 2004), tuna (Pal and Ellis 2010) or turkey (Pal and Ellis 2010). Furthermore, whey proteins have
been shown to have a faster rate of digestion and absorption than other sources of protein, eliciting a rapid peak in plasma amino acid concentrations, which may contribute to their satiating effect (Boirie et al. 1997; Hall et al. 2003). Current evidence also suggests that increases in plasma amino acid concentrations, although not reflective of the amino acid composition of the food consumed, are associated with decreases in appetite (Mikkelsen et al. 2000; Anderson et al. 2004; Veldhorst et al. 2008). Moreover, the differences in magnitude of satiety are associated with the amino acid profile of the food source (Luhovyy et al. 2007; Veldhorst et al. 2008). Overall, studies investigating the effects of protein source on appetite and energy intake have generally reported whey to be more potent than other proteins. In light of this evidence, the effects of intraduodenal and oral protein in lean and obese individuals was examined using whey protein as the protein source for all studies described in this thesis.
<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Meal type, energy content</th>
<th>Test meal composition</th>
<th>Comparative test meals composition</th>
<th>Time to next meal</th>
<th>Satiety outcomes</th>
<th>Subsequent energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batterham, 2006</td>
<td>19 males (10 normal weight, 9 obese)</td>
<td>Pasta (normal vs high-protein), tomato sauce, dessert 1088 ± 2 kcal</td>
<td>65 % P, 17 % C, 17 % F</td>
<td>HC: 65 % C, 17 % P, 17 % F</td>
<td>Not measured</td>
<td>Lean: HP significantly reduced hunger</td>
<td>Not measured</td>
</tr>
<tr>
<td>Belza, 2013</td>
<td>25 males (13 normal weight, 12 overweight and obese [BMI 25.2-37 kg/m²])</td>
<td>Pork rice and mushroom pate; covertly manipulated 720 kcal + 300 mL water</td>
<td>50 % P, 30 % F, 20 % C</td>
<td>MHP: 25 % P, 30 % F, 45 % C</td>
<td>4 h</td>
<td>Significant dose dependent increases in</td>
<td>NP: 997 ± 77 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>88.4 g</td>
<td>NP: 24 g</td>
<td></td>
<td>satiety and fullness</td>
<td>MHP: 986 ± 72 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HP: 830 ± 79 kcal</td>
</tr>
<tr>
<td>Brennan, 2012</td>
<td>32 males (16 normal weight, 16 obese [BMI 30-35.6 kg/m²])</td>
<td>Pasta, mince and tomato sauce, vanilla yoghurt; covertly manipulated; ~10 % total daily energy requirements (TDE)</td>
<td>45 % P, 25 % F, 30 % C</td>
<td>AP: 30 % P, 30 % F, 40 % C</td>
<td>3 hours</td>
<td>Lean: HP significantly reduced hunger</td>
<td>Lean: HP: 926 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.35 g/kg bw</td>
<td>HC/LP: 10 % P, 30 % F, 60 % C</td>
<td></td>
<td>and increased fullness</td>
<td>HF: 989 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HF: 15 % P, 55 % F, 30 % C</td>
<td></td>
<td></td>
<td>AP: 1079 kcal</td>
</tr>
<tr>
<td>Crovetti, 1998</td>
<td>10 normal weight women</td>
<td>Isocaloric (557 ± 9 kcal)</td>
<td>68 % P, 19 % F, 13 % C</td>
<td>HC: 10 % P, 21 % F, 69 % C</td>
<td>7 hours</td>
<td>HP significantly increased satiety</td>
<td>HP: 1192 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>97 g</td>
<td>HF: 9 % P, 70 % F, 21 % C</td>
<td></td>
<td></td>
<td>HC: 1130 kcal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HC: 14 g</td>
<td></td>
<td></td>
<td>HF: 1043 kcal (NS)</td>
</tr>
</tbody>
</table>
### Table 3.1 continued: studies examining acute effects of high-protein meals on appetite and energy intake

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Meal type, energy content</th>
<th>Test meal composition</th>
<th>Comparative test meals</th>
<th>Time to next meal</th>
<th>Satiety outcomes</th>
<th>Subsequent energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnson, 1993</td>
<td>14 (8 females, 6 males) BMI 18.5-27.1 kg/m²</td>
<td>150 and 300 kcal preloads</td>
<td>72 % P, 18 % F, 0 % C</td>
<td>HC: 12 % P, 3 % F, 86 % C</td>
<td>90 min</td>
<td>HP significantly increased satiety</td>
<td>150 kcal: HP 753 kcal, HF 897 kcal, HC 877 kcal P&lt;0.05 vs HF, HC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HP: chicken</td>
<td>300 kcal preload: 57 g</td>
<td>HF: 2 % P, 89 % F, 10 % C</td>
<td></td>
<td></td>
<td>300 kcal: HP 641 kcal, HF 814 kcal, HC 674 kcal P&lt;0.05 vs HF, HC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC: pasta, tomato sauce, onion, herbs</td>
<td>150 kcal preload: 28.5 g</td>
<td></td>
<td></td>
<td></td>
<td>150 kcal: HP 753 kcal, HF 897 kcal, HC 877 kcal P&lt;0.05 vs HF, HC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF: whipped cream, blueberries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150 kcal: HP 641 kcal, HF 814 kcal, HC 674 kcal P&lt;0.05 vs HF, HC</td>
</tr>
<tr>
<td>Latner, 1999</td>
<td>12 females; BMI 19-29 kg/m²</td>
<td>Isocaloric (450 kcal) sensory-matched liquid preloads</td>
<td>71 % P, 19 % F, 10 % C</td>
<td>HC: 99% C; Mixed: 35.7 % P, 9.6 % F, 55.1 % C</td>
<td>4.5 hours</td>
<td>HP significantly reduced hunger</td>
<td>HP: 943 kcal, HC: 1239 kcal Mixed:1034 kcal P&lt;0.05 vs HC</td>
</tr>
<tr>
<td>Ortinau, 2014</td>
<td>12 normal weight females</td>
<td>Isocaloric (160kcal) HP: yoghurt, HF(1): crackers, HF(2): chocolate</td>
<td>36 % P, 0 % F, 6 % C</td>
<td>HF(1): 0 % P, 32 % F, 68 % C; HF(2): 6.6 % P, 30.1 % F, 63.3 % C</td>
<td></td>
<td></td>
<td>HP: HF(1): HF(2): P&lt;0.05 vs HF(2) P=0.08 VS HF(1)</td>
</tr>
<tr>
<td>Poppitt, 1998</td>
<td>12 normal weight females</td>
<td>Isocaloric (476 kcal); Fish and potato pie, gin and tonic flavoured drink, covertly manipulated, sensory-matched</td>
<td>60 % P, 20 % F, 20 % C</td>
<td>HC: 12 % P, 20 % F, 68 % C</td>
<td>1.5 hours</td>
<td>HP significantly increased satiety</td>
<td>HP: 523 kcal, HC 596 kcal HF 607 kcal HA 660 kcal P&lt;0.05 vs. HC, HF, HA</td>
</tr>
</tbody>
</table>
Table 3.1 continued: studies examining acute effects of high-protein meals on appetite and energy intake

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Meal type, energy content</th>
<th>Test meal</th>
<th>Comparative test meals</th>
<th>Time to next meal</th>
<th>Satiety outcomes</th>
<th>Subsequent energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porrini, 1997</td>
<td>14 normal weight males</td>
<td>Omelette, covertly manipulated, <strong>HP</strong>: 273.5 kcal <strong>HF</strong>: 284.3 kcal</td>
<td>54 % P, 45 % F, 1% C</td>
<td>15 % P, 79 % F, 6 % C</td>
<td>10.5 g</td>
<td>2 hours</td>
<td>HP 1385 kcal <strong>HF</strong>: 1596 kcal (NS)</td>
</tr>
<tr>
<td>Porrini, 1995</td>
<td>12 normal weight males</td>
<td><strong>HC</strong>: Baked macaroni (960 kcal), <strong>HP</strong>: Meatballs in sauce (880 kcal)</td>
<td>56 % P, 25 % F, 19 % C</td>
<td>17 % P, 27 % F, 56 % C</td>
<td>41 g</td>
<td>2 hours</td>
<td>HP significantly increased satiety <strong>HC</strong>: 808 kcal <strong>P&lt;0.01 vs HC</strong></td>
</tr>
<tr>
<td>Potier, 2010</td>
<td>56 normal weight (12 men, 44 women)</td>
<td>Isocaloric hot chocolate drink (245 kcal), covertly manipulated</td>
<td>50 g protein</td>
<td><strong>HC</strong>: 50 g maltodextrin <strong>HF</strong>: 22.3 g oil</td>
<td>50 g</td>
<td>1 hour</td>
<td>No differences in satiety <strong>HC</strong>: 590 kcal <strong>HF</strong>: 609 kcal <strong>P&lt;0.05 HC vs. HF</strong></td>
</tr>
<tr>
<td>Raben, 2003</td>
<td>19 normal weight (9 women, 10 men)</td>
<td>Unmatched food components (described in text); isocaloric (595 kcal for females; 714 kcal for males)</td>
<td>32 % P, 31 % F, 37 % C</td>
<td><strong>HC</strong>: 12 % P, 24 % F, 64 % C; <strong>HF</strong>: 12 % P, 64 % F, 24 % C; <strong>HA</strong>: 12 % P, 24 % F, 43 % C, 23 % Al.</td>
<td>Not reported</td>
<td>5 hours</td>
<td>No differences in energy intake (actual figures not reported)</td>
</tr>
</tbody>
</table>
### Table 3.1 continued: studies examining acute effects of high-protein meals on appetite and energy intake

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Meal type, energy content</th>
<th>Test meal</th>
<th>Comparative test meals</th>
<th>Time to next meal</th>
<th>Satiety outcomes</th>
<th>Subsequent energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolls, 1988</td>
<td>10 normal weight females</td>
<td>300 kcal&lt;br&gt;HP: chicken&lt;br&gt;HC (starch): pasta&lt;br&gt;HF: cream cheese&lt;br&gt;HC (sucrose): Turkish delight&lt;br&gt;HC/HF: chocolate</td>
<td>74 % P&lt;br&gt;26 % F&lt;br&gt;0 % C</td>
<td>HC(st): 9 % P,&lt;br&gt;8 % F,&lt;br&gt;70 % C&lt;br&gt;HF: 3 % P,&lt;br&gt;97 % F,&lt;br&gt;0 % C&lt;br&gt;HC(su): 2 % P,&lt;br&gt;0 % F,&lt;br&gt;91 % C&lt;br&gt;HC/HF: 5 % P,&lt;br&gt;8 % F,&lt;br&gt;60 % C</td>
<td>2 hours</td>
<td>HP significantly increased fullness and decreased hunger</td>
<td>P &lt; 0.05 HP and HC(st) vs. all (actual intakes not reported)</td>
</tr>
<tr>
<td>Smeets, 2008</td>
<td>30 (19 women, 11 men) BMI 20-30 kg/m²</td>
<td>Pasta, sausages, tomato sauce, covertly manipulated; ~15 % TDE</td>
<td>Not reported&lt;br&gt;25 % P&lt;br&gt;30 % F&lt;br&gt;45 % C</td>
<td>Not reported&lt;br&gt;10 % P,&lt;br&gt;30 % F,&lt;br&gt;60 % C</td>
<td>4.5 hours</td>
<td>HP significantly increased satiety</td>
<td>Not measured</td>
</tr>
<tr>
<td>Veldhorst, 2009a</td>
<td>25 normal weight subjects</td>
<td>Custard, 20 % TDE (602 ± 17 kcal)</td>
<td>25 % P&lt;br&gt;55 % C&lt;br&gt;20 % F</td>
<td>10 % P,&lt;br&gt;35 % F,&lt;br&gt;55 % C</td>
<td>38 g</td>
<td>LP increased satiety</td>
<td>NS (actual intakes not reported)</td>
</tr>
<tr>
<td>Veldhorst, 2009b</td>
<td>25 normal weight subjects</td>
<td>Custard, 20 % TDE (602 ± 17 kcal)</td>
<td>25 % P&lt;br&gt;55 % C&lt;br&gt;20 % F</td>
<td>10 % P,&lt;br&gt;35 % F,&lt;br&gt;55 % C</td>
<td>38 g</td>
<td>HP increased fullness and satiety</td>
<td>NS</td>
</tr>
</tbody>
</table>

Al, alcohol; BW, body weight; C carbohydrate; F, fat; HA, high alcohol; HC, high carbohydrate; HC(st), high carbohydrate (starch); HC(su), high carbohydrate (sucrose); HF, high fat; HP, high protein; MHP, moderate-high protein; NP, normal protein; P, protein.
3.4 Longer-term effects of high-protein diets on appetite, food intake and body weight

In addition to the acute, meal-induced effects of protein on appetite and energy intake, chronic consumption of a diet higher in protein, under both energy-restricted and *ad libitum* conditions has been shown to have advantageous effects on the suppression of appetite and subsequent energy intake, as well as weight loss, retention of fat free mass, and improvements in body composition. The following sections will review the evidence for the effects of both *ad libitum*, and energy-restricted, high-protein diets.

3.4.1 Effects of high-protein diets on appetite and food intake

In the context of energy balance, a diet with a protein intake of 10-15 % of total daily energy intake is considered a standard- or adequate-protein diet, while > 15 % is considered a high-protein diet. High-protein diets with ≥ 30 % total daily energy intake from protein, when compared with a normal-protein diet, and administered for durations ranging between 24 hours and 5 days, have been shown to result in consistently higher satiety ratings throughout the day (Westerterp-Plantenga *et al.* 1999; Lejeune *et al.* 2006; Westerterp-Plantenga *et al.* 2009a). For example, in the controlled environment of a respiration chamber, healthy, lean women in energy balance were allocated to either a high-protein diet (30 % protein, 40 % carbohydrate, 30 % fat) or adequate-protein diet (10 % protein, 60 % carbohydrate, 30 % fat) over a period of 4 days. Throughout the day, satiety and fullness were rated higher, hunger and appetite rated lower, and energy intake was reduced with the high-protein diet (Westerterp-Plantenga *et al.* 1999). Similar effects (using a diet with macronutrient compositions identical to those used in women) have also been reported in lean men (Westerterp-Plantenga *et al.* 2009a). In a similar respiration chamber study, lean women fed either a high-protein (30 %), or adequate protein (10 %)/high carbohydrate, fat matched (30...
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%), diet over 4 days, reported an increased 24-hour satiety, and decreased hunger and energy intake, over the duration of the study period (Lejeune et al. 2006). In what is considered to be a landmark trial, Weigle et al explored the effects of both an isocaloric, and ad libitum, high-protein diet on weight loss, appetite and energy intake over a 4 month period. Subjects consumed, sequentially, a weight-maintaining, adequate-protein diet (15 % protein, 35 % fat and 50 % carbohydrate) for 2 weeks, followed by 2 weeks of an isoenergetic high-protein diet (30 % protein, 20 % fat, 50 % carbohydrate). They then consumed an ad libitum diet with 30 % protein, 20 % fat, 50 % carbohydrate for 12 weeks. Satiety was markedly increased with the isocaloric high-protein diet, while energy intake was spontaneously reduced by ~ 441 kcal on the ad libitum phase of the diet (Weigle et al. 2005). Together, these results suggest that increasing the protein content of the diet may have potent anorectic effects, and can result in spontaneous reductions in energy intake.

3.4.2 Effects of high-protein ad-libitum, and energy restricted, diets on weight loss and changes in body composition

In energy-restricted diets, where the overall macronutrient balance is manipulated, it is not uncommon for the absolute protein content (i.e. as grams of protein) to remain the same, but for the percentage contribution of protein content to reach up to 40 % of total daily energy intake, as fat and carbohydrate are generally the macronutrients that are reduced to induce a negative energy balance.

3.4.2.1 Effects of high-protein ad-libitum diets

Longer-term, high-protein, ad libitum trials are limited, however, in the trial by Weigle et al, subjects lost an average of 4.9 kg at the end of the 16 weeks, as a result of reduced hunger and sustained feelings of fullness, and decreased energy intake (Figure 3.4) (Weigle et al.
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2005). Skov et al randomised 60 free living overweight men and women to either a high-protein (25 % total intake) or moderate-protein (12 % total intake) ad libitum diet for 6 months. Subjects in the high-protein group lost considerably more weight (8.8 kg vs. 5.1 kg) and fat (7.6 kg vs 4.3 kg) compared with the low protein diet, and consumed, on average, \( \sim 450 \) kcal less per day (Skov et al. 1999b). In an extension of this study, the authors followed up the participants for an additional 6 months, with less stringent intervention. While there were no differences between the high-protein and moderate-protein groups for weight loss (6.2 kg vs. 4.3 kg) the high-protein group had a 10% greater reduction in intra-abdominal adipose tissue (Due et al. 2004).

In the large scale, multi-centre DIOGENES trial, the role of protein intake in weight maintenance after weight loss has also been examined (Aller et al. 2014). After an 8 week LCD weight loss phase, subjects were randomised to one of five weight maintenance diets, i) low-protein, low-GI (LP/LGI), ii) low-protein, high-GI (LP/HGI), iii) high-protein, high-GI (HP/HGI), iv) high-protein, low-GI (HP/LGI) or v) a diet according to healthy eating guidelines. Subjects consuming a high-protein diet regained significantly less weight, and had more favourable changes in body composition, including a lower proportion of weight regain as fat mass and a higher proportion as fat-free mass (Aller et al. 2014). These data also confirm previous observations that weight maintenance after weight loss is greater with higher-protein ad libitum diets (Westerterp-Plantenga et al. 2004; Lejeune et al. 2005).
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Figure 3.3: Mean total daily energy intake (○) and body weight (●) for 19 healthy subjects plotted against day of study. Subjects undertook a 2 week run-in diet comprised of 15% protein, 35% fat and 50% carbohydrate, followed by a 2 week isocaloric diet where energy from protein was increased from 15 to 30%, and fat decreased to 20%. Finally, the subjects were admitted to a 12 week ad libitum phase, where the composition of the diet remained the same, but subjects were instructed to consume only as much of the diet as they wished, and foods were supplied to provide 15% more energy than required for weight maintenance. The bars represent SEs (Weigle et al. 2005).

3.4.2.2 Effects of high-protein energy-restricted diets

During diet-induced weight loss, loss of fat free mass (FFM) typically accounts for approximately 20% of total weight loss (i.e. 200 g /1 kg) (Krieger et al. 2006). FFM, particularly skeletal muscle, is a major contributor to the basal metabolic rate (BMR) and resting energy expenditure (REE), and is integral in protein metabolism. In overweight and obese individuals trying to lose weight, the loss of FFM as a result of acute energy restriction may down-regulate a number of metabolic processes, including protein turnover and
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The extent to which these processes are diminished is dependent on the degree of energy restriction, and result in an increase in whole-body proteolysis, amino acid oxidation and nitrogen excretion (Pasiakos et al. 2010). This effect decreases and plateaus as the body adapts to conserve energy and protein stores (Stein et al. 1991; Pasiakos et al. 2010).

Thus, in the context of weight loss, protein appears to be an integral component of an energy-restricted diet to offset the demand for metabolic substrates, maintain energy expenditure through the retention of FFM, and optimise protein utilisation (Pasiakos et al. 2010). There is an abundance of studies to suggest that high-protein diets, with an intake above the recommended intake of 0.8 g/kg/d may offer the advantage of increased body fat mass loss, and retention of FFM, when compared with a moderate- or low-protein diet (Skov et al. 1999b; Farnsworth et al. 2003; Layman et al. 2003; Krieger et al. 2006; Leidy et al. 2007; Wycherley et al. 2012a; Wycherley et al. 2012b; Soenen et al. 2013). For example, in the 52 week study by Wycherley et al (2012), subjects on a high-protein diet (31-33 % protein) lost approximately 1.5 kg more body weight than those on a standard-protein (20-21 % protein) diet. In addition, the subjects on the high-protein diet lost more body fat (-27 % vs. -22 %), and less lean mass (-4 % vs. -6 %), than those on the standard protein diet (Wycherley et al. 2012a). A recent meta-analysis, which selected studies with energy-matched high- and standard-protein arms, conducted for up to 21 weeks, reported a significant additional weight loss of 0.79 kg following a high-protein, compared with low-protein diet (Wycherley et al. 2012b).
In a meta-regression analysis by Krieger et al, the authors reported that the degree of FFM retention during weight loss through energy restriction tended to increase with successive quartiles of protein intake. In studies of less than 12 weeks duration, high-protein diets (>1.05g/kg/d) were associated with retention of an additional 0.6 kg FFM, while in studies >12 weeks, this was increased to retention of an additional 1.21 kg (Krieger et al. 2006). Taken together, these studies suggest that dietary protein, over and above the RDA of 0.8g/kg/d may exert a protective effect on the retention of FFM during energy restriction, and that this protective effect is generally increased with increased amounts of protein in the diet. Studies examining the effects of high-protein diets on fat loss, and total weight loss, under hypoenergetic, isoenergetic and ad libitum conditions are reviewed in Table 3.2.

In contrast, a number of studies have reported no difference in weight loss between high-protein and adequate or low-protein diets (Piatti et al. 1994; Luscombe et al. 2002; Farnsworth et al. 2003; Layman et al. 2003) (Table 3.2). A recent review of diets manipulating the amount of protein has put forward two alternate explanations for the discrepancies of the success of diets, in the theories of protein spread and protein change (Bosse and Dixon 2012). In their protein spread theory, Bosse et al proposed that, to be classed as “successful”, there must be sufficient spread, or difference, in g/kg/day of protein intake between “high-protein” intervention groups and controls. Specifically, a high-protein diet should have 58.4 % more protein than a control diet to observe significant changes in body composition (i.e. loss of fat mass and maintenance or gain of lean mass) and anthropometry (i.e. weight loss), compared to ~39 % spread in “unsuccessful” studies. For example, in the study by Brehm et al (Table 3.2) protein intake in the high-protein diet (1.19 g/kg/d) was ~74 % greater than that of the standard protein diet (0.88 g/kg/d), and resulted in significantly greater fat and total weight loss in response to the high protein diet. In contrast,
the study by Larsen et al had a spread of ~16% between the high- and standard-protein diets (1.32 g/kg/d, 1.11 g/kg/d respectively), and fat and total weight loss did not differ between the two diets.

In addition, the protein change theory postulates that for a study to be “successful” there must be an increase in protein intake, as g/kg/day, from baseline to intervention of ~29% (i.e. from 1 g/kg/d to >1.3 g/kg/d), compared with the ~5% increase shown in unsuccessful studies. Of importance to note, is that, in the “unsuccessful” studies included in this review (i.e. those that did not observe changes in anthropometry), groups allocated to the high-protein diet did consume more protein than control; however during the intervention period they actually consumed less protein than their habitual intake, which may explain the lack of any observable change. Further research is required to establish whether there are thresholds for protein change and spread, and how baseline protein levels may affect outcomes of dietary interventions.
### Table 3.2: Effects of high-protein diets on weight loss and body composition

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Duration</th>
<th>Diet</th>
<th>Baseline diet</th>
<th>Study diet</th>
<th>Mean weight loss (kg) ± SEM</th>
<th>Mean fat loss (kg) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baba, 1999</td>
<td>OB, HI</td>
<td>4 weeks, ER (80% of REE)</td>
<td>HP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brehm, 2005</td>
<td>OB</td>
<td>16 weeks, Ad libitum diet vs. SP ER (25% ER</td>
<td>HP</td>
<td>9069</td>
<td>1.00</td>
<td>16</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HP (25% ER)</td>
<td>9110</td>
<td>0.88</td>
<td>14</td>
<td>51</td>
</tr>
<tr>
<td>Claessens, 2009</td>
<td>OB</td>
<td>17-18 weeks</td>
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Table 3.2 continued: Effects of high-protein diets on weight loss and body composition

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ER, energy restricted; HI, hyperinsulinemic; HP, high-protein; NR, not reported; OB, obese; OW, overweight; PHF, high-protein high-fat; SP, standard protein, WM, weight maintenance; Values are means ± SEM (unless otherwise stated). *weight regain during weight maintenance period; **diet recorded at end of 52 weeks, values are means ± SD. *Italicised data indicate prescribed diet (where no actual diet data was available);^^ HP diet supplemented with 50 g whey protein, SP with 50g maltodextrin.
3.5 Metabolic effects of dietary protein

Dietary protein also has positive effects on physiological mechanisms that modulate body weight, glycaemic control and energy expenditure (particularly diet-induced thermogenesis) which may also affect satiety. The effects of high-protein diets on these outcomes, and the mechanisms underlying them, are discussed in the following sections.

3.5.1 Effects of protein on glycaemic control

Dietary protein exerts unique effects on blood glucose profile both acutely, and improves glycaemic control over the long term. Acutely, protein stimulates the release of both insulin and glucagon, without concomitant changes in peripheral blood glucose; however the mechanisms underlying this phenomenon are complex. Originally, it was hypothesised that the stimulation of insulin by protein would induce cellular uptake of glucose, thereby reducing circulating levels of glucose following a meal. However, protein also stimulates glucagon, which promotes an increase in glycogenolysis and gluconeogenesis to offset the effects of insulin. In addition, augmented secretion of GI peptides, in particular the incretin hormones GLP-1 and GIP, as well as the slowing of gastric emptying, play a role in the glycaemic response to protein (Lan-Pidhainy and Wolever 2010). Whey protein is also unique in that it has potent effects on inhibiting the peptidase dipeptidyl peptidase IV (DPP-IV). DPP-IV hydrolyses incretin hormones to their inactive forms, which results in elevated blood glucose (Tulipano et al. 2011a). Whey has been shown to stimulate GLP-1 release and to inhibit DPP-IV in both rodents and humans, resulting in an increased and prolonged insulin response (Drucker 2006b; Gunnarsson et al. 2006; Tulipano et al. 2011a). A detailed discussion of GLP-1 and GIP will be provided in Chapter 4.
In both healthy and diabetic subjects, protein has been shown to induce a potent insulin response, which may be dependent on the insulin sensitivity of the individual. For example, in healthy individuals, Brand-Miller et al. reported that, following ingestion of a lean beef steak providing ~75g protein, insulinaemia was induced, while blood glucose remained relatively stable (Brand-Miller et al. 2000). Krezowski et al. have shown that in healthy subjects the insulin response to 50 g of protein (beef) was modest compared with 50 g of glucose, while co-ingestion of protein and glucose had an additive effect on insulin release (Krezowski et al. 1986). In contrast, the same group has shown that in type 2 diabetics, a 50 g load of animal protein produced an equivalent insulin response, with no increase in blood glucose, compared with a 50 g load of glucose. When co-ingested, protein and glucose had a synergistic effect on insulin release, and actually reduced the blood glucose response (Nuttall et al. 1984). The synergistic effect of protein when co-ingested with 50 g of carbohydrate has also been replicated in type II diabetics in response to 25 g of cottage cheese, turkey, gelatine, fish and soy, but not egg white (Gannon et al. 1988). In contrast, others have reported no attenuation of the hypoglycaemic effects of protein in insulin resistant, non-diabetic subjects (Lan-Pidhainy and Wolever 2010), however these differences may be attributed to the sources of protein (i.e. soy vs. lean beef vs. whey protein), and the degree of insulin resistance in different study populations.

Over the longer term (i.e. up to 12 months), high-protein diets also appear to have beneficial effects on glycaemic control. A number of studies have reported favourable changes in markers of glycaemia, including blood glucose and glycated haemoglobin (HbA1c) levels (as a measure of long term blood glucose control), oral glucose tolerance and insulin sensitivity (Piatti et al. 1994; McAuley et al. 2005). For example, a study by McAuley et al. (described in Table 3.2), reported that after 8 weeks on a high-protein or high-protein/high-
Dietary protein and weight loss

fat diet, insulin sensitivity was improved compared with a high-carbohydrate diet in overweight, insulin resistant women (McAuley et al. 2005). Piatti et al also reported that, in obese, glucose tolerant women, 3 weeks on a very low calorie (800kcal/d) high-protein diet, resulted in improved glucose disposal and oxidation (measured during a euglycaemic clamp) compared with a high-carbohydrate diet (Piatti et al. 1994). In elderly T2DM populations, the addition of essential amino acids to the diet has also been shown to improve metabolic control, including lowering fasting blood glucose and serum insulin levels, and lowering levels of HbA1c, presumably as a result of the insulinotropic effect of the amino acids (Solerte et al. 2004; Solerte et al. 2008). Collectively, this evidence suggests that increased protein in the diet, both acutely (i.e. in meals) and in the long term has beneficial effects on glycaemia, and can improve glycaemic control in both insulin resistant and type 2 diabetics.

3.5.2 Effects of protein on energy expenditure

Total daily energy expenditure (TDEE) comprises of three main processes: basal metabolic rate (i.e. the energy cost of maintaining bodily function at rest (BMR), active metabolic rate (i.e. the energy expenditure resulting from daily activity (AMR) and diet-induced thermogenesis (DIT). DIT is the increase in energy expenditure above baseline following consumption of food, and is associated with digestion, absorption, storage and disposal of the nutrients ingested (Halton and Hu 2004). Overall, DIT is responsible for ~10 % of total daily energy expenditure in humans; however this varies dependent on the macronutrient ingested (Westerterp 2004). For example, DIT from protein results in an energy cost of 25-30 %, compared with carbohydrate, which has been reported to be between 5 and 15 % of energy consumed (Westerterp et al. 1999). DIT from fat remains under contention, with some studies reporting a lower effect compared to carbohydrate (i.e. 2-3 % of energy consumed) (Swaminathan et al. 1985), while others report no difference (Nair et al. 1983).
A considerable amount of literature exists for the effects of protein, in both very short-term (i.e. 1-10 hours) and short-term (i.e. 12-36 hours) settings on DIT. For example, in subjects fed isocaloric meals high in fat, carbohydrate or protein, DIT has been shown to be higher after 120 min (Swaminathan et al. 1985) and up to 7 hours (Crovetti et al. 1998) following the high-protein meals compared with the high-carbohydrate and high-fat meals. The DIT that results from increased protein in the diet may increase TDEE, while the fall in TDEE that is normally observed with weight loss can be blunted by the DIT of protein (Baba et al. 1999; Luscombe-Marsh et al. 2005; Lejeune et al. 2006).

Importantly, the higher DIT of protein has also been associated with the satiating capacity of protein. In a 36-h study conducted in a respiratory chamber, women fed in energy balance had a higher DIT (14.6 ±2.9 %) with a high-protein diet (29 % protein, 61 % carbohydrate, 10 % fat), compared with DIT (10.5 ±3.8 %) with a high-fat diet (9 % protein, 30 % carbohydrate, 61 % fat). The high-protein diet was also associated with higher satiety both during meals, and across a 24 hour period (Westerterp-Plantenga et al. 1999). While it is important to recognise the effects of protein on inducing energy expenditure, and the relationship between DIT and satiety, the thermic effect of protein alone is not sufficient to promote significant weight loss and improvement in obesity outcomes, without additional dietary and lifestyle changes.

### 3.6 Risks and adverse effects of high-protein diets

Despite the considerable evidence for high-protein diets in promoting positive changes in body composition, appetite regulation and weight loss, a number of undesirable side effects and metabolic risks have also been reported. For example, a high protein intake, particularly from red meat, has been associated with high total and saturated fat intakes. In addition some
epidemiological studies have shown associations between high intakes of protein, namely animal proteins, with increased risks of osteoporosis and renal disease, while other studies report no association or a negative association; hence, there remains substantial controversy surrounding such associations.

Dietary protein and bone

While there is a consensus that moderate levels of protein intake (i.e. 1.0 – 1.5 g/kg/d) do not adversely affect calcium levels and skeletal metabolism (Spencer et al. 1983; Heaney 2000; Dawson-Hughes and Harris 2002; Kerstetter et al. 2003), some data have suggested that high-protein diets, especially with a large proportion of animal proteins, may induce a low-grade metabolic-acidosis, resulting in the degradation of bone to neutralise this effect (Sebastian et al. 1994; Frassetto et al. 1998), which in turn may increase renal calcium excretion (Hegsted and Linkswiler 1981; Kerstetter et al. 1997). However, using stable dual-isotopes for calcium, a number of studies have identified that the observed rise in urinary calcium is concomitant with increased intestinal calcium absorption (Cao et al. 2011). That is, the increased appearance of calcium in the urine does not necessarily indicate increased resorption from bone, but likely reflects increased turnover from several sources.

In their review, Eisenstein et al reported that dietary protein appears to have a calciuretic effect at loads of 2 g/kg/d, when compared with control diets of 0.7-1.0 g/kg/d (Eisenstein et al. 2002). A recent meta-analysis has confirmed that there are beneficial effects, albeit small, of both animal and vegetable proteins on bone health (Darling et al. 2009). Moreover, there is considerable epidemiological evidence that suggests that higher total, and animal, protein intakes are associated with increased bone mineral density, and lowered risk of osteoporosis and fractures in both normal subjects, and those supplemented with calcium and vitamin D
Dietary protein and weight loss

Chapter 3

(Hannan et al. 2000; Dawson-Hughes and Harris 2002). Protein intakes of up to 1.5 g/kg/d appear to maintain calcium homeostasis (Spencer et al. 1983; Heaney 2000; Dawson-Hughes and Harris 2002; Kerstetter et al. 2003), while protein intakes of up to 2.0 g/kg/d do not appear to have deleterious effects on bone. Conversely, low to moderate protein diets appear to have adverse effects on calcium metabolism and skeletal health, and studies have now identified impaired intestinal calcium absorption as a factor in inducing secondary hyperparathyroidism and hypocalciuria when protein intake is restricted to <0.8 g/kg/d (Kerstetter et al. 1998; Kerstetter et al. 2000). Collectively, these data suggest that moderate-to high-protein diets do not have deleterious effects on bone, and may in fact enhance bone health, while low-protein intakes may result in impaired calcium metabolism.

Dietary protein and renal function

Consumption of amounts of protein, particularly animal protein at up to 2 g/kg/d, have been associated with an increased renal acid load, which may lead to the development of kidney stones (Reddy et al. 2002), compensatory increases in renal acid and ammonia excretion, metabolic acidosis and an increased risk of tubule injury (van den Berg et al. 2011). In a prospective study of ~45,000 men, aged between 40 and 75 years who were followed for 4 years, animal protein intake was associated with risks of symptomatic kidney stone formation (Curhan et al. 1993). In contrast, a review by Eisenstein and Roberts established that in normal, healthy individuals without pre-existing renal disease, there is minimal evidence for adverse effects of high-protein diets on renal function (Eisenstein et al. 2002). While some short-term studies have reported that high-protein intakes result in hyperfiltration (Hegsted and Linkswiler 1981; Brandle et al. 1996), this can be explained by structural changes in the healthy kidney, including glomerular and tubular changes (Brandle et al. 1996), and increases in renal mass, that result in increased glomerular filtration rate (Skov et al. 1999a). In
healthy, obese individuals without history of renal disease, no deleterious effects have been observed on renal function following high-protein weight loss diets of up to 2 years (Brinkworth et al. 2010; Friedman et al. 2012). In addition, in the 11 year follow-up period of the Nurse’s Health Study, during which protein intake was accounted for over 4 years, average protein intakes of up to 1.1 g/kg/d were not associated with reduced renal function in healthy women without pre-existing renal disease (Knight et al. 2003). In women with mild renal insufficiency, an average protein intake of up to 1.0 g/kg/d was significantly associated with increases in estimated glomerular filtration rate and an accelerated decline in renal function (Knight et al. 2003). In people with pre-existing renal disease, or at risk of renal disease (i.e. type II diabetics), limiting dietary protein to 0.6-0.8 g/kg/d is thought to slow progression of the disease, particularly in end-stage chronic kidney disease (Chauveau and Aparicio 2011), and may lower mortality rate (Pedrini et al. 1996). In conclusion, the data suggests that in healthy individuals, protein intakes of up to 2.2 g/kg/d have minimal negative effects on renal function for periods of up to 2 years. In individuals with renal insufficiency or disease, high-protein diets should be undertaken with extreme caution, and under close medical supervision.

3.7 Concluding remarks

Protein is a critical component of the diet of humans, and has a number of physiological roles. Considerable evidence suggests that protein is important in regulating total energy intake, glycaemic control and body weight. It is well established that protein has potent effects on appetite, where, kcal for kcal, protein is more satiating than fat or carbohydrate. This satiating effect of protein has generated considerable interest in the value of protein in weight-loss diets, to inhibit hunger and improve weight loss and diet compliance. Also of significance to human health, the evidence largely indicates that high-protein diets may also
improve a number of health-related outcomes, retention of fat free mass and changes in body composition. Despite the considerable evidence for superior effects of protein on appetite sensation and energy intake, the mechanisms underlying these effects are not well described. While it has been demonstrated that DIT from protein contributes to some extent, the pre-and post-absorptive satiety signals generated, in particular, by the GI tract, have not been well explored. Chapter 4 will examine the current evidence for the role of the GI tract in determining appetitive responses, and will highlight the current paucity of information surrounding the GI responses to protein, and how these may relate to the satiating capacity of protein.
Chapter 4: The Role of The Gastrointestinal Tract In The Regulation of Glycaemia, Appetite And Energy Intake

4.1 Introduction

Human eating patterns are influenced by a number of factors. Multiple physiological mechanisms interact to maintain a balance between energy intake and energy expenditure. GI factors are being increasingly recognised as a key part of this complex interplay. Nutrients interact with receptors along the length of the GI tract, as well as in the liver and other organs to produce neural and hormonal feedback signals, that are relayed to the brain. These signals determine a number of outcomes, including how the ingested nutrients are stored and utilised, the postprandial glycaemic response, initiation and termination of meals, and the amount of food consumed (Woods et al. 1998).

A number of inter-related GI motor and hormonal functions are critical to the regulation of food intake. Specifically, GI motor factors include antropyloroduodenal (APD) motility (Azpiroz and Malagelada 1987b; Heddle et al. 1988a), which regulates gastric emptying (Sepple and Read 1989) and intragastric meal distribution, through changes in proximal and distal gastric motility. GI hormones include the orexigenic hormone, ghrelin (released from the stomach) (Overduin et al. 2005), and the anorexigenic hormones, including, but not limited to, CCK (Lilja et al. 1984; Cook et al. 1997), GLP-1 (Herrmann et al. 1995; Little et al. 2005; Little et al. 2006a), PYY (MacIntosh et al. 1999; Onaga et al. 2002) and GIP (Thor et al. 1987), released from various regions of the small intestine. The postprandial glycaemic response is also modulated by changes in gastric emptying and GI hormone release, along with the release of insulin and glucagon. This chapter will first describe the anatomy of the upper GI tract. It will then focus on the role of inter-related GI motor and hormonal factors
that are critical in the regulation of glycaemia and energy intake, in response to ingestion of fat, carbohydrate and protein, both in isolation, and in mixed form. This chapter will also present the limited evidence regarding how upper GI function may be altered in obesity.

4.2 Anatomy and function of the upper gastrointestinal tract

4.2.1 Oral cavity

The oral cavity is the site of initial nutrient detection, whereby ingested material, including food and potential toxins, interact with receptors and is immediately “tasted”. From an evolutionary point of view, tasting of ingested matter is vital for health and survival. For example, sweet and savoury tastes promote the intake of energy-rich foods, which provide a nutritional benefit. In contrast, bitterness is closely associated with the presence of toxins, which should be avoided (Lan-Pidhainy and Wolever 2010).

The perception of taste begins on the tongue, where epithelial-derived taste receptor cells detect chemical cues from ingested matter (Lindemann 1996; Gardner et al. 2007). Taste buds are onion-shaped clusters of ~50 to 150 taste receptor cells that are distributed across the surface of the tongue and the soft palate. Each taste bud features a taste pore-projections at the apical surface of the taste bud-through which the tastant, makes contact with, and stimulates, taste cell receptors (Figure 4.1). These signals are transmitted from the basal nerve endings via the facial nerve to the nucleus tractus solitarius in the brainstem where taste is perceived. These signals also participate in the cephalic phase of digestion, by initiating anticipatory release of GI enzymes and hormones, and stimulating gall bladder contraction, relaxation of the Sphincter of Oddi and receptive gastric relaxation (Cuomo and Sarnelli 2004).
Once food is masticated in the oral cavity and swallowed, the ingested nutrients pass through the oesophagus, a muscular tube lined with stratified squamous epithelium, into the stomach, by peristalsis. The stomach is a J-shaped, sac-like chamber connecting the oesophagus and duodenum, the first section of the small intestine (Figure 4.2). The stomach is divided anatomically into three distinct regions, the fundus, the corpus, and the antrum.
The stomach is comprised of two muscular layers, the inner, circular muscle layer, and the outer, longitudinal muscle layer. A third oblique muscle layer covers the fundus. Functionally, the stomach can be divided into two regions, i) the proximal region (comprised of the fundus and corpus) and ii) the distal compartment, which includes the antrum (Figure 4.2). The thin, smooth, oblique muscle layer of the proximal stomach allows this area to act as a reservoir for ingested food. The proximal region also generates low frequency, sustained contractions to maintain a basal pressure within the stomach, and to propel food towards the distal stomach and the small intestine, thus contributing to gastric emptying. The distal area of the stomach includes the antrum and the pylorus, and is characterised by a thickening of the circular muscle towards the pylorus, which aids in the mixing and grinding of solid food into smaller particles. The distal region is also characterised by peristaltic patterns of muscular contraction that increase in amplitude as they migrate towards the pylorus, propelling chyme into the duodenum (Burks et al. 1985; Cullen and Kelly 1993).

4.2.3 Pylorus

The pylorus is a short, 2-cm region, which connects the antrum of the stomach to the duodenum. The pylorus is a muscular sphincter, that is integral in regulating the flow of gastric digesta from the stomach into the small intestine; transpyloric flow can only occur when the pylorus is open (Horowitz et al. 1994). Two types of motility patterns are characteristic of the pylorus. Tonic contractions, known as basal pyloric pressure (BPP), maintain the closure of the pylorus against flow of digesta from the antrum. In contrast, isolated pyloric pressure waves, which intermittently close the gastroduodenal junction, regulate the pulsatile flow of gastric contents from the stomach into the small intestine in response to intestinal feedback (Keinke et al. 1984; Horowitz et al. 1994).
4.2.4 Small intestine

The small intestine is a muscular tube of approximately 5 m in length, and is divided into three distinct regions; the most proximal region, the duodenum (~25 cm long), the middle region, the jejunum (~2m long), and the most distal region, the ileum (~3m long) (Figure 4.2). The small intestine is the primary site for absorption of nutrients and the origin of a number of feedback signals that interact to regulate the transit of food through the gut. These signals modulate gastric emptying by relaxing the proximal stomach, inhibiting antral contractions and stimulating tonic and phasic pyloric contractions.

4.3 Motor function of the upper gastrointestinal tract

Upper GI motility alternates between two distinct patterns: i) the interdigestive migrating motor complex (MMC), which occurs in the fasted state; and ii) the fed motility pattern which characterises the postprandial state and is initiated following food ingestion. The following sections describe patterns of gastric emptying, as well as motility patterns in the stomach, pylorus and small intestine during both the fasted and postprandial states.

4.3.1 Fasting motor patterns

During fasting, the MMC represents the cyclical pattern of motility exhibited by the GI tract. The MMC can be divided into specific phases of activity; i) phase I is a period of quiescence, characterised by few, or no, contraction and lasts for approximately 40-60 min. Phase I is followed by ii) phase II, during which intermittent contractions increase progressively in frequency, with the latent periods between contractions gradually shortening over the 20-40 min of phase II activity; until occurrence of iii) phase III, characterised by a period of contractions occurring at maximum frequency and amplitude, i.e. 3 contractions/min in the stomach and ~12 contractions/min in the duodenum that migrate through the GI tract to the
terminal ileum (Code and Marlett 1975). The phase III period lasts between 5 and 10 min, and is followed by a short transition period (phase IV) (Takahashi 2012), before the activity returns to phase I (Dooley et al. 1992). The MMC propagates aborally through the GI tract, ensuring that any food remaining in the stomach or small intestine is propelled to more distal regions, or expelled from the body (Dooley et al. 1992).

4.3.2 Postprandial motor patterns

The arrival of nutrients in the stomach and small intestine triggers a number of interrelated changes in GI motor function, which convert fasting motility to the ‘fed’ or postprandial motor pattern. Following a meal, two motor responses occur in the proximal stomach. First, receptive relaxation occurs, whereby, after swallowing, intragastric pressure is transiently decreased. Secondly, the proximal stomach relaxes to accommodate and store the ingested food, without an increase in intragastric pressure, termed adaptive relaxation (Azpiroz and Malagelada 1987a). Gastric distension interrupts MMC activity in both the stomach and proximal small intestine, while fluid and/or nutrient in the small intestine interrupt MMC activity across the entire small intestine (Code and Marlett 1975). In the antrum, ingested food is mixed with gastric secretions and broken down into chyme by chemical and mechanical digestion in the distal stomach. These antral contractions are propulsive, moving the chyme from the stomach into the duodenum between phasic and tonic pyloric contractions. Gastric emptying occurs only when the pylorus is open; in the fed state the pylorus remains closed for prolonged periods to promote gastric mixing and trituration (Horowitz et al. 1994). The chyme is ultimately delivered into the small intestine at an overall rate of 1-4 kcal/min in healthy humans, (Hunt et al. 1985), which is optimal for digestion and absorption (Horowitz et al. 1994).
4.3.3 Gastric emptying

The rate, and pattern, of gastric emptying of ingested nutrients is modulated by a number of factors, including the characteristics of the ingested meal, and the activity of other regions of the GI tract. Gastric emptying is predominantly pulsatile, rather than continuous, and individual flow pulses are varied, so that the resultant flow can be retrograde or antegrade. Patterns of transpyloric flow are regulated by coordinated motor activity in the proximal and distal stomach, pylorus, and small intestine. The coordinated activity of the stomach and pylorus act as a brake to slow gastric emptying, ensuring food is delivered at a rate that allows optimal digestion and absorption of the ingested food.

The physical state and macronutrient composition of the meal affect rates of gastric emptying. Nutritive liquids and liquefied solids empty from the stomach more slowly, and in a linear fashion, while non-nutritive liquids empty rapidly in a non-linear, mono-exponential fashion (Horowitz et al. 1994). The emptying of digestible solids is characterised by a lag phase of between 10 and 30 min before the first substantial emptying occurs. During this lag phase, mechanical trituration reduces particles to < 2 mm diameter (Meyer 1980), and the food is gradually redistributed from the proximal to distal stomach (Horowitz et al. 1994). The duration of the lag is influenced by the rate of redistribution of the solid matter, which results in an emptying pattern of the solid portion of a meal that resembles that of a high-calorie meal, i.e. in a linear manner (Park and Camilleri 2005).

4.4 Effects of macronutrients on gastrointestinal motor function

Administration of “pure” carbohydrate or fat directly into the small intestine affects gastric emptying, intragastric meal distribution and APD motility (Chapman et al. 1999; Feinle et al.)
2000; Pilichiewicz et al. 2007a; Pilichiewicz et al. 2007b), while the effects of protein on GI motility have, until recently, been unclear.

### 4.4.1 Effects of macronutrients on gastric emptying

It has been established that fat and carbohydrate, when consumed orally, empty from the stomach at a rate of 1-4 kcal/minute (Brener et al. 1983; Horowitz et al. 1993). While the rate of emptying of protein has been examined, information remains limited. While some studies report that the gastric emptying rate of protein is similar to that of fat and carbohydrate (Goetze et al. 2007), others have suggested that gastric emptying of protein is slowed in a load-dependent manner, so that increased loads of protein are emptied at a similar overall rate (Calbet and MacLean 1997; Khoshoo and Brown 2002; Maughan et al. 2004). Further research is necessary to elucidate the overall gastric emptying rate of protein, and to establish the relationship between gastric emptying of protein, GI hormone release, glycaemia and energy intake.

### 4.4.2 Effects of macronutrients on antropyloroduodenal motility

The infusion of intraduodenal lipid has been shown to stimulate isolated pyloric pressure waves (IPPWs), and suppress antral and duodenal activity (Pilichiewicz et al. 2006; Pilichiewicz et al. 2007b), in a load-dependent manner. Similarly, intraduodenal glucose stimulates IPPWs and suppresses antral and duodenal motility (Heddle et al. 1988d) with the degree of stimulation or suppression dependent on load (Pilichiewicz et al. 2007a). Intraduodenal lipid appears to be more potent than glucose, since administration of fat has been shown to stimulate pyloric contractile activity (Cook et al. 1997) and slow gastric emptying (Heddle et al. 1989) more strongly than an isocaloric amount of glucose (Cook et al. 1997; Andrews et al. 1998). Direct comparison of equicaloric loads of intraduodenal
lipid, or glucose (3 kcal/min), and combinations of the two (2 kcal/min lipid + 1 kcal/min maltodextrin or 1 kcal/min lipid + 2 kcal/min maltodextrin) has confirmed this (Seimon et al. 2009a). Pure lipid more potently modified antropyloroduodenal motility and GI hormone release, while reducing the proportion of lipid in the infusion resulted in comparatively diminished gastrointestinal motor and hormonal responses.

In contrast, the effects of protein on GI motility are poorly defined, with a few studies hitherto examining only the effects of single amino acids and amino acid mixtures on APD motility. Edelbroek et al observed that in healthy men, intraduodenal L-tryptophan, compared to D-tryptophan, had a greater effect on the stimulation of basal pyloric and duodenal motility (Edelbroek et al. 1994). In addition, Gielkens et al administered intravenous amino acid mixtures, and observed a greater suppression of antral motility and slowed duodeno-caecal transit in healthy men, compared with an IV saline infusion (Gielkens et al. 1999). More recently, Steinert et al have reported differential effects of small doses of L-glutamine, L-phenylalanine and leucine on GI motility. In the first study, they infused L-glutamine and L-phenylalanine separately for 90 minutes at rates of 0.15 and 0.45 kcal/min. Interestingly, only L-phenylalanine affected GI motility (as antral and pyloric pressures), and only at the highest dose, however both doses stimulated CCK release. In contrast, L-glutamine had no effects on GI motility or hormone release (Steinert et al. 2015a). In the second study, the reported that leucine, an abundant branched chain amino acid, infused at 0.45 kcal/min suppresses antral motility, and increased plasma CCK (Steinert et al. 2015b). In contrast, 0.15 kcal/min only slightly increased plasma CCK concentrations. Interestingly, the 0.45 kcal/min load reduced energy intake at a buffet meal by ~ 13%. Taken together, these data suggest that individual amino acids (as part of whole proteins typically consumed
in the diet) may exert different and specific effects on aspects of GI function. However, it remains unclear how whole proteins may modulate GI motility.

4.4.3 The role of gastrointestinal motor function in determining the glycaemic response and energy intake

The rate of arrival of ingested nutrient to the small intestine, through co-ordinated APD motility to regulate gastric emptying, plays a substantial role in mediating the postprandial glycaemic response. The rate of gastric emptying of carbohydrate is a well-established determinant of postprandial glycaemia (Horowitz et al. 1993; Jones et al. 1996; Rayner et al. 2001; O'Donovan et al. 2004), in both healthy individuals (Horowitz et al. 1993) and patients with type 2 diabetes (Jones et al. 1996). In fact, gastric emptying accounts for approximately 35% of the variation in peak blood glucose concentrations after a standardised 75 g glucose load. The rate of gastric emptying also determines the magnitude of the response of a number of GI hormones released from the small intestine, which in turn provide feedback to further modulate gastric emptying, and the release of other GI hormones.

GI motility also plays a key role in determining appetite responses and energy intake. For example, the slowing of gastric emptying prolongs gastric distension, and is associated with increased feelings of “fullness” and reduced energy intake. A close, indirect relationship between antral distension and feelings of fullness has been described (Jones et al. 1997b), while food intake at a buffet meal has been shown to be inversely related to antral filling from the previous meal (Sturm et al. 2004). In humans, distension of the proximal and distal stomach results in slowed gastric emptying, and has been shown to increase perceptions of fullness (Feinle et al. 1997; Jones et al. 1997a) and suppress energy intake (Kissileff et al. 2003; Sturm et al. 2004). Furthermore, a recent pooled data analysis of data generated within
our department has identified that, following intraduodenal infusions of lipid and carbohydrate; IPPWs are an independent predictor of subsequent energy intake in healthy, lean males. Thus, GI motility plays a key role in determining glycaemic responses to nutrient, as well as appetite and energy responses, which are also driven by the release of a number of GI hormones, as detailed in the following sections.

### 4.5 Gastrointestinal hormone release

The release of a number of GI peptides is modulated by the presence of nutrients in the GI tract. The anorexigenic hormone, ghrelin, which is released primarily from the stomach (Kojima et al. 1999) to initiate eating, is suppressed by feeding (Cummings et al. 2001; Wren et al. 2001a). In contrast, the release of orexigenic hormones, including the release of CCK (Lilja et al. 1984; Matzinger et al. 2000) from the proximal, and the “incretin” hormones GLP-1 (Flint et al. 1998; Gutzwiller et al. 1999), and GIP, as well as PYY and glucagon from the distal, small intestine, is stimulated by nutrients. Collectively, these hormones have relatively well-defined, and interrelated, effects on GI motility, glycaemia and appetite, including initiation of a meal and meal size; these effects are also dependent on the macronutrient composition of a single meal. The individual GI hormones, their effects on GI motility, the release of other hormones, glycaemia, and appetite and energy intake are described in detail in the subsequent sections, and in Table 4.1.
Table 4.1: GI hormones and their described roles within the body

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Released from</th>
<th>In response to</th>
<th>Stomach</th>
<th>Small intestine</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Adipose stores</th>
<th>Brain</th>
<th>Additional effects</th>
<th>Suppressed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>stomach</td>
<td>↓ blood glucose</td>
<td>↓ antral phase III activity</td>
<td>↑ GE</td>
<td>↑ plasma glucose</td>
<td>↓ serum insulin</td>
<td>↑ appetite</td>
<td>↑ meal ingestion</td>
<td>Meal ingestion, CCK, PYY, insulin</td>
<td></td>
</tr>
<tr>
<td>CCK</td>
<td>L cells</td>
<td>↑ PYY</td>
<td>↑ enzyme release</td>
<td>↓ appetite</td>
<td>↑ gall bladder contraction; ↓ energy intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Duodenum</td>
<td>↓ pyloric motility, GE</td>
<td>↓ duodenal motility</td>
<td>↓ jejunal motility</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>Fat, protein*, CCK</td>
<td>↑ enzyme release</td>
<td>↓ appetite</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PYY</td>
<td>L cells</td>
<td>↓ gastric motility</td>
<td>Mediates ileal brake</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ileum, large intestine</td>
<td>↑ enzyme release</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLP-1</td>
<td>L cells</td>
<td>↓ duodenal motility</td>
<td>↑ duodenal motility</td>
<td>↓ appetite</td>
<td>DPP-IV</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Distal small intestine</td>
<td>↑ enzyme release</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GIP</td>
<td>K cells</td>
<td>↑ insulin release and release, ↑ glucagon release</td>
<td>↑ fatty acid synthesis ↑ fatty acid to triglyceride conversion</td>
<td>DPP-IV</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Duodenum</td>
<td>↑ duodenal motility</td>
<td>↓ duodenal motility</td>
<td></td>
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<tr>
<td>Glucagon</td>
<td>Pancreatic α cells</td>
<td>↑ duodenal activity frequency, ↑ length MMC</td>
<td>↑ glycogenolysis ↓ glycolysis</td>
<td>↑ lipolysis</td>
<td>May ↓ energy intake</td>
<td>GLP-1, GIP</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Fasting Exercise blood glucose Protein</td>
<td>↓ antral contractility</td>
<td></td>
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<tr>
<td>Insulin</td>
<td>Pancreatic β cells</td>
<td>↑ blood glucose ↑ plasma amino acids GLP-1, GIP</td>
<td>↓ duodenal PIII</td>
<td>↑ nutrient storage</td>
<td>Crosses blood brain barrier, ↓ energy intake</td>
<td>↑s cell proliferation ↑ uptake of glucose, amino acids, fatty acids</td>
<td>↓ blood glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*macronutrients listed in respective order of potency of effect; CCK, cholecystokinin; CHO, carbohydrate; DPP-IV, dipeptidyl peptidase IV; GE, gastric emptying; GIP glucose-dependent insulino tropic peptide; GLP-1, glucagon-like peptide-1; PIII, phase 3 activity; PYY, peptide-YY.
4.5.1 Ghrelin

Ghrelin is a 28-amino acid peptide, secreted from the fundic region of the stomach by oxyntic cells (Kojima et al. 1999). The major active human isoform of ghrelin, acyl ghrelin, is characterised by an n-octanoyl side-chain at the serine 3 position. This side chain is integral for the biological action of ghrelin as an endogenous ligand for the growth-hormone secretagogue receptor type 1a (GHS-R1a) (Kojima et al. 1999; Date et al. 2000). Other ghrelin isoforms include octanoyl ghrelin-(1-27), decanoyl ghrelin, decanoyl ghrelin-(1-27), as well as the non-active moieties, des-acyl ghrelin and des-acyl ghrelin-(1-27) (Hosoda et al. 2003). Ghrelin-secreting P/D1 cells are the second-most prolific cells in the gastric mucosa, suggesting an integral role in gastric physiology (Dixit et al. 2004; Locatelli et al. 2005). Ghrelin has also been shown to be secreted in smaller amounts from the islets of Langerhans in the pancreas, and from both alpha- and beta-cells (Wierup et al. 2002). Ghrelin has no relevant homology to any other circulating GI peptides (Locatelli et al. 2005), and, in contrast to other gut peptides, ghrelin concentrations are high during fasting, and suppressed rather than stimulated, by food (Cummings et al. 2001). Studies using pyloric occlusion and small intestinal infusion models in animals, and intraduodenal infusion in humans, have established that the suppression of ghrelin is dependent on feedback arising from the interaction of nutrients with the small intestine, rather than the stomach (Williams et al. 2003; Overduin et al. 2005; Parker et al. 2005). Critically, the magnitude of the post-meal inhibition of ghrelin release has been shown to be directly proportional to the energy content of the meal, so that a more calorific meal suppresses ghrelin release for longer (Callahan et al. 2004).

4.5.1.1 Effects of ghrelin on gastrointestinal motility and hormone release

Exogenous ghrelin has been shown to have a prokinetic effect on gastric emptying in rodents (Dornonville de la Cour et al. 2004), and in both healthy humans (Levin et al. 2006) and
patients with diabetic gastroparesis (Murray et al. 2005). Specifically, intravenous administration of ghrelin induces phase III activity in the stomach (Tack et al. 2006), increases proximal stomach tone (Tack et al. 2006), accelerates gastric emptying, and stimulates hunger (Levin et al. 2006). In rodents, intraperitoneal administration of the ghrelin receptor (GHS-R1a) antagonist, “14f”, dose-dependently slowed gastric emptying, suggesting that endogenous ghrelin plays a role in regulating gastric emptying, at least in the rat (Puleo et al. 2012). Since ghrelin is suppressed, rather than stimulated following meal ingestion (Monteleone et al. 2003), and comparative ghrelin receptor antagonist studies in humans have not been conducted, the relevance of the effect of ghrelin on the rate of gastric emptying and GI motility remains unclear.

4.5.1.2 Effects of ghrelin on postprandial glycaemia

Data on the effects of ghrelin on insulin and glycaemic control remain inconsistent. Negative correlations have been reported between ghrelin and body fatness, and ghrelin and insulin concentrations (Tschop et al. 2001; Poykko et al. 2003), with a positive correlation between ghrelin and insulin sensitivity (Poykko et al. 2003). Given that T2DM is characterised by insulin resistance, these data suggest that lower ghrelin levels may play a role in the aetiology of diabetes (Poykko et al. 2003). Under experimental conditions, “14f” has been shown to improve intraperitoneal glucose tolerance in rats, to a similar magnitude as a common insulin secretagogue (Puleo et al. 2012). In humans, intravenous infusion of ghrelin at rates up to 1.5 nmol/kg/hour has been shown to impair glucose clearance rates and insulin release acutely (Broglio et al. 2001; Tong et al. 2010). Overall, these data suggest that ghrelin may be involved in glycaemic control through negative-feedback regulation of insulin secretion and glucose disposal, however, further research is required, particularly since the effects of endogenous ghrelin on the blood glucose and insulin response are not established in humans.
4.5.1.3 Effects of ghrelin on appetite and energy intake

Exogenous ghrelin has been shown to increase energy intake in both rodents and humans (Wren et al. 2001a; Wren et al. 2001b). For example, in rats, 7 days of chronic ghrelin administration resulted in increased weight and adiposity (Wren et al. 2001b), while in humans, a 260-min infusion of ghrelin at 5 pmol/kg/min decreased fullness, increased hunger and increased energy intake at a subsequent meal by ~ 28 %, compared with a saline control (Wren et al. 2001a). Ghrelin exerts its action by activating the hypothalamic neuropeptide Y-Y1 (NPY-Y1) and agouti-related protein (AGRP) pathways which provide central stimuli for increasing energy intake and reducing energy expenditure (Dixit et al. 2004).

In healthy humans, it has been demonstrated that pre-prandial increases in ghrelin are related to hunger scores when meals are initiated voluntarily, suggesting a role for ghrelin in meal initiation in the absence of time- and food-specific cues (Cummings et al. 2004). Moreover, ghrelin release is typically upregulated during chronic energy restriction (i.e. anorexia nervosa, weight loss) and down-regulated in positive energy balance (i.e. obesity) (Ariyasu et al. 2001). In obese humans, fasting ghrelin concentrations have been shown to be reduced, and the postprandial decline of plasma ghrelin concentrations diminished, or absent (Tschop et al. 2001). In diet-induced obese mice, daily administration of a GHS-R1a antagonist reduced food intake and resulted in ~15 % weight loss compared with pair-fed controls, while in healthy normal weight and ob/ob mice GHS-R antagonists decreased energy intake, reduced gastric emptying and decreased body weight gain in ob/ob mice (Asakawa et al. 2003). Collectively, these data suggest that, in both rodents and humans, ghrelin plays a key role in driving eating behaviour, and weight regain. The GHS- receptor is also a primary target of growth hormone in humans. While the role of GHS-R agonists in relation to aging have been explored (Smith et al. 2007), the integral relationship between the GHS-R receptor
and growth hormone function means evidence for the effects of GHS-R receptor agonists on feeding behaviour is limited. Inverse agonists of the ghrelin receptor are also currently being explored as potential interventions in obesity, however evidence for these is limited (Holst et al. 2006; Bhattacharya et al. 2014). Since human responses to ghrelin receptor antagonism may differ compared with rodent models, further research is required to establish the exact physiological role of ghrelin in feeding behaviour in humans.

4.5.2 Cholecystokinin (CCK)

Cholecystokinin is synthesised in the “I” cells of the small intestinal mucosa, primarily in the duodenum and to some extent, the jejunum (Table 4.1). Of the multiple forms of CCK, CCK-8, CCK-22, CCK-58 and CCK-33/39 are the primary biologically active forms of CCK found in the human brain, intestine and circulation (Eysselein et al. 1990; Wang and Cui 2007). CCK peaks after 15–30 min of meal ingestion in response to the presence of digestive products of fat and protein (Larsson and Rehfeld 1978; Liddle et al. 1985; Lieverse et al. 1994a; Foltz et al. 2008), and to a lesser extent carbohydrate (Parker et al. 2005), in the small intestinal lumen. CCK releasing cells are also present in enteric vagal afferent neurones, and sections of the central nervous system, including the thalamus, hypothalamus, basal ganglia and dorsal hindbrain, where CCK acts as a neurotransmitter (Crawley and Beinfeld 1983).

Studies administering exogenous CCK have shown effects on a number of functions within the GI tract, including the regulation of gastric emptying (Liddle et al. 1986) and GI motility (Meyer et al. 1989; Brennan et al. 2008), stimulation of gall bladder contraction (Liddle et al. 1985; Beglinger et al. 1992), stimulation of pancreatic enzyme release (Jansen and Lamers 1983; Beglinger et al. 1992), and suppression of energy intake (Kissileff et al. 1981; Kissileff et al. 2003).
4.5.2.1  **Effects of CCK on gastrointestinal motility and hormone release**

In both animals (Moran and McHugh 1982; Moran and McHugh 1988) and humans (Liddle et al. 1986; Kleibeuker et al. 1988), exogenous administration of CCK slows gastric emptying (Liddle et al. 1986; McHugh and Moran 1986), suppresses antral and duodenal motility (Rayner et al. 2000; Brennan et al. 2005) stimulates pyloric pressures (Rayner et al. 2000; Brennan et al. 2005) and jejunal motility, slows gastric emptying (Fried et al. 1991), and suppresses colonic motility (Kellow et al. 1987).

Studies using loxiglumide, a CCK₁ receptor antagonist, have established a key role for CCK in slowing gastric emptying and GI transit (Fried et al. 1991; Katschinski et al. 1996). For example, administration of loxiglumide results in an acceleration of gastric emptying and stimulation of antral motility following a 500-mL liquid fat meal (Intralipid) (Schwizer et al. 1997), and the inhibitory effects of duodenal fat on gastric motility (Feinle et al. 1996) have been shown to be abolished by the administration of loxiglumide. Moreover, administration of loxiglumide during a 150-min duodenal infusion of a mixed-nutrient liquid meal decreased total numbers of antral, pyloric and duodenal contractions by 44 %, 74 % and 41 % respectively (Katschinski et al. 1996).

CCK also appears to play a role in modulating the release of some GI hormones. For example, intraduodenal infusion of a long-chain fatty acid suppressed ghrelin release and stimulated PYY and CCK release. When loxiglumide was administered concurrently to the ID infusion, the suppression of ghrelin secretion was reversed and the PYY response abolished (Degen et al. 2007). In addition, IV administration of CCK-8 stimulated PYY release, and suppressed ghrelin release, when compared with a saline control (Brennan et al. 2007; Degen et al. 2007). Finally, both IV infusion of CCK-8, and consumption of a mixed
meal, stimulated pancreatic polypeptide release; when loxiglumide was administered, both the meal- and CCK-8 induced effects on pancreatic polypeptide were abolished. Taken together, these data suggest that all of these effects are mediated by CCK via the CCK₁ receptor.

4.5.2.2 Effects of CCK on postprandial glycaemia

CCK does not appear to have direct effects on the modulation of postprandial glycaemia. In humans, intravenous administration of CCK at levels normally circulating postprandially has no effect on glucose-stimulated insulin secretion (Fieseler et al. 1995a). In studies where exogenous CCK was administered following an orally-administered mixed meal or glucose load (Liddle et al. 1988; Schick et al. 1991; MacIntosh et al. 2001), CCK blunted the insulin response, and lowered blood glucose, however this was probably the result of slowing of gastric emptying. In this respect, CCK appears to indirectly regulate blood glucose concentrations, by modulating gastric emptying into the small intestine, which, in the case of carbohydrate is associated with reduced glucose delivery.

4.5.2.3 Effects of CCK on appetite and energy intake

It is well established that exogenous administration of CCK suppresses appetite and energy intake. Early investigations in rodents using intraperitoneal administration of the biologically active, sulphated octapeptide of CCK (CCK-8) reported a dose-dependent suppression of energy intake (Gibbs et al. 1973). More recently, intraperitoneal injection of both CCK-8 and CCK-58 has been shown to reduce meal size similarly, while only CCK-58 increased the inter-meal interval (Overduin et al. 2014; Sayegh et al. 2014). This suggests that, at least in rats, CCK isomers do not have equal bioactivity; however it is unclear whether the same
holds true for humans. The mechanism by which CCK induces these effects in rodents is thought to be through liberation of gastric stores of leptin (Bado et al. 1998).

In humans, intravenous CCK-8 and CCK-33 both increased fullness, decreased hunger and suppressed subsequent energy intake in healthy young (Kissileff et al. 1981; Brennan et al. 2005), older (MacIntosh et al. 2001) and obese (Pi-Sunyer et al. 1982) individuals. These changes likely result from the concurrent changes in GI motor and hormone function that mimic the effects of intraduodenal nutrient (i.e. suppression of APD motility). While some studies have reported that exogenous administration of CCK can induce nausea at high doses, which may impact the energy intake reported, others have shown a reduction in energy intake following exogenous CCK, in the absence of nausea (Brennan et al. 2008).

Limited studies suggest that endogenous CCK plays a role in the regulation of appetite and energy intake. For example in one study, infusion of the CCK1 receptor antagonist, loxiglumide, attenuated the inhibitory effects of fat on subsequent energy intake, resulting in increased food intake (Lieverse et al. 1994a; Matzinger et al. 1999). In another, loxiglumide administered for one hour prior to, and during, ingestion of a meal increased energy intake, albeit modestly, by around 10%, and increased feelings of hunger compared with a saline infusion (Beglinger et al. 2001). These observations demonstrate that endogenous CCK is involved in appetite regulation through CCK1 receptor mechanisms.

### 4.5.3 Peptide YY (PYY)

PYY is a 36-amino acid peptide, and part of the pancreatic polypeptide family. PYY is secreted by endocrine “L” cells, predominantly in the ileum and large intestine as PYY\(_{(3-36)}\) and PYY\(_{(1-36)}\) (Adrian et al. 1985), the latter of which is rapidly degraded by DPP-IV to
PYY\textsubscript{(3-36)}. PYY\textsubscript{(3-36)} is the active, and most abundant, isoform in the human circulation (Grandt \textit{et al.} 1994; Ueno \textit{et al.} 2008) (Table 4.1). PYY\textsubscript{(1-36)} is a known agonist of the Y1 and Y2 receptors of the NPY receptor family, and is reported to have orexigenic effects (Corp \textit{et al.} 1990), while PYY\textsubscript{(3-36)} is an agonist of the Y2 receptor, with anorexigenic effects (Batterham \textit{et al.} 2002). Release of PYY in the GI tract is dependent on the caloric load of the ingested meal (Ekblad and Sundler 2002), and occurs in response to fat (Onaga \textit{et al.} 2002; Pilichiewicz \textit{et al.} 2006), particularly fatty acids (Pappas \textit{et al.} 1986), and protein (Fu-Cheng \textit{et al.} 1997; Veldhorst \textit{et al.} 2008), but not carbohydrate (Groger \textit{et al.} 1997). The release of PYY has also been shown to occur in response to neurohumoral signals arising from the small intestine, including CCK (Lin \textit{et al.} 2000; Brennan \textit{et al.} 2007; Brennan \textit{et al.} 2008).

4.5.3.1 Effects of PYY on gastrointestinal motility and hormone release

Exogenous administration of PYY in both humans and animals modulates GI motility. In rhesus monkeys, intramuscular injection of PYY\textsubscript{(3-36)} has been shown to slow gastric emptying of saline in a dose-dependent manner (Moran \textit{et al.} 2005b). In humans, intravenous infusion of PYY\textsubscript{(3-36)} at 0.18 or 0.51 pmol/kg/min also slows gastric emptying and mouth-to-caecum transit dose-dependently (Savage \textit{et al.} 1987). Because PYY-releasing cells are located primarily in the distal small intestine, and the secretion of PYY is related to the fat-induced inhibition of distal gut motility, it has been suggested that PYY is a primary mediator of the ileal brake – the slowing of gastric emptying induced by distal small intestinal feedback in response to fat (Lin \textit{et al.} 1997). In addition to inhibiting GI motility, endogenous PYY release has been shown to inhibit colonic chloride secretion, as well as pancreatic exocrine and insulin release, (Ballantyne 2006) and neuropeptide Y (NPY), release (Lluis \textit{et al.} 1987a; Lluis \textit{et al.} 1987b; Ueno \textit{et al.} 2008).
4.5.3.2 Effects of PYY on postprandial glycaemia

Knowledge of the effects of PYY on postprandial glycaemia is limited. Since PYY is released in proportion to the amount of calories, as well as the amount of fat or protein (but not carbohydrate) present in a meal, it does not appear to have direct effects on glycaemic control. A number of in vivo studies have shown that PYY administered at supraphysiological concentrations inhibits insulin secretion in rat, mouse and dog pancreas. In humans, intravenous infusion of PYY$^{(3-36)}$ at 0.8 pmol/kg/min has been found to increase plasma free fatty acids, as well as post-prandial, but not pre-prandial, insulin and glucose concentrations (Sloth et al. 2007b). This effect was likely due to increased lipolysis increasing circulating free fatty acids, and thus acutely increased insulin resistance.

4.5.3.3 Effects of PYY on appetite and energy intake

A number of studies have examined the effects of PYY on appetite and energy intake, however the results are conflicting. For example, central administration of PYY has been shown to stimulate feeding in rats (Hagan 2002). In humans, subcutaneous administration of PYY$^{(1-36)}$ at up to 200 pmol/kg lean body mass, or of PYY$^{(3-36)}$ at up to 100 pmol/kg lean body mass had no effect on energy intake, although PYY$^{(3-36)}$ did increase perceptions of satiety and decreased perceptions of hunger and prospective consumption (Sloth et al. 2007a). In contrast, peripheral administration of PYY$^{(3-36)}$ has been shown by others to reduce energy intake in both rodents, and lean and obese humans (Batterham et al. 2002; Batterham and Bloom 2003; Batterham et al. 2003). Batterham et al have reported that PYY, administered at 2 nmol per m$^2$ of body surface area (which resulted in peak concentrations of ~53 pmol/L in lean, and 57 pmol/L in obese men) reduced energy intake by ~ 30% in both groups (Batterham et al. 2003). A more recent study infusing a range of doses of PYY$^{(3-36)}$ (0.2, 0.4 and 0.8 pmol/kg/min [resulting in peak concentrations of ~ 63, 88 and 150 pmol/L]) also
reported a dose-dependent suppression of energy intake. In response to the highest dose (0.8 pmol/kg/min), which resulted in supraphysiological concentrations of plasma PYY, suppression of energy intake was ~32%, however nausea was a considerable side effect (Degen et al. 2005). There is evidence that a threshold for plasma PYY concentrations to suppress energy intake exists; and it appears that shortly beyond this threshold, adverse effects (i.e. nausea) occur (Degen et al. 2005; le Roux et al. 2006). It is possible that the lack of effects Sloth et al observed on energy intake occurred as a result of administering a single, subcutaneous bolus of PYY\(_{1-36}\) and PYY\(_{3-36}\), which resulted in a longer, slower appearance of PYY in the plasma, rather than the continuous IV infusion utilised by others. They also used a dose-escalation design, whereby the dose of PYY administered was increased at subsequent study visits, which may have influenced their findings.

Studies using specific PYY receptor antagonists to evaluate the effects of endogenous PYY on appetite and energy intake have not been performed in humans due to a lack of availability and, thus, the role of endogenous PYY in appetite and energy intake regulation in humans remains unknown.

4.5.4 Glucagon-like peptide-1 (GLP-1)

(GLP-1 is a 33-amino acid peptide product of the glucagon gene. The glucagon gene is expressed in the pancreas and the small intestinal mucosal endocrine cells. The glucagon gene displays two additional glucagon-like sequences at its carboxyl terminus, and its primary translations product, proglucagon, is cleaved to release two peptides, GLP-1 and GLP-2, which have a high sequence homology to glucagon (Bell et al. 1983) (Table 4.1). which GLP-1 is released from L cells in the distal small intestinal mucosa in response to fat (Feinle et al. 2003), protein (Herrmann et al. 1995) and carbohydrate (Feinle et al. 2002a;
Pilichiewicz et al. 2007a). Bioactive GLP-1 is generated from GLP-1(1-37), and exists most abundantly as GLP-1(7-36) amide, and GLP-1(7-37). Both forms of GLP-1 are rapidly biodegraded by DPP-IV to the inactive forms GLP-1(9-36)amide and GLP-1(9-37), which are unable to access the GLP-1 receptor, and lack insulinotropic activity (Mentlein et al. 1993; Drucker 2006a). As such, only ~10% of the GLP-1 that is released reaches the systemic circulation (Deacon 2004). GLP-1 functions as an incretin hormone, stimulating insulin, and suppressing glucagon, release, which increases insulin-dependent glucose clearance in the periphery to maintain normoglycaemia (Gutniak et al. 1992; D'Alessio et al. 1994).

4.5.4.1 Effects of GLP-1 on gastrointestinal motility and hormone release

GLP-1 has been shown to slow gastric emptying in healthy (Nauck et al. 1997; Delgado-Aros et al. 2002; Little et al. 2006b), type 2 diabetic (Meier et al. 2003), and obese subjects (Naslund et al. 1998b; Flint et al. 2001). Exogenous administration of GLP-1 results in relaxation of the proximal stomach, inhibition of antral and duodenal motility and the stimulation of tonic and phasic pyloric pressure waves, in a load-dependent manner. A study conducted in our department reported that GLP-1 administered at 0.3 and 0.9 pmol/kg/min slowed gastric emptying of both liquids and solids (Little et al. 2006b). Others have demonstrated that intravenous administration of GLP-1 at 0.4 and 1.2 pmol/kg/min significantly inhibited the number and amplitude of antral and duodenal contractions, dose-dependently increased pyloric tone and significantly stimulated isolated pyloric pressure waves (Schirra et al. 2000). In addition, intravenous GLP-1 also enhanced the pyloric tone stimulated by duodenal infusion of lipid (Schirra et al. 2000).

A limited number of studies have examined the effects of endogenous GLP-1 on GI motor function, using the specific GLP-1 receptor antagonist, exendin(9-39). The administration of
exendin(9-39) has been shown to block the effects of GLP-1 on the slowing of gastric emptying in rodents (Tolessa et al. 1998), and to attenuate tonic and phasic pyloric motility in response to intraduodenal glucose in humans (Schirra et al. 2006).

4.5.4.2 Effect of GLP-1 on postprandial glycaemia

GLP-1 plays a significant role in regulating postprandial blood glucose. The effect of GLP-1 on blood glucose, insulin and glucagon release has generated considerable interest in the potential of GLP-1 (Schirra et al. 1998a; Schirra et al. 1998b), and specific GLP-1 analogues and receptor agonists, to improve the regulation of blood glucose concentrations in type II diabetes patients (Nauck 1998; Meier et al. 2003). This may be particularly important, as the insulinotropic effects of GIP are not maintained in type II diabetes (Nauck et al. 1993), and GIP has no effect on gastric emptying (Meier et al. 2004). Exogenous administration of GLP-1 lowers glycaemia by stimulating the release of insulin and inhibiting the release of glucagon (Schirra et al. 2006). Using exendin(9-39), Schirra et al. were able to demonstrate the key role of GLP-1 in incretin activity and glycaemia. When subjects were administered exendin(9-39) under fasting conditions blood glucose concentrations were increased, insulin concentrations remained unchanged, and plasma glucagon concentrations were reduced (Schirra et al. 2006). Moreover, when endogenous GLP-1 was stimulated by duodenal glucose, exendin(9-39) reduced plasma insulin concentrations, and resulted in elevated blood glucose concentrations.

While in fasted healthy individuals, exogenous GLP-1 stimulates insulin secretion (Kreymann et al. 1987), when GLP-1 is co-administered with a meal insulin levels appear to decrease (Nauck et al. 1997). Additional observations that postprandial administration of GLP-1 results in reduced, rather than augmented, insulin release, in both healthy (Nauck et
and type II diabetic subjects (Meier et al. 2003) suggest that GLP-1 may exert its glucose lowering effects by slowing gastric emptying (Nauck et al. 1997). A recent study, whereby the inhibitory effect of GLP-1 on gastric emptying was reversed using the prokinetic drug erythromycin, supports this notion, in that, despite increased insulin and GIP responses, erythromycin administration was associated with attenuation of the hypoglycaemic effects of GLP-1 (Meier et al. 2005).

4.5.4.3 Effects of GLP-1 on appetite and energy intake

The role of GLP-1 in the regulation of appetite and energy intake is somewhat inconsistent. For example, intravenous administration of GLP-1 at doses up 1.2 pmol/kg/min has been shown to reduce energy intake in humans in some studies (Gutzwiller et al. 1999; Naslund et al. 1999), but not others (Long et al. 1999; Brennan et al. 2005). Rodent studies have shown that intravenous GLP-1 decreases food intake (Turton et al. 1996), and the effects of endogenous GLP-1 food intake are attenuated by the GLP-1 receptor antagonist exendin(9-39) (Turton et al. 1996). When the actions of GLP-1 were blocked by exendin(9-39), food intake did not change in fasted rats, but was doubled in fed rats, and the feeding response to neuropeptide Y, an appetite-stimulant, was augmented (Turton et al. 1996). In humans, recent studies have demonstrated that GLP-1 receptor blockade fails to suppress satiety following a standardised breakfast (Melhorn et al. 2014), but has modest effects following ingestion of a liquid glucose preload (Steinert et al. 2014). Moreover, in both studies, the expected increase in voluntary food intake did not occur following GLP-1 receptor blockade. While the evidence in rats supports the notion that GLP-1 is a physiological regulator of satiety, these recent studies suggest that GLP-1 may not have a major physiological role in food intake in humans (Melhorn et al. 2014; Steinert et al. 2014). It cannot be ruled out however, that in these studies additional mechanisms, including GI hormones, may have
overridden the possible effect of GLP-1 receptor antagonism on satiation, and further work is required to elucidate the role of GLP-1 in the regulation of energy intake in humans.

4.5.5 Glucose-dependent insulinotropic peptide (GIP)

GIP was first identified for its ability to inhibit gastric secretions (Pederson et al. 1975), hence its classical name, ‘gastric inhibitory peptide’. GIP is encoded by the GIP gene, and circulates as a 42-amino acid biologically active peptide, secreted from K cells in the proximal small intestine, particularly the duodenum, in response to nutrients, especially carbohydrate (Ebert and Creutzfeldt 1980) and protein (Carr et al. 2008). The primary role of GIP in the body is as an incretin hormone, stimulating pancreatic insulin release in the presence of increased glucose concentrations, thus, regulating the glycaemic response (Tseng et al. 1996). GIP release is also stimulated by fat, but only weakly, and not sufficiently to stimulate insulin release (Ross and Dupre 1978). GIP exerts a greater proportion of the incretin effect than GLP-1 (Ahren 2013), since GLP-1 is rapidly degraded by DPP-IV and only ~10% of what is released reaches the systemic circulation (Deacon 2004). GIP also has direct insulinotropic effects on pancreatic beta cells, but does not exhibit the same effects on gastric emptying as GLP-1 (Yamada and Seino 2004). GIP release in response to a meal is proportional to both the caloric value, and rate of delivery of the nutrient to the small intestine, with the rate of nutrient absorption playing a key role in the modulation of GIP release (Creutzfeldt 1979).

4.5.5.1 Effects of GIP on gastrointestinal motility and hormone release

The effects of GIP on GI motility, particularly in humans, remain unclear. In rats, exogenous GIP has been shown to increase intestinal transit time (Ogawa et al. 2011). In dogs, exogenous GIP infused at supraphysiological doses suppressed MMC activity, which
reappeared shortly after cessation of the infusion (Thor et al. 1987). In humans, exogenous GIP administered at 2 pmol/kg/min (which resulted in supraphysiological GIP concentrations) has been found to have no effect on gastric emptying (Meier et al. 2004), while others have shown that exogenous GIP administered at 2 and 5 pmol/kg/min modestly accelerates gastric emptying (Edholm et al. 2010). That such high doses of exogenous GIP are required to produce only small effects on gastric emptying suggests GIP is not integral in modulating gastric motility.

4.5.5.2 Effects of GIP on postprandial glycaemia

GIP stimulates pancreatic beta-cell proliferation and postprandial insulin release. Intravenous GIP, when administered simultaneously with glucose, has been shown to stimulate insulin release, suggesting that the insulinotropic effect of GIP is dependent on the presence of glucose (Dupre et al. 1973). In the rat, administration of the GIP receptor antagonist, ANT-GIP (GIP (7–30)-NH₂), reduced insulin secretion by ~72 % without affecting the insulinotropic effects of GLP-1 or glucose, suggesting that GIP plays a major role in modulating the postprandial glycaemic response (Tseng et al. 1996).

GIP, administered at supraphysiological doses (20ng/kg/min) intravenously for 180 min immediately after a meal, has been shown to override the glucagonostatic effects of GLP-1 in patients with type II diabetes (Chia et al. 2009). During the infusion, glucagon release was increased, and postprandial blood glucose increased under already hyperglycaemic conditions (Chia et al. 2009). In a second study in patients with type 2 diabetes, co-administration of GIP and GLP-1 also showed that GIP antagonises the normal glucagon-lowering effect of GLP-1 (Mentis et al. 2011), however, the mechanisms underlying this remain unknown. Exogenous GIP administration has also shown that the insulinotropic effect of GIP is reduced
in type 2 diabetes (Nauck et al. 2004). This demonstrates the critical role of GIP in the regulation of glycaemia, as the loss of the insulinotropic effect of GIP characterises type II diabetes, while the effects of GLP-1 are preserved (Nauck et al. 2004).

4.5.5.3 Effects of GIP on appetite and intake

The effects of GIP on appetite and energy intake are unclear. GIP has been implicated in lipid metabolism, including the regulation of insulin-stimulated incorporation of fatty acid into triglycerides, stimulation of lipoprotein lipase activity and the stimulation of fatty acid synthesis (Gautier et al. 2008). In rats, GIP receptor blockade using a daily injection of the receptor antagonist (Pro³GIP) had no effect on body weight or energy intake over 14 days (Parker et al. 2007), suggesting that the effects of GIP on appetite are limited.

4.5.6 Glucagon

Glucagon is a 29-amino acid peptide, secreted from the pancreas in response to increases in blood glucose (Bromer et al. 1957). Glucagon is synthesised primarily in the A-cells located on the edges of the Islets of Langerhans (Baum et al. 1962), from the 160-amino acid precursor, proglucagon.

4.5.6.1 Effects of glucagon on gastrointestinal motility and hormone release

The evidence describing the effects of glucagon on GI motility and hormone release are inconsistent, both between, and within, species. For example, in dogs, intravenous administration of glucagon suppressed antral contractility, with no effect on duodenal motility (Shibata et al. 2001). In humans, intravenous exogenous glucagon has been shown in some studies, but not others, to delay gastric emptying of both a glucose (Chernish et al. 1978), and mixed nutrient (Jonderko et al. 1988; Jonderko et al. 1989) meal. Studies have
also shown that injection of a bolus dose of glucagon suppresses antral and duodenal contractility (Gregersen et al. 1988), abolishes jejunal contractility (Patel et al. 1979) and increases the duration of the MMC, which is associated with a decreased frequency of MMC cycles (Larsen et al. 1986).

4.5.6.2 Effects of glucagon on postprandial glycaemia

The primary role of glucagon in the body is to raise blood glucose during fasting, exercise and hypoglycaemia, by stimulating glycogenolysis and gluconeogenesis from lactate, pyruvate, glycerol and specific amino acids (Claus et al. 1983). Changes in the ratio of circulating insulin to glucagon shifts lipid metabolism from storage to release (Steinberg et al. 1959). Glucagon also acts to oppose the action of insulin in the liver to regulate glucose metabolism postprandially (Stalmans 1983) and may also directly stimulate insulin release (Samols et al. 1966) through its own receptors expressed on pancreatic β-cells, at least in non-diabetic individuals (Kawai et al. 1995; Moens et al. 1998). Hyperglucagonaemia is implicated in hyperglycaemia, and thus may play a role in the pathophysiology of diabetes.

4.5.6.3 Effects of glucagon on appetite and energy intake

Evidence for the effects of glucagon on appetite and energy intake is limited. In rats, intraperitoneal injection of glucagon suppressed food intake in a dose-dependent manner (Geary et al. 1986; Le Sauter and Geary 1991), while another study has shown that glucagon suppresses food intake in orally-fed, but not sham-fed rats, suggesting that a gastric, or post-gastric stimulus is required for glucagon to suppress energy intake (Le Sauter and Geary 1987). Using a specific antibody for glucagon, evidence in rats suggests that endogenous glucagon released in response to meal-related nutrients may account for up to 50 % of the meals satiating capacity (Langhans et al. 1982; Le Sauter et al. 1991). In humans evidence is
scarcer. Glucagon or CCK infused alone intravenously reduced the size of a subsequent meal. Conversely, when glucagon and CCK were infused concurrently, no additive effect on energy intake suppression was seen, suggesting some degree of antagonism (Geary et al. 1992).

### 4.5.7 Insulin

Insulin is a pancreatic peptide, synthesised in β-cells within the Islets of Langerhans, and insulin’s primary role within the body is to maintain glucose homeostasis. Insulin crosses the blood-brain barrier to directly access hypothalamic neurons, and thus regulate energy homeostasis (Pardridge et al. 1985; Banks et al. 1997). Insulin is released in response to an increase in plasma glucose concentrations, as well as by the presence of plasma amino acids (Floyd et al. 1966b; Floyd et al. 1966a; Knopf et al. 1966). The magnitude of insulin release in response to carbohydrate is critically dependent on the release of the incretin hormones, GLP-1 and GIP (Lavin et al. 1998; Pilichiewicz et al. 2007a). The understanding of the role of insulin in the regulation of GI motility and energy intake in humans is limited, since studies have not been able to distinguish between the effects of insulin, and the effects of hypoglycaemia, on these outcomes.

#### 4.5.7.1 Effects of insulin on gastrointestinal motility and hormone release

In dogs, intravenous infusion of insulin has been shown to stimulate gastric motility (Regan and Barnes 1933; Nelsen et al. 1966), and this effect is thought to be due to stimulation of the vagus nerve by hypoglycaemia (Walker et al. 1974). In humans, the effects of insulin on gastric emptying and GI motility are far more variable. For example, under euglycaemic conditions, hyperinsulinaemia has been shown to slightly, but significantly, slow gastric emptying of both solids and liquids in healthy humans (Eliasson et al. 1995; Kong et al.
1998). In contrast, hyperinsulinaemia has little to no effect on gastric emptying in people with type–I and II diabetes (Kong et al. 1999). Hyperinsulinaemia has also been shown to inhibit antral phase III activity, to shorten periods of duodenal phase III activity (Bjornsson et al. 1995), and increase MMC frequency under fasting conditions (Gielkens et al. 1997). In addition, in healthy individuals, hyperinsulinaemia appears to inhibit jejunal motility in the fed state, and slow small intestinal transit time in one study (Kong et al. 1998), but not another (Hasler et al. 1995). No significant changes in the number of antral, pyloric or duodenal pressure waves, or basal pyloric pressures, were observed in a 45-minute period after insulin was administered (i.e. hypoglycaemia) when compared with a euglycaemic state (saline infusion) (Fraser et al. 1991). As such, the effects of insulin appear to depend on both the insulin sensitivity of the individual, as well as the absolute amount of insulin in the circulation. Hyperinsulinaemic euglycaemic clamp studies may help to elucidate this.

### 4.5.7.2 Effects of insulin on postprandial glycaemia

An initial rise in blood glucose resulting from intestinal absorption triggers insulin release. Insulin acts to remove glucose from the blood to peripheral tissues at a dynamic rate, which maintains blood glucose levels within an acceptable physiological range of ~4-7 mM. Insulin stimulates cell growth and proliferation, the uptake of glucose, amino acids and fatty acids from the blood into cells and the processes that regulate glycogen, protein and lipid synthesis, and inhibits hepatic gluconeogenesis, glycogenolysis, lipolysis and protein degradation (Saltiel and Kahn 2001). Insulin resistance or deficiency results in dysregulation of these processes, and produces elevations in both fasting and postprandial glucose and lipid levels (Saltiel and Kahn 2001).
4.5.7.3 Effects of insulin on appetite and energy intake

The role of insulin in the regulation of appetite and food intake remains unclear. In animals, in the absence of hypoglycaemia, increased levels of circulating insulin appear to suppress energy intake (Nicolaidis and Rowland 1976; Woods et al. 1984). In humans, insulin infusion during euglycaemia had no effect on appetite sensations, suggesting that insulin does not enhance satiety, at least under these conditions (Chapman et al. 1998). In contrast, endogenous increases in insulin, occurring in response to a meal, have been demonstrated to be related inversely to energy intake at a subsequent meal, at least in lean subjects (Speechly and Buffenstein 2000; Verdich et al. 2001), and in response to a protein meal in lean and overweight, but not obese, subjects (Bowen et al. 2006).

4.5.8 Effects of macronutrients on gastrointestinal hormone release

Fat, carbohydrate and protein exert macronutrient-specific effects on the magnitude and pattern of secretion of GI hormones after a meal. This section will explore the comparative effects of fat, carbohydrate and protein on ghrelin, CCK, GLP-1, GIP and PYY release.

In both acute (i.e. single meal) (Monteleone et al. 2003) and longer-term (up to 2 weeks) (Weigle et al. 2003) studies, the meal-related suppression of ghrelin release has been shown to be greater in response to high-carbohydrate and high-protein, compared with high-fat meals/diets. A high-protein breakfast decreased postprandial ghrelin secretion more than a high-carbohydrate breakfast (Blom et al. 2006), however, no effects on ad libitum energy intake were observed. Others have reported that a high-protein meal led to lower ghrelin levels at 180 minutes compared with high-carbohydrate and high-fat meals in healthy adults, suggesting that dietary protein might enhance longer-term postprandial ghrelin suppression and satiety (Tannous dit El Khoury et al. 2006). In contrast, several studies have shown that
while carbohydrate and fat ingestion suppressed ghrelin release, ingestion of a protein-rich meal stimulated ghrelin levels (Erdmann et al. 2003; Erdmann et al. 2004). Others have found no relationship between ghrelin and energy intake in response to protein (Moran et al. 2005a), thus the relationship between ghrelin release, protein and energy intake remains unclear.

Concentrations of plasma CCK are more potently stimulated by intraduodenal lipid compared with carbohydrate in both healthy young and older men (Chapman et al. 1999; Feinle et al. 2002c). In addition, intraduodenal (Mearadji et al. 2001; Seimon et al. 2009b) and oral (Liddle et al. 1985) fat and protein have been shown to be significantly more potent than carbohydrate in stimulating the secretion of CCK. Intraduodenal leucine and L-phenylalanine have also been shown to stimulate CCK release (Steinert et al. 2015a; Steinert et al. 2015b). Oral administration of liquid protein preloads to lean and overweight men stimulated and prolonged CCK elevation more than glucose. In obese men, whey and casein milkshakes compared to glucose- or lactose-based drinks, increased plasma CCK by 71%, for up to 90 minutes after consumption of the meal (Bowen et al. 2006). In another study, a whey protein milkshake, as well as glucose and fructose drinks, increased CCK to a similar peak at 30 minutes. However while the CCK response to the glucose and fructose drinks gradually declined following this, the whey protein drink produced a second peak at t= 120 min (Bowen et al. 2007). This result is congruent with other studies, which have reported a unique CCK response to milk proteins, such that levels reach an initial peak around 15-20 minutes following ingestion, before falling slightly and peaking again at approximately 90 minutes (Fernstrom et al. 1979; Bowen et al. 2006; Brennan et al. 2012); the physiological relevance of this dual peak is not clear.
GLP-1 release is comparably stimulated by both oral and intraduodenal lipid and glucose (Feinle et al. 2002b; Adam and Westerterp-Plantenga 2005). Raben et al reported that GLP-1 responses were greatest after a high-protein meal, compared to meals rich in fat, carbohydrate or alcohol (Raben et al. 2003). In addition, Blom et al showed that a whey protein isolate enriched meal stimulated GLP-1 secretion more than a high-carbohydrate meal (Blom et al. 2006). Bowen et al also reported that 50g of whey protein stimulated GLP-1 (and CCK) release more than glucose and fructose, however the increases in GLP-1 and CCK did not translate to a greater suppression of energy intake at a subsequent ad libitum meal, possibly due to the size of the liquid preload (~250 kcal), and the duration between the preload and the ad libitum meal overriding the effects of appetite hormones (Bowen et al. 2007).

Oral ingestion of carbohydrate or fat has been shown to potently increase postprandial GIP release (Elliott et al. 1993), while protein appears to have lesser effects (Karamanlis et al. 2007). Co-ingestion of fat with carbohydrate or protein has been shown to augment the GIP response, with GIP responses more potent following carbohydrate+fat compared with protein+fat (Collier and O'Dea 1983), while co-ingestion of protein with glucose has been shown to blunt the GIP response to glucose (Karamanlis et al. 2007). In dogs, an intraduodenal infusion of mixed amino acids (i.e. digestion products of protein) or medium chain triglyceride stimulated GIP release, but not as potently as a long chain triglyceride, or glucose, infusion (O'Dorisio et al. 1976). In humans, some studies have reported a stimulatory effect of single amino acids, particularly glutamine, on GIP release (Greenfield et al. 2009; Chang et al. 2013). For example, both oral (Greenfield et al. 2009) and intraduodenal (Chang et al. 2013) administration of the amino acid glutamine has been shown to stimulate GIP release in both healthy and type 2 diabetic individuals, however the effect of oral glutamine on GIP is modest, compared with glucose (Greenfield et al. 2009).
Fat has been shown to be a more potent stimulus for PYY secretion compared with protein or carbohydrate respectively in an oral administration study (Adrian et al. 1985) and compared with glucose, when administered intraduodenally (Seimon et al. 2009b). Layer et al (1995) reported that the ileal infusion of lipid resulted in a significant increase in PYY release, while infusion of carbohydrate or protein produced no change in plasma PYY concentrations (Layer et al. 1995). In contrast, others have reported that protein and carbohydrate meals more potently stimulate PYY release than fat (Pedersen-Bjergaard et al. 1996). For example, Batterham et al have reported that a high-protein mixed-meal stimulated the largest increase in PYY when compared with a high-fat and high-carbohydrate meal. Brennan et al reported similar effects, whereby in lean subjects, the magnitude of the increase in PYY from baseline was greater in response to a high-protein and tended to be higher in response to a high-carbohydrate/low-protein, compared with a high-fat, mixed meal (Brennan et al. 2012).

Collectively, the above studies identify a number of inconsistencies in the evidence for the effects of protein on individual, yet interrelated, GI hormones. These may be partially attributable to methodological differences, including oral vs. intragastric, intraduodenal and ileal administration of nutrient, differences in composition of meals and different study populations. As the magnitude and secretory patterns of GI hormones in response to protein have not yet been clearly established, investigation is required to further characterise these, to determine their relationship to changes in appetite and energy intake, and to establish how these may be altered in obesity. In particular, this provides an opportunity to undertake novel research in the effects of modes of administration (oral versus small intestinal) of protein on these hormones and the relationships with energy intake and glycaemic control. This will aid in establishing the mechanisms by which protein exerts its appetite-suppressive effects, to ultimately assist in developing robust nutritional strategies to target obesity.
4.6 Perturbations of gastrointestinal function in obesity

Recent, albeit limited, research has suggested that GI motility and hormone release are disturbed in overweight and obese individuals. Given that gut motility and hormone release play a critical role in determining glycaemic regulation, appetite and energy intake, it is crucial to understand how GI function differs between lean and obese populations, and how these factors may contribute to the aetiology and maintenance of obesity.

4.6.1 Changes in gastric emptying in obesity

A number of studies have evaluated gastric emptying in obese humans, however the data remain inconclusive. For example, rates of gastric emptying in obese individuals have been reported to be slower (Horowitz et al. 1983; Maddox et al. 1989; Jackson et al. 2004), similar to (Zahorska-Markiewicz et al. 1986; Wisen and Johansson 1992; French et al. 1993; Glasbrenner et al. 1993; Hutson and Wald 1993; Verdich et al. 2000; Hellmig et al. 2006; Buchholz et al. 2013; Seimon et al. 2013) or faster (Wright et al. 1983; Gryback et al. 1996; Tosetti et al. 1996b; Näslund et al. 1998; Cardoso-Junior et al. 2007) than in lean individuals. The reasons for these differences are unclear, however, it is possible that they may be partly attributable to differences in methodologies between studies, including small numbers, which may have reduced the power to detect statistical differences beyond inter-individual variability (Wisen and Johansson 1992), differences in meal composition or meal consistency (e.g. solid (Tosetti et al. 1996a) compared with semi-solid (Maddox et al. 1989; French et al. 1993; Buchholz et al. 2013) or liquid (Wisen and Johansson 1992; Seimon et al. 2013) meals), types of food to which radionuclides were added (i.e. egg omelette (Tosetti et al. 1996a) eggs, toast and orange juice (Jackson et al. 2004), Ensure (a liquid mixed nutrient drink) (Seimon et al. 2013) or soups with covertly manipulated composition (French et al. 1993)), and the techniques used to assess gastric emptying (e.g. scintigraphy (Wright et al. 1983)).
1983; Maddox et al. 1989), breath testing (Jackson et al. 2004) or multiple-marker dilution method (Wisen and Johansson 1992). Different studies have also used different selection criteria for “obese” individuals, i.e. moderately (BMI < 40 kg/m²) (Wright et al. 1983; Jackson et al. 2004) compared with morbidly (BMI > 40 kg/m²) obese (Naslund et al. 1998a; Cardoso-Junior et al. 2007). Finally, age, gender, the habitual diet of individuals (Cunningham et al. 1991a; Cunningham et al. 1991b), the presence of additional co-morbidities, including diabetes, and weight stability (Park and Camilleri 2005) can also influence gastric emptying rates.

The rate of gastric emptying is dynamic, and is modulated in response to both the macronutrient composition, and size, of the meal. Under experimental settings, when intragastric pressure is increased, simulating a large meal, the pressure gradient between the stomach and small intestine is increased, which accelerates gastric emptying (Strunz and Grossman 1978; Geliebter et al. 1986). In a stomach with increased gastric capacity, a set meal volume may result in a smaller increase in intragastric pressure, slowing gastric emptying (Geliebter et al. 1992). Gastric distension stimulates gastric stretch receptors which activate hypothalamic neurons via vagal signalling (Paintal 1953), and induces feelings of satiety (Iggo 1955). In the case of a larger gastric capacity, a larger volume of food may be required to activate this signalling pathway, resulting in overconsumption.

It has been demonstrated that gastric capacity and maximal tolerable volume are greater in obese than lean subjects when tested with a water-filled intragastric latex balloon (Geliebter 1988; Geliebter et al. 1988), without differences in subsequent energy intake (Geliebter et al. 1988). In contrast, studies using a barostat have reported that basal gastric tone, postprandial gastric accommodation and perceptions of distension do not differ between lean and obese
individuals (Klatt et al. 1997). It is important to note that there are differences in compliance between a latex balloon, and heavier, less-compliant polyethylene bags, and the use of an electronically controlled barostat for pressure and volume distension, compared with manual inflation to a set volume, and these methodological differences may partially explain the differences in outcomes (Park and Camilleri 2005). Using the minimally invasive technique of single-photon emission computed tomography imaging, studies have reported no differences in fasting or postprandial gastric volume in response to a liquid mixed-nutrient meal, between obese and lean, however these authors did report a greater fasting volume of the distal stomach in obese, compared with lean subjects (Kim et al. 2001). While one study has reported no association between an increased BMI and fasting gastric volume (Delgado-Aros et al. 2004), another has shown an association between increased BMI and maximal tolerable volume of a liquid, so that overweight and obese subjects consumed ~225 kcal more at maximal satiation than lean individuals (Park and Camilleri 2005). Collectively, these data demonstrate the paucity of conclusive literature describing the changes in gastric capacity and gastric volume in obesity. If, indeed, these are increased in obesity, it may provide a potential target for improving meal-related satiety, and aiding in weight loss.

4.6.2 Changes in gastrointestinal motor function in obesity

To date, studies examining whether interdigestive motility differs between obese and healthy, lean individuals, and the clinical relevance of these differences, are limited. One study has reported disturbed fasting motility, including a shortened phase I, increased duration of phase II, and less frequent occurrence of phase IIIIs of the MMC, with a greater proportion of phase III contractions occurring more distally in the obese, when compared with lean individuals (Pieramico et al. 1992). It is unclear whether these changes are involved in the perpetuation of obesity, or if they occur as a result of being overweight. Some authors have suggested that
changes in GI motility may occur as a result of reduced sensitivity to GI hormones that promote motility, including CCK (Gallagher et al. 2009), however this notion requires further investigation.

Few studies have evaluated the potential differences in postprandial GI motor responses to macronutrients in the obese, compared with lean individuals. Moreover, if such differences do exist, limited data is available to establish whether they may be implicated in obesity, or if they can be modified. A recent study from our department assessed oral and GI sensitivities to oleic acid in lean and overweight/obese men. The authors reported a diminished number of IPPWs in response to a 90 min intraduodenal infusion of oleic acid (0.78 kcal/min) in the obese compared with lean participants (Stewart et al. 2011), suggesting that GI sensing of oleic acid, at least at this concentration, may be compromised in obesity. In another study, following a 120-min intraduodenal infusion of a long-chain triglyceride emulsion (Intralipid®, 2.86 kcal/min) BPP was greater in lean compared with obese individuals at baseline, and after 5 days of 30 % energy restriction (Seimon et al. 2014). Interestingly, after 12, but not 4 weeks, of 30 % energy restriction, BPP was significantly increased in the obese participants compared with baseline, and after 5 days of energy restriction (Seimon et al. 2014). This suggests that long-term energy restriction may at least partially restore GI sensitivity to intraluminal fat in obese individuals. In light of this evidence, it appears that obese individuals may be less sensitive to fat or lipid. In contrast, there is very limited evidence examining whether the GI motor responses to protein are maintained, or compromised in obesity, and hence further investigation is required.
4.6.3 Changes in the release of gastrointestinal hormones in obesity

There is considerable evidence that the secretion of GI hormones, including ghrelin, CCK, PYY, the incretin hormones, GLP-1 and GIP, as well as the secretion of insulin, may be altered both in the fasted state, and postprandially, in the obese. For example, fasting ghrelin concentrations have been reported to be inversely related to BMI (Tschop et al. 2001; Shiiya et al. 2002). In addition, since fasting ghrelin concentrations are lower in obese individuals, they cannot be suppressed by a meal to the same extent as in lean individuals (English et al. 2002). Interestingly, circulating ghrelin levels have been shown to increase during weight loss (Cummings et al. 2002), possibly as a compensatory mechanism to stimulate food intake and maintain the body’s energy stores.

When compared with lean individuals, fasting CCK concentrations do not appear to differ in obese individuals (French et al. 1993; Lieverse et al. 1994b; Seimon et al. 2014), while postprandial plasma CCK concentrations have been shown to be greater in obese individuals by some (French et al. 1993), but not others (Lieverse et al. 1994b; Seimon et al. 2014). Since CCK is a known anorexigenic hormone, an increase in plasma CCK concentrations in the obese may reflect a decreased sensitivity to CCK, which may be partly responsible for reduced satiety. In a recent study, CCK concentrations were significantly reduced in response to 10 weeks of a ~500 kcal VLCD, and these changes were maintained for 1 year after weight loss (Sumithran et al. 2011), which provides a potential mechanistic explanation for the difficulties involved with maintaining weight loss long term.

Available data suggest that both fasting (Batterham et al. 2003; Paddon-Jones et al. 2006) and postprandial (Paddon-Jones et al. 2006) plasma PYY concentrations are reduced in obese individuals. Batterham et al also reported that the diminished fasting and postprandial PYY
concentrations in obese occur in the presence of an increased energy intake (Batterham et al. 2003). This suggests an impaired release of PYY in the obese, which has implications in that the energy content of a meal required to stimulate sufficient PYY to induce an anorectic response may be higher, resulting in overconsumption.

The outcomes of studies investigating concentrations of incretin hormones in obesity are inconclusive. Fasting GLP-1 levels have been shown to be reduced (Lugari et al. 2004) or similar (Mersebach et al. 2003), in obese compared with lean. In addition, plasma GLP-1 responses to oral carbohydrate, or a mixed-nutrient meal, but not fat, were reported to be attenuated in obese, by some (Ranganath et al. 1996; Verdich et al. 2001), but not others (Seimon et al. 2013). It may be that the differences in GLP-1 release observed between studies reflects differences in gastric emptying between lean and obese, rather than an impaired stimulation of GLP-1 release, since gastric emptying rate is critical in determining the magnitude of the incretin response. Using an intraduodenal infusion of fat and carbohydrate, to deliver nutrient directly to the small intestine at a pre-determined rate and thus eliminate the effects of gastric emptying, it has been shown that plasma GLP-1 concentrations between healthy lean and obese subjects did not differ (Feinle et al. 2002a).

Changes in the release of plasma GIP concentrations in obese, compared with lean individuals is also inconclusive. For example, some studies have shown plasma GIP concentration to be greater during fasting, and in response to meal ingestion, in obese compared with lean (Stubbs 1996; Vilsboll et al. 2003), while other studies have found GIP concentrations to be similar (Verdich et al. 2001) or reduced (Wikarek et al. 2014).
Both fasting and postprandial concentrations of glucagon have been shown to be similar in lean and obese non-diabetic patients in some studies (Gossain et al. 1974), but not others (Arafat et al. 2013). Postprandial glucagon concentrations were also observed to be higher in obese, non-diabetic participants, compared with obese, type II diabetics (Kozawa et al. 2013). In contrast, despite fasting hyperglycaemia, fasting glucagon levels were higher, and were not suppressed, during a glucose infusion in diabetic subjects (Gossain et al. 1974).

Fasting insulin concentrations have consistently been shown to be elevated in non-diabetic obese, compared with healthy lean individuals (Prager et al. 1987; Korek et al. 2013). In addition, the magnitude of the insulin response to both a high-fat and high-carbohydrate meal is augmented in obesity, inducing hyperinsulinaemia (Korek et al. 2013). This hyperinsulinaemia has been associated by some (Flanagan et al. 2003), but not others (Caixas et al. 2002), with the attenuation of the postprandial reduction of ghrelin levels in obese individuals, which may perpetuate hyperphagia, and thus, weight gain, however the mechanisms underlying this remain unclear.

In summary, it appears that both fasting levels, and the release of GI hormones in response to nutrient are perturbed, albeit to varying degrees, in obese, compared with lean, individuals. The differences in the patterns of secretion, or the sensitivity to, GI hormones following meal ingestion may play a role in the attenuated satiety, increased energy intake and changes in glycaemic control that are commonly observed in obesity. These changes in GI function may be a result of over-eating, and this hypothesis is supported by studies evaluating the effects of experimental dietary over- and under-exposure. While the changes in GI motor and hormone responses to fat in obesity have been explored, evidence for changes in the GI responses to protein is scarce. Despite the known potent effects of protein on appetite and energy intake,
the physiological mechanisms, specifically in the GI tract are not well known. Moreover, the GI responses to protein, and how these differ between lean and obese individuals has not been thoroughly examined. Hence, the physiological mechanisms through which protein exerts its superior effects on appetite, how these mechanisms may be affected by obesity, and their role in modulating weight loss during a high-protein diet, are unclear.

### 4.7 Concluding remarks

This chapter has reviewed the anatomy of the GI tract and the role of GI factors, and the interactions between these parameters with the regulation of glycaemia and appetite and energy intake, in both lean and obese humans. It is well-known that the modulation of GI hormone function plays a key role in determining postprandial glycaemia, and in combination with GI motility, is involved in modulating appetite and energy intake responses. While the GI responses to fat and carbohydrate are well established, limited research to date has characterised the effects of protein on these GI mechanisms, both in health and obesity.

Examining the GI motor and hormonal responses to protein, and how these influence appetite and energy intake may help to explain the superior appetite-suppressive effects of protein. Importantly, an understanding of the GI mechanisms underlying the effects of protein on appetite and energy intake may aid in the development of targeted nutritional interventions for the treatment and prevention of obesity.
The studies described in subsequent chapters of this thesis (5-9), address the following aims:

i) To evaluate gastric emptying, GI hormone release and/or suppression, glycaemic responses, appetite and energy intake in response to oral protein loads in lean males (Chapter 6).

ii) To evaluate the effects of loads of intraduodenal protein on antropyloroduodenal motility, hormone release/suppression, glycaemic responses, appetite and acute energy intake in lean (Chapter 7) and obese (Chapter 8) males, and

iii) To compare the effects of intraduodenal protein, and lipid, alone and in combination on antropyloroduodenal motility, hormone release and/or suppression, glycaemic responses, appetite and acute energy intake in lean males (Chapter 9).
Chapter 5: Subjects And Methodologies

5.1 Introduction

This chapter describes the techniques that were utilised in the studies described in Chapter 6-9. All of the techniques are state-of-the-art, and well established in our laboratory, including high resolution manometry for the measurement of APD motility (Heddle et al. 1988c), 3-dimensional ultrasound for the measurement of gastric emptying (Gentilcore et al. 2006; Vanis et al. 2011), radioimmunoassay for the analysis of plasma concentrations of GI and glucoregulatory hormones (Feltrin et al. 2004; Feltrin et al. 2006), visual analogue scale (VAS) questionnaires for the assessment of appetite perceptions (Parker et al. 2004), and a standardised, cold, buffet-style meal for assessment of acute energy intake (Feltrin et al. 2004).

5.2 Subjects

5.2.1 Study participants

For all studies, healthy male subjects, aged 18–60 years, were enrolled. Lean subjects were of normal body weight for their height, with a BMI of 19–25 kg/m². Obese subjects, included in the study described in Chapters 8, had a BMI of 30.0–35 kg/m². Lean and obese subjects were matched for age. The number of subjects required for each study was determined using power calculations based on previous studies, as outlined in the individual chapters.

5.2.2 Subject recruitment

Subjects were recruited through advertisement in local newspapers, flyers throughout the Universities of Adelaide and South Australia, Flinders University, the Royal Adelaide
Hospital, and from an existing database of volunteers who had previously participated in research studies within our Department.

5.2.3 Common exclusion criteria

Prior to enrolment in a study, all subjects underwent a screening visit in our laboratory to determine their eligibility for the study. Subjects were questioned to exclude individuals who: had a history of GI surgery or disease, took medication which affected GI motor function, appetite or body weight, had significant illnesses (i.e. diabetes, cardiovascular disease, epilepsy), evidence of drug use or abuse, consumption of > 2 standard alcoholic drinks (20 g alcohol) per day, or > 5 standard alcoholic drinks per week, were smokers, had chronic food allergies or were lactose-intolerant. Healthy, lean subjects were also required to be unrestrained eaters (i.e. score ≤ 12 in the eating restraint component (Factor 1) of the “Three-Factor Eating Questionnaire” (Appendix I) (Stunkard and Messick 1985). While the degree of eating restraint was assessed and recorded in the overweight and obese subjects, it was not used as an exclusion, as overweight and obese subjects were expected to have some degree of eating restraint. A 10-mL blood sample was also taken at screening, and subjects excluded if their iron stores were outside the following ranges: Ferritin 20-300 µg/L; Iron 8-30 µmol/L; Transferrin 2.0-4.0 g/L; Transferrin Saturation 10-55 %.

Subjects in the study described in Chapter 8 were also excluded when their fasting blood glucose and/or glycated haemoglobin (HbA1c), which is a marker of long term (3 month) blood glucose control, were outside the following ranges: Blood glucose ≥ 6.0 mmol/L; HbA1c ≥ 6.0 %.
5.2.4 Ethics committee approval

All protocols were approved by the Royal Adelaide Hospital Research Ethics Committee. Each subject provided written, informed consent prior to their enrolment in the study and all experiments were carried out in accordance with the Declaration of Helsinki. Each trial was registered on the Australia and New Zealand Clinical Trial Registry, and their registration numbers are provided in their respective chapters. Written, informed consent was obtained from each subject prior to participation in a study. All subjects understood that their participation in the research was voluntary, and that they were free to withdraw from participation in the study at any time. All subjects were offered an honorarium of $15 per hour for participation in the study assessing gastric emptying of protein (Chapter 6) and $18 per hour for participation in all studies involving an intraduodenal catheter (Chapter 7-9), as approved by the Royal Adelaide Hospital Ethics Committee.

5.3 Assessment of gastrointestinal motor function

5.3.1 Three-dimensional ultrasonography

3D ultrasonography measurements (Chapter 6) were performed using a Logiq™ 9 ultrasonography system (GE Healthcare Technologies, Sydney, NSW, Australia) with TruScan Architecture (i.e. in-built magnetically sensed 3D). For 3D positioning and orientation measurement (POM), a transmitter was placed immediately behind, and to the left of the subject, at the level of the stomach, so that the subject was positioned between the transmitter and the ultrasound scanner, and a snap-on sensor attached to a 3.5C broad spectrum 2.5-4 MHz convex transducer (Tefera et al. 2002). As the transmitter produces a spatially varying magnetic field, which is easily distorted by ferrous and conductive metals, all metal objects were removed from the subject and from the area immediately between the POM transmitter and sensor (Liao et al. 2004).
5.3.1.1 Three-dimensional image acquisition

For 3D data acquisition, 3D sweeps of the total stomach were taken to evaluate total gastric volume. Subjects were instructed to hold their breath at the end of inspiration, not move, while the stomach was scanned using a continuous translational movement along its long axis. Scans started proximally at the left subcostal margin, where the transducer was tilted cranially to image the superior stomach, then moved distally to the gastroduodenal junction to produce transverse sections of the entire stomach (Gilja et al. 1997). Total scanning time for each image was ~10 seconds, and two scans were taken at each time point. When a gastric contraction was observed, the acquisition was paused until the contractile wave passed.

5.3.1.2 Image analysis

The raw data, as original scan planes, were copied to CD-ROM and transferred to a Windows workstation. The images were used for 3D reconstructions of the stomach using EchoPAC-3D software® (GE Vingmed Sound, Horten, Norway) (Figure 5.1). Total gastric volumes were derived at each time point and expressed as percentages of volumes at t= 0 min (volume immediately following drink consumption) with total gastric volume at t= 0 defined as 100% . Gastric emptying profiles were constructed, and the time at which 50 % of the meal had emptied from the stomach (50 % emptying time, T50) was derived.
Chapter 5

5.3.2 High-resolution manometry

High-resolution perfusion manometry is a well-established technique, used to measure changes in pressure in the GI lumen (Dent 1976; Heddle et al. 1988b), and relies on the occurrence of contractions that occlude the lumen around a multi-lumen manometric catheter. The catheter is perfused with degassed water through side-holes, and changes in pressure within regions of interest in the GI tract are transmitted via the water column within the catheter, to external transducers, where they are digitised and relayed to a software program.

5.3.2.1 Catheter design

The silicon rubber manometric catheter (100 cm total length, 3.5 mm outer diameter, Dentsleeve International Ltd, Mui Scientific, Ontario, Canada) consisted of 16 side-holes (0.1 mm in diameter) separated by 1.5 cm intervals. Six side-holes (channels 1–6) were positioned in the antrum, a 4.5 cm pyloric sleeve sensor (channel 7), with two channels present on the back of the sleeve (channels 8 and 9), was positioned across the pylorus, and seven side-holes (channels 10–16) were positioned in the duodenum. An additional side-hole

Figure 5.1: Ultrasonic image of the stomach, demonstrating A) region-of-interest; and B) 3D reconstructed volumetric image of the stomach (Gentilcore et al. 2006).
(1 mm in diameter) was positioned 11.75 cm distal to the pylorus, and this was used for the administration of the intraduodenal infusions.

5.3.2.2 Nasoduodenal intubation and manometry

Subjects were intubated with the catheter via an anaesthetised nostril (Lignocaine 5%, Orion Laboratories Pty Ltd, Calcatta, WA, Australia) into the stomach, and the catheter allowed to pass into the duodenum by peristalsis (Heddle et al. 1989). The maximum intubation length depended on the height of the individual, but did not exceed 75 cm. A sterile saline-filled reference electrode (20 gauge intravenous cannula) was inserted subcutaneously in the subject’s forearm to continuously measure the transmucosal potential difference (TMPD) at the most distal antral (channel 6) and the most proximal duodenal (channel 10) channels to maintain correct positioning of the catheter. The catheter was positioned with the sleeve sensor straddling the pylorus, registered by known TMPDs (channel 6 equal to, or more negative than -20 mV; channel 10 equal to, or more positive than -15 mV), with a difference between the two channels of at least 15 mV (Heddle et al. 1988a). All channels were perfused with degassed, distilled water at a rate of 0.15 mL/min, except for the two TMPD channels, which were perfused with degassed 0.9% saline (Heddle et al. 1989). Once the catheter was correctly positioned, fasting motility was monitored until the occurrence of a phase III of the MMC. At the end of phase III, a 15-min baseline was recording during phase I of the MMC, (which is a period of motor quiescence), after which, all study interventions began.
Figure 5.2: Schematic representation of the silicone-rubber manometric catheter used for intraduodenal nutrient infusion, incorporating six antral and seven duodenal side-holes, spaced 1.5 cm apart, a pyloric sleeve sensor and infusion port (Chapters 7-9).

5.3.2.3 Data acquisition and analyses

Manometric pressures were digitised and recorded on a computer-based system running commercially available software (Flexisoft®, Version 3, Oakfield Instruments, A/Prof GS Hebbard, Melbourne, Australia, written in Labview 3.1.1 (National Instruments)) and stored for subsequent analysis. Antropyloroduodenal (APD) pressures were analysed for the i) number and amplitude of antral and duodenal pressure waves (PWs), ii) basal pyloric pressure and iii) number and amplitude of IPPWs. Phasic PWs were defined by an amplitude of $\geq 10$ mmHg, with a minimum interval between peaks of 15 seconds for antral and pyloric PWs, and 3 seconds for duodenal waves (Samsom et al. 1998). Basal pyloric pressure (“tone”, or tonic pyloric pressure) was calculated for each minute by subtracting the pressure (excluding phasic pressures) recorded at the most distal antral channel, from the mean basal pyloric pressure recorded at the sleeve (Heddle et al. 1988b) using custom-written software (Prof. A Smout, University Medical Centre, Amsterdam, Netherlands). Numbers and
amplitudes of antral and duodenal pressures were used to calculate antral and duodenal motility indices (MIs) over 60 min, using the following equation: \( MI = \ln[(\text{sum of amplitudes} \times \text{no. phasic PWs}) + 1] \) (Camilleri and Malagelada 1984).

### 5.4 Evaluation of gastrointestinal and appetite responses to oral and intraduodenal nutrient

Orally administered nutrients were used to assess gastric emptying, intragastric meal distribution, GI hormones, appetite and energy intake. Intraduodenal infusion allowed assessment of GI function, appetite and energy intake without orosensory and gastric influences such as gastric distension and gastric emptying.

#### 5.4.1 Oral protein preloads

Three protein preloads, matched for sensory aspects, were used in the study described in **Chapter 6**; a saline control, and two containing a moderate (30 g), or high (70 g), amount of protein. The preloads were prepared in the Departmental research kitchen on the morning of the study. The base ingredients were distilled water, sodium chloride, lime flavoured low-calorie cordial (Bickford’s Australia Pty Ltd, South Australia) and whey protein isolate (ClearPro®, Fonterra Research Centre, Palmerston North, New Zealand). The amount of whey protein was modified to provide the three different loads, and osmolarity of the drinks was matched with the addition of sodium chloride to the solutions. The preloads were served in a wrapped cup with a straw to eliminate visual assessment of the different drinks.

#### 5.4.2 Intraduodenal infusions

In **Chapters 7-9**, intraduodenal infusions were administered using a manometric catheter as described (5.3.2.1). All infusion solutions were delivered into the small intestine at a rate of
4 mL/min (3kcal/min) for 90 minutes in Chapter 9, so the total volume infused in all study conditions was 360 mL, with a total energy content of 270 kcal. In Chapters 7 and 8, different protein loads were infused at a rate of 4 mL/min, for 60 minutes, so the total volume infused was 240 mL.

5.4.2.1 Lipid infusions
A commercially available lipid emulsion, Intralipid® (20 %, 300 mOsm/kg, 2 kcal/mL, Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia), which consists of long-chain triglycerides from soy bean oil (50 g/500 mL), egg phospholipids (1.2 g/500 mL) and glycerol anhydrous (2.25 g/500 mL), was used as the lipid infusion in Chapter 9. Intralipid® was diluted with distilled water to achieve the load required (3 kcal/min), and sodium chloride was added to match the osmolarity of the lipid solutions to the other infusions, as specified. Intralipid® was selected as the lipid emulsion, since the fatty acids comprising it, specifically linoleic, oleic and palmitic acids, resemble fatty acids that are common in the diet. Intralipid® has been used extensively in previous studies by our group, and others, evaluating the effects of lipid on GI function, appetite and energy intake, and is generally well tolerated (Chapman et al. 1999; Feinle et al. 2000; Pilichiewicz et al. 2006; Seimon et al. 2009a).

5.4.2.2 Protein infusions
A whey protein hydrolysate, (WPH 821 [18.1 % hydrolysate]; Fonterra Research Centre, Palmerston North, New Zealand) was kindly donated by Fonterra to be used as the protein source in Chapters 7 and 8, while a commercially available whey protein hydrolysate (Hydrolysed Whey Protein Isolate DH17 Ultra [18.5 % hydrolysate], MyoPure, Muscle Brand Pty Ltd, NSW, Australia) was used in Chapter 9. In all studies, dissolved in distilled
water, and sodium chloride added to match solutions for osmolarity. Accordingly, solutions in Chapters 7 and 8 had an osmolarity of 680 mOsm/L, and 480 mOsm/L in Chapter 9. Whey protein was selected as it is thought to have the most potent appetite-suppressive effects of dietary protein sources (Luhovyy et al. 2007). Hydrolysed whey was selected as it is more likely to resemble partially digested protein entering the duodenum after oral ingestion (Silk et al. 1985).

5.4.2.3 Lipid:protein combination infusions

Protein stock solutions were prepared by dissolving 28 (1 kcal/min for L2P1 infusion) or 56 (2 kcal/min for L1P2 infusion) g whey protein hydrolysate powder in distilled water, to a volume of 140 or 280 mL, respectively. 105 mL 20% Intralipid, and 87.5 mL 1.8% NaCl solution were added to the P1, and 52.5 mL 20% Intralipid and 175 mL 1.7% NaCl solution to the P2, stock solutions respectively, to give the L2P1 and L1P2 solutions a total load of 3 kcal/min.

5.5 Assessment of plasma hormone, blood glucose and total amino acid concentrations

For continuous blood sampling, an intravenous cannula was inserted into an antecubital vein. Venous blood samples (10-15 mL) were collected in ice-chilled ethylenediaminetetraacetic acid (EDTA)-treated tubes and centrifuged immediately at 3200 rpm for 15 min at 4°C to obtain plasma. Plasma samples were then frozen at –80°C for later analysis. For the studies described in Chapters 7 and 9, 4 mL venous blood samples for the measurement of serum insulin were collected in serum-Z tubes containing clotting beads. Radioimmunoassays were used to measure plasma concentrations of ghrelin, CCK, PYY, GLP-1, GIP, and glucagon,
and serum/plasma insulin. Samples from individual subjects were measured in the same run. Intra- and inter-assay coefficients of variation (CVs) are specified within the chapters.

### 5.5.1 Plasma ghrelin

For the studies described in Chapters 6 and 7, plasma ghrelin concentrations (pmol/L) were measured by radioimmunoassay, using a method modified from that previously published (Parker et al. 2005). The radiolabel (NEX388) was purchased from Perkin Elmer. Briefly, the sensitivity of the curve was improved by modification to a disequilibrium assay. The standard and samples were incubated with the antibody for 3-4 days prior to incubation with the radiolabel for a further 24 hours at 4°C. The minimum detectable concentration was 11.8 pmol/L.

### 5.5.2 Plasma peptide tyrosine tyrosine (PYY)

Plasma PYY (pmol/L) was measured by radioimmunoassay using an antiserum raised in rabbits (kindly donated by Dr. B Otto, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Munich, Germany) against human PYY(1-36) (Sigma-Aldrich), thus, the assay measures both PYY(1-36) and PYY(3-36). The antiserum showed <0.001 % cross-reactivity with human pancreatic polypeptide or sulphated CCK-8 and 0.0025 % cross-reactivity with human neuropeptide Y. Standards (1.6-50 fmol/tube) or samples (200 µL plasma) were incubated in 200 µl assay buffer (50 mM NaPO₄, 10 mM EDTA, 2 g/L gelatine, 0.1 g/L Na-Azide, pH 7.4) and a 1/12000 dilution of antiserum for 24 hours, followed by an incubation with 100 µl of 10000 cpm tracer (NEX3410, Perkin Elmer) for 24 hours. Antibody-bound tracer was separated from free tracer by second antibody precipitation, followed by incubation for 2 hours at room temperature and centrifugation at 4000 rpm for 20 minutes.
The supernatant was discarded, and the pellets were counted in a gamma counter (Brennan et al. 2008). The minimum detectable concentration was 1.5 pmol/L.

### 5.5.3 Plasma cholecystokinin (CCK)

Plasma CCK-8 concentrations (pmol/L) were measured after ethanol extraction using an adapted radioimmunoassay (Santangelo et al. 1998). Standards were prepared using synthetic sulphated CCK-8 (Sigma-Aldrich, St Louis, MO, USA) and an antibody (C2581, Lot 105H4852, Sigma-Aldrich). Sulphated CCK-8 $^{125}$I, labelled with Bolton and Hunter reagent (Perkin Elmer, Boston, MA, USA) was used as tracer. The antibody-bound fraction was separated by the addition of dextran-coated charcoal containing gelatine and the radioactivity determined in the supernatants following centrifugation. The minimum detectable concentration was 1 pmol/L.

### 5.5.4 Plasma glucagon-like peptide-1 (GLP-1)

Plasma GLP-1 concentrations (pmol/L) were determined after ethanol extraction, using a radioimmunoassay kit (GLPIT-36HK, Millipore, Billerica, MA) (Pilichiewicz et al. 2007a). The antibody used does not cross-react with glucagon, gastric inhibitory peptide, or other gut or pancreatic peptides, and it measures intact GLP-1(7–36) amide as well as the degraded form, GLP-1(9–36) amide. The minimum detectable concentration was 3 pmol/L.

### 5.5.5 Plasma glucose-dependent insulinoaotropic peptide (GIP)

Total GIP (pmol/L) was measured by radioimmunoassay with the use of modifications of a previously published method (Wishart et al. 1992). Specifically, the standard curve was prepared in buffer, rather than extracted, charcoal-stripped serum, and the radio-iodinated label was supplied by Perkin Elmer. The minimum detectable concentration was 2 pmol/L.
5.5.6 Plasma glucagon

Plasma glucagon concentrations (pmol/L) were measured by RIA (GL-32K, Millipore, Billerica, MA). The antibody used does not cross-react with insulin, proinsulin, C-peptide, somatostatin, or pancreatic polypeptide and has <0.1% cross-reactivity with oxyntomodulin. The minimum detectable concentration was 5.7 pmol/L.

5.5.7 Plasma/serum insulin

For Chapters 7 and 9, insulin concentrations were obtained from serum. For Chapters 6 and 8, insulin concentrations were obtained from plasma. Serum and plasma insulin concentrations (mU/L) were measured by ELISA (10-1113, Mercodia, Uppsala, Sweden) (Pilichiewicz et al. 2007a). The minimum detectable concentration was 1.0 mU/L.

5.5.8 Blood glucose concentrations

Venous blood glucose concentrations (mmol/L) were measured immediately by the glucose oxidase method using a portable glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA) (Horowitz et al. 1991). The accuracy of this method has been confirmed in our laboratory using the hexokinase technique (Horowitz et al. 1991).

5.5.9 Total amino acid concentrations

Plasma total amino acid (TAA) concentrations (mmol/L) were analysed in Chapter 6 using precolumn derivatisation with 6-aminquinolyl-N hydroxysuccimimidyl carbamate (AQC). The derivatives were then separated and quantified by reversed-phase high-performance liquid chromatography (HPLC). The AAs were detected by fluorescence. Before derivatisation, 100 µl of plasma samples were diluted 1:1 with internal standard solution (Norvaline) and deproteinised by ultra-filtration through a membrane with 10 kDa nominal
molecular weight cut-off (Ultrafree MC with PL-10 membrane, Millipore, MA, USA). AAs contained in the filtrate (100 µl) were labelled using the Waters AccQTag™ chemistry and analysed using a Waters Acquity™ UPLC system (Waters Corporation, MA, USA). The analysis was performed at the Australian Proteome Analysis’s Facility established under the Australian Government’s National Collaborative Research Infrastructure Strategy (NCRIS). All analyses were performed by the same technician.

### 5.6 Assessment of appetite and eating behaviour

#### 5.6.1 Visual analogue scale questionnaire

Validated visual analogue scale questionnaires (VAS) are the most commonly used tool to measure subjective appetite perceptions and GI symptoms during or after a feeding challenge (Flint et al. 2000; Parker et al. 2004). Perceptions of appetite, including hunger, fullness, desire-to-eat and prospective consumption, as well as GI symptoms, including nausea and bloating, were measured. Other perceptions, including drowsiness, tiredness, happiness and anxiety, were included to distract subjects from the main purpose of the questionnaire, but were not evaluated. The VAS comprised of 100 mm horizontal lines with strengths of perceptions anchored at each end, describing the extremes of each sensation (i.e. 0 mm “I am not hungry at all”, 100-mm “I am extremely hungry”). Subjects were asked to place a vertical mark along the scale, corresponding with the strength of the sensation they felt at the time point at which the VAS was administered (Appendix II). VAS questionnaires were administered at baseline, and at 15 minute intervals throughout the studies.

#### 5.6.2 Buffet meal

A commonly used method for quantifying the amount of food a subject consumes after a specific treatment, meal or preload is *ad libitum* consumption from a buffet meal (Feltrin et
The buffet meal contained a variety of food items, as detailed in Table 5.1, and the amount of food offered was in excess of what the subject was expected to consume (Feltrin et al. 2004). The buffet meal was presented at a fixed time point, immediately after the final ultrasound image was taken (3 hours after the preload) (Chapter 6), or immediately after the cessation of intraduodenal infusions (Chapters 7-9). Subjects were instructed to consume freely from it until they were comfortably full; the buffet was removed after 30 minutes. All food items were weighed before and after presentation to the subject, and energy consumption (kcal), amount eaten (g) and macronutrient intake of fat, carbohydrate and protein (absolute (g) and % of total) were calculated using commercially available software (Foodworks Version 3.01, Xyris Software (Australia) Pty Ltd, Highgate Hill, QLD, Australia) (Feltrin et al. 2004).
Table 5.1: Foods offered in buffet meals

<table>
<thead>
<tr>
<th>Food items</th>
<th>Amount served</th>
<th>Energy content, kcal</th>
<th>Fat, g</th>
<th>Carbohydrate, g</th>
<th>Protein, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wholemeal bread, 4 slices</td>
<td>125</td>
<td>307.6</td>
<td>4.4</td>
<td>50.0</td>
<td>12.6</td>
</tr>
<tr>
<td>White bread, 4 slices</td>
<td>125</td>
<td>304.6</td>
<td>2.5</td>
<td>57.8</td>
<td>10.5</td>
</tr>
<tr>
<td>Ham, sliced</td>
<td>100</td>
<td>108.2</td>
<td>3.6</td>
<td>0.0</td>
<td>18.8</td>
</tr>
<tr>
<td>Chicken, sliced</td>
<td>100</td>
<td>161.7</td>
<td>7.0</td>
<td>0.0</td>
<td>24.6</td>
</tr>
<tr>
<td>Cheese, sliced</td>
<td>100</td>
<td>403.5</td>
<td>33.3</td>
<td>1.0</td>
<td>25.8</td>
</tr>
<tr>
<td>Tomato, sliced</td>
<td>100</td>
<td>13.4</td>
<td>0.1</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Cucumber, sliced</td>
<td>100</td>
<td>10.5</td>
<td>0.1</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Lettuce</td>
<td>100</td>
<td>6.5</td>
<td>0.1</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Strawberry yoghurt</td>
<td>200</td>
<td>184.0</td>
<td>3.8</td>
<td>26.9</td>
<td>10.2</td>
</tr>
<tr>
<td>Fruit salad</td>
<td>150</td>
<td>89.2</td>
<td>0.1</td>
<td>30.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Chocolate custard</td>
<td>150</td>
<td>158.0</td>
<td>5.2</td>
<td>22.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Apple</td>
<td>200</td>
<td>100.8</td>
<td>0.2</td>
<td>25.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Banana</td>
<td>200</td>
<td>171.0</td>
<td>0.2</td>
<td>39.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Orange juice, unsweetened</td>
<td>300</td>
<td>118.2</td>
<td>1.0</td>
<td>25.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Iced coffee</td>
<td>500</td>
<td>355.9</td>
<td>8.5</td>
<td>51.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Water</td>
<td>600</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Margarine</td>
<td>21</td>
<td>129.5</td>
<td>14.6</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>21</td>
<td>77.8</td>
<td>6.8</td>
<td>4.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Milky Way®, 1 bar</td>
<td>13</td>
<td>58.2</td>
<td>1.2</td>
<td>9.8</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>3205</strong></td>
<td><strong>2758</strong></td>
<td>93.5</td>
<td><strong>349.3</strong></td>
<td><strong>134.7</strong></td>
</tr>
</tbody>
</table>

*aSunblest, Tiptop, Australia; bHans Deli leg ham, Woolworths, Australia; cVirginia chicken, Woolworths, Australia; dCoon tasty cheese slices, Australian Cooperative Foods Ltd., Australia; eYoplait, National Foods Ltd., Australia; fGoulburn Valley, SPC Ardmona Operations Ltd., Australia; gYogo, National Foods Ltd., Australia; hDaily Juice Company, Australia; iFarmers Union, Balemar Pty Ltd., Australia; jFlora, Unilever Australasia, Australia; kKraft, Kraft Foods Ltd., Australia; lMars Inc, Virginia, USA.
5.6.3 Percentage compensation

The percent compensation (\% compensation) describes the accuracy with which an individual is able to adjust their subsequent energy intake following a preload/infusion to maintain energy balance. \% compensation for a given preload/infusion was calculated as the difference between energy intake from the *ad libitum* buffet meal on the control day and in response to the test infusant/meal, divided by the difference in the energy content of the test infusant and the control, multiplied by 100 \% (i.e. \% compensation = \((EI_{\text{buffet; control infusant}} - EI_{\text{buffet; test infusant}}) / (\text{Energy content}_{\text{test infusant}} - \text{Energy content}_{\text{control infusant}})\) x 100) (Johnson and Birch 1994), so that an index of 100 \% represents full compensation of the caloric load of the infusion.

5.6.4 Three-factor eating questionnaire

Since particular eating behaviours may influence the response of individuals to treatments, in terms of energy intake, it was important to identify, and exclude, individuals with restrictive eating behaviour. The Three Factor Eating Questionnaire, devised by Stunkard and Messick (1985) (Stunkard and Messick 1985) assesses three factors of human eating behaviour; i) cognitive restraint of eating, ii) disinhibition of eating, and iii) hunger. The questionnaire contains fifty one items; twenty one of which assess Factor I, sixteen assessing Factor II and Fourteen assessing factor III (see Appendix I for scoring and distribution of factors of this questionnaire). The factor used as an exclusion criterion in this thesis is Factor I, which characterises “restrained eating”, and describes the tendency of individuals to restrict their food intake as a means of controlling body weight. A score of \(\leq 12\) for Factor I in the Three Factor Eating Questionnaire was used as a cut-off point – healthy subjects were excluded from participating in these studies if their score was \(> 12\), as this classified them as restrictive.
eaters. As described earlier, in obese subjects eating restraint was assessed, but not used as an exclusion, as they were expected to exhibit some degree of eating restraint.

5.7 Statistical analysis

Statistical tests used in each study are described in individual chapters. Data were analysed using commercially available statistical software (SPSS Statistics version 19 (SPSS Inc., Chicago, IL, USA). In accordance with statistical hierarchies, significant effects have been reported as treatment*time interactions, treatment and/or time effects, in that order. Thus, when a treatment or time effect is reported, no treatment*time interaction was observed. In all studies, data are presented as mean values ± standard error of the mean (SEM), and a P value <0.05 was considered statistically significant in all analyses. Relationships between parameters of choice were assessed using linear within-subject correlations (r) where appropriate (Bland and Altman 1995b).
## Statement of Authorship

| Title of Paper | Acute load-dependent effects of oral whey protein on gastric emptying, gut hormone release, glycemia, appetite, and energy intake in healthy men. |
| Publication Status | ✔ Published |

### Principal Author

| Name of Principal Author (Candidate) | Amy Theresia Hutchison |
| Contribution to the Paper | Designed the research, conducted the research, analysed the data and performed statistical analysis. Contributed to data interpretation and wrote the manuscript. |

| Overall percentage (%) | 30% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |

| Signature: | Date: 25/11/15 |

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

| Name of Co-Author | Diana Piscitelli |
| Contribution to the Paper | Analysed the data; assisted with data interpretation and preparation of the manuscript. |

| Signature: | Date: 23 November 2015 |

| Name of Co-Author | Michael Horowitz |
| Contribution to the Paper | Designed the research, contributed to data interpretation and preparation of the manuscript. |

<p>| Signature: | Date: 24/11/15 |</p>
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<th>Name of Co-Author</th>
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<tr>
<td>Karen L. Jones</td>
<td>Contributed to data interpretation and preparation of the manuscript.</td>
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<td>Peter M. Clifton</td>
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<td>Scott Stanfield</td>
<td>Conducted hormone analysis.</td>
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<td>Trygve Haanen</td>
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<td>Name of Co-Author</td>
<td>Natalie Lancombe-Murah</td>
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<tr>
<td>-------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Contribution to the Paper</td>
<td>Designed the research, analyzed data and performed statistical analysis. Contributed to data interpretation and writing of the manuscript.</td>
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This publication is included on pages 125 - 150 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.3945/ajcn.115.117556
## Statement of Authorship

**Title of Paper**
Intraduodenal protein modulates antropyloroduodenal motility, hormone release, glycemia, appetite, and energy intake in lean men.

**Publication Status**
- Published
- Accepted for Publication
- Submitted for Publication
- Unpublished and Unsubmitted work written in manuscript style

**Publication Details**

## Principal Author

**Name of Principal Author (Candidate)**
Amy Therese Hutchison (RYAN)

**Contribution to the Paper**
Conducted the research, analysed the data and performed statistical analysis. Contributed to data interpretation and wrote the manuscript.

**Overall percentage (%)**
30%

**Certification:**
This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would contrain its inclusion in this thesis. I am the primary author of this paper.

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## Co-Author Contributions

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Christine Feisle-Bisset

**Contribution to the Paper**
Designed the research, contributed to data interpretation and preparation of the manuscript.

**Signature**

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**Name of Co-Author**
Anisima Kallas

**Contribution to the Paper**
Conducted the research.

**Signature**

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**Date**
25/11/2015

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**Date**
19.11.2015
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<td>Judith M Wishart</td>
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<td>Peter M Clifton</td>
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<td>Michael Horowitz</td>
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<td>26/1/2015</td>
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<td>Natalie Leacombe-Marsh</td>
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| Publication Status | ☑ Published  
☑ Submitted for Publication  
☑ Unpublished and Unsubmitted work written in manuscript style |

### Principal Author

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<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Amy Therese Hutchison</th>
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<tr>
<td>Contribution to the Paper</td>
<td>Designed the research, conducted the research, analysed the data and performed statistical analysis. Contributed to data interpretation and wrote the manuscript.</td>
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<td>Overall percentage (%)</td>
<td>35%</td>
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<td>Conducted hormone analysis.</td>
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<td>Michael Horowitz</td>
<td>Designed the research, contributed to data interpretation and preparation of the manuscript.</td>
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<tr>
<td>Peter M Clifton</td>
<td>Contributed to data interpretation and preparation of the manuscript.</td>
</tr>
<tr>
<td>Natalie Lascombe-March</td>
<td>Designed the research. Contributed to data interpretation and preparation of the manuscript.</td>
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http://dx.doi.org/10.3945/ajcn.115.114538
# Statement of Authorship

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| Name of Principal Author (Candidate) | Amy Therese Hutchison (RYAN) |
| Contribution to the Paper | Conducted the research, analysed the data and performed statistical analysis. Contributed to data interpretation and wrote the manuscript. |
| Overall percentage (%) | 30% |

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| Name of Co-Author | Natalie Lascombe-March |
| Contribution to the Paper | Designed and conducted the research. Contributed to data interpretation and preparation of the manuscript. |

Signature: [signature]

Date: 25.11.15

| Name of Co-Author | Alexander A Sales |
| Contribution to the Paper | Conducted the research and analysed data. |

Signature: [signature]

Date: 25.11.2015
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Chapter 10: Conclusions

The studies reported in this thesis have examined key aspects of the inter-related gastrointestinal mechanisms involved in the regulation of glycaemia, appetite and energy intake in response to protein in lean and obese individuals. Three broad aims of the research were: i) to assess gastrointestinal motor and hormone, glycaemic, appetite and energy intake responses, to increasing loads of protein administered both orally and intraduodenally in lean individuals, ii) to determine the comparative effects of equicaloric loads of protein and lipid, and combinations of protein and lipid on gastrointestinal motility, hormone release, glycaemia, appetite and energy intake in healthy, lean individuals and iii) to establish whether obese individuals remain sensitive to the effects of intraduodenal protein, and to explore the comparative effects of protein on gastrointestinal function, blood glucose control, appetite and energy intake in obese, compared with lean, individuals.

The study in Chapter 6 was carried out to establish the effects of increasing loads of protein, consumed orally, on gastric emptying, gastrointestinal hormone release, appetite and energy intake. We demonstrated that the gastric emptying rate of protein was comparable between 30 g and 70 g loads, when expressed as kilocalories per minute. Furthermore, the study showed that protein empties from the stomach at rates within the range of 1-4 kcal/min, as has been previously established for gastric emptying of fat and carbohydrate. Congruent with this, we observed similar patterns and magnitudes of release of CCK, GLP-1, GIP, and the suppression of ghrelin, in the first ~45 min following both protein loads. However, beyond this, the 70 g load was associated with a sustained GI hormone release, and reduced blood glucose, concomitant with a prolonged exposure to nutrient, compared with the 30 g load. Despite this, energy intake at the buffet meal was similar. Collectively, these data suggest
that the gastric emptying rate of protein is independent of the amount of protein ingested, and importantly, that a threshold amount of protein of ~30 g is sufficient to modify GI hormone, glycaemic and appetitive responses, with no further benefit conferred by higher doses; at least in lean healthy individuals.

**Chapter 7** investigated the effects of increasing loads of protein, infused intraduodenally to standardise the rate of delivery to the small intestine, eliminating orosensory and gastric influences, in lean, healthy individuals. The study demonstrated that a 60 min infusion of whey protein had load-dependent effects on the suppression of antral motility, and the stimulation of isolated pyloric pressure waves, plasma CCK and GLP-1 and insulin. We also demonstrated that the highest load of intraduodenal protein, 3 kcal/min (delivering 48 g of protein), reduced blood glucose, while maintaining normoglycaemia. Importantly, we found that whey protein, administered intraduodenally, had very potent effects on the suppression of energy intake, in that the magnitude of the reduction of energy intake at the buffet meal exceeded the caloric content of the infusion. Moreover, when considered in light of the findings from Chapter 6, these data add weight to the notion that ~30-48 g of protein is probably optimal to reduce energy intake at the next meal. These findings are of interest for the development of nutrition-based interventions for appetite control and weight loss, in that a moderate amount of protein may be able to be incorporated into a snack, or “pre-load”, to reduce subsequent energy intake.

The study in **Chapter 8** explored the hypothesis that, in the obese, gastrointestinal sensitivity to protein remains intact, and obese individuals would have similar responses to intraduodenal protein, compared with lean individuals. We found that, in obese individuals, protein exerted load-dependent effects on GI motor and hormonal function. When the
responses were compared between lean and obese, following equicaloric protein loads (3 kcal/min), GI motor and CCK and GLP-1 responses were similar. Energy intake was only marginally higher following the infusion in obese individuals. Of interest, we observed that blood glucose was reduced by a similar magnitude in both groups; however, the obese group had significantly greater insulin, and a comparatively blunted GIP, response. This may be indicative of early disruption of the insulin-incretin axis in apparently healthy, obese individuals. Whether this may also be accompanied by changes in gastric emptying, and the long-term implications of the elevated insulin responses, require further investigation. In contrast to responses to intraduodenal lipid, which appear to be blunted in the obese, the gastrointestinal tract appears to remain responsive to protein, which highlights a potential mechanism for targeting obesity with diet-based interventions that contain a moderately increased content of high-quality dairy-based protein.

In light of the potent effects of the 3kcal/min protein load observed in Chapter 7 and the known effects of lipid on gastrointestinal motility and hormone release, Chapter 9 evaluated the comparative effects of equicaloric (3kcal/min) loads of lipid and protein, and combinations of the two, on gastrointestinal function, appetite and energy intake in healthy, lean individuals. The study established that protein and lipid infused intraduodenally had differential effects on gastrointestinal motor and hormone function. Specifically, lipid more potently stimulated pyloric pressures, CCK and GLP-1, while protein had significantly greater effects on insulin and glucagon release than lipid. Despite this, protein and lipid similarly suppressed energy intake, suggesting that while GI mechanisms appear to play a key role in the appetite-suppressive effects of lipid, additional mechanisms may be involved with respect to protein. These mechanisms require further investigation. Interestingly, protein and lipid administered in 2:1 and 1:2 combinations did not have additive effects on
any of the outcomes measured. This suggests it is highly probable that there is a critical threshold, or rate of delivery, of each nutrient in the small intestine, to elicit an effect.

Some considerations must be taken into account when interpreting the data presented in this thesis. Only males were studied as they have been reported to be are more sensitive to dietary manipulation than females. Whey protein was selected as the source of protein in all studies, as the literature suggests it has more potent effects on appetite and energy intake than other protein sources, and it is one of the most commonly consumed proteins in Australia and other Western countries; however, the findings cannot necessarily be extended to other protein sources. Finally, while we sought to characterise a number of physiological responses to protein in healthy lean humans, we would expect modest differences in some GI mechanisms between obese compared with lean humans, as was demonstrated in Chapter 8. Future directions should consider the effects of different sources of protein on GI motor and hormone function, glycaemia and appetite, and whether these sources, alone or incorporated (in different ratios) into a mixed-nutrient meal, might be useful in modulating energy intake. The potential for whey protein for modifying blood glucose control, particularly in individuals with type 2 diabetes, also warrants further investigation. Finally, if nutrition-based therapies to modulate appetite for the purposes of weight management are to be developed further, future studies should consider the differences in GI function between lean and overweight individuals, and the implications for this on appetite and energy intake.

Collectively, the data have provided novel insights into the roles that gastrointestinal motor and hormone responses to dietary protein play in the regulation of blood glucose, appetite and energy intake in healthy lean and obese subjects. These observations provide substantial and new information about a number of gastrointestinal mechanisms through which high-protein
diets may be effective in maintaining satiety, and thus reducing body weight over the long-term. The data represent an important contribution to the current knowledge of basic appetite and glucoregulatory physiology, and have established several key gastrointestinal mechanisms underlying the effects of protein in the diet. As such, the findings in this thesis provide a substantial foundation for the development of more tailored dietary interventions as potential treatments for obesity and obesity-related diseases.
Appendices

Appendix I: 3 factor Eating Questionnaire and scoring

Factor 1 (F1) = eating restraint
Factor 2 (F2) = hunger
Factor 3 (F3) = disinhibition

Questions under each factor are denoted at the end of the question; answers that score in that factor are circled.
Part 1: Read each of the following 36 statements carefully. If you agree with the statement or feel that it is true as applied to you, answer true by circling the (T). If you disagree with the statement, or feel that it is false as applied to you, answer false by circling the (F). Be certain to answer all of the questions.

1. When I smell a freshly baked pizza, I find it very difficult to keep from eating, even if I have just finished a meal. (F)
2. I usually eat too much at social occasions, like parties and picnics. (F)
3. I am usually so hungry that I eat more than three times a day. (F)
4. When I have eaten my quota of calories/fat, I am usually good about not eating any more. (F)
5. Dieting is so hard for me because I just get too hungry. (F)
6. I deliberately take small helpings as a means of controlling my weight. (F)
7. Sometimes things just taste so good that I keep on eating even when I am no longer hungry. (F)
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. (F)
9. When I feel anxious, I find myself eating. (F)
10. Life is too short to worry about dieting. (T)
11. Since my weight goes up and down, I have gone on reducing diets more than once. (F)
12. I often feel so hungry that I just have to eat something. (F)
13. When I am with someone who is overeating, I usually overeat too. (F)
14. I have a pretty good idea of the number of calories/grams of fat in common foods. (F)
15. Sometimes when I start eating, I just can’t seem to stop. (F)
16. It is not difficult for me to leave something on my plate. (F)
17. At certain times of the day, I get hungry because I have got used to eating then. (F)
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. (F)
19. Being with someone who is eating often makes me hungry enough to eat also. (F)
<p>| | | |</p>
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<tr>
<td>20. When I feel blue, I often overeat. (F2)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>21. I enjoy eating too much to spoil it by counting calories, counting grams of fat or watching my weight. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>22. When I see a real delicacy, I often get so hungry that I have to eat right away. (F3)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>23. I often stop eating when I am not really full as a conscious means of limiting the amount I eat. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>24. I get so hungry that my stomach often seems like a bottomless pit. (F3)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>25. My weight has hardly changed at all in the last ten years. (F2)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>26. I am always hungry, so it is hard for me to stop eating before I finish the food on my plate. (F3)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>27. When I feel lonely, I console myself by eating. (F2)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>28. I consciously hold back at meals in order to not gain weight. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
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<td>29. I sometimes get very hungry late in the evening or at night (F3)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>30. I eat anything I want anytime I want. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
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<td>31. Without even thinking about it, I take a long time to eat. (F2)</td>
<td>(T)</td>
<td>(F)</td>
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<td>32. I count calories/grams of fat as a conscious means of controlling my weight. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>33. I do not eat some foods because they make me fat. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>34. I am always hungry enough to eat at any time. (F3)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>35. I pay a great deal of attention to changes in my figure. (F1)</td>
<td>(T)</td>
<td>(F)</td>
</tr>
<tr>
<td>36. While on a diet, if I eat a food that is not allowed, I often then splurge and eat other high calorie foods. (F2)</td>
<td>(T)</td>
<td>(F)</td>
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</table>
Part 2: Each question in this section is followed by a number of options. After reading each question carefully, choose one option which most applies to you, and circle the appropriate answer.

37. How often are you dieting in a conscious effort to control your weight?
   - rarely
   - sometimes
   - usually
   - always

38. Would a weight fluctuation of 3 kg affect the way you live your life?
   - not at all
   - slightly
   - moderately
   - very much

39. How often do you feel hungry?
   - only at meal times
   - sometimes between meals
   - often between meals
   - almost always

40. Do your feelings of guilt about overeating help you to control your food intake?
   - never
   - rarely
   - often
   - always

41. How difficult would it be for you to stop eating halfway through dinner and not eat for the next four hours?
   - easy
   - slightly difficult
   - moderately difficult
   - very difficult

42. How conscious are you of what you are eating?
   - not at all
   - slightly
   - moderately
   - extremely

43. How frequently do you avoid ‘buying large’ on tempting foods?
   - almost never
   - seldom
   - usually
   - almost always

44. How likely are you to shop for low calorie or low fat foods?
   - Unlikely
   - slightly likely
   - moderately likely
   - very likely

45. Do you eat sensibly in front of others and splurge alone?
   - never
   - rarely
   - often
   - always

46. How likely are you to consciously eat slowly in order to cut down on how much you eat?
   - unlikely
   - slightly likely
   - moderately likely
   - very likely
Appendix I

47. How frequently do you skip dessert 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 even though you are not hungry?

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

50. To what extent does this statement describe your eating behaviour? ‘I start dieting in the morning, but because of any 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

51. On a scale of 1 to 6, where 1 means no restraint in eating (eat whatever you want, whenever you want it) and 6 means total restraint (constantly limiting food intake and never ‘giving in’), what number would you give yourself?

1. eat whatever you want, whenever you want it
2. usually eat whatever you want, whenever you want it
3. often eat whatever you want, whenever you want it
4. often limit food intake, but often ‘give in’
5. usually limit food intake, rarely ‘give in’
6. constantly limit food intake, never ‘give in’
Appendix II

Appendix II: Visual Analog Scale

Name (Initials):  Visit:  Time:
Please indicate how you are feeling at this moment by placing a vertical line at the appropriate point on each horizontal scale below. Furthest LEFT means you do not feel the sensation in question, furthest RIGHT means you feel it very much. Please, mark all scales.

I feel nauseated

I feel drowsy

I feel bloated

I feel anxious

I feel hungry

I feel full

I feel happy

I feel energetic

How strong is your desire to eat?

I feel comfortable

How much food do you think you can eat?
## Appendix III: Characteristics of study participants in Chapter 6

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<th>ID</th>
<th>Age</th>
<th>Weight (kg)</th>
<th>Height (m)</th>
<th>BMI</th>
<th>Waist (cm)</th>
<th>Restraint score</th>
<th>Exercise:</th>
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<tr>
<td>1</td>
<td>20</td>
<td>67.2</td>
<td>1.87</td>
<td>19.2</td>
<td>74</td>
<td>1</td>
<td>no planned exercise (asthma)</td>
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<tr>
<td>2</td>
<td>22</td>
<td>59.3</td>
<td>1.71</td>
<td>20.2</td>
<td>70</td>
<td>5</td>
<td>weights 3x/wk., running 3x/wk.</td>
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<td>3</td>
<td>22</td>
<td>70.8</td>
<td>1.70</td>
<td>24.4</td>
<td>85</td>
<td>7</td>
<td>bike 1 hr 4x wk.</td>
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<tr>
<td>4</td>
<td>20</td>
<td>85.5</td>
<td>1.85</td>
<td>24.9</td>
<td>86</td>
<td>9</td>
<td>No planned exercise</td>
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<tr>
<td>5</td>
<td>21</td>
<td>77.2</td>
<td>1.83</td>
<td>23.0</td>
<td>85</td>
<td>10</td>
<td>Gym 30 min 4x/wk.; soccer 1 hr/wk.</td>
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<td>6</td>
<td>18</td>
<td>68.2</td>
<td>1.81</td>
<td>20.8</td>
<td>75</td>
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<td>powerlifting 1.5 hr 2x/wk.; parkour</td>
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<td>7</td>
<td>30</td>
<td>62</td>
<td>1.75</td>
<td>20.2</td>
<td>84</td>
<td>3</td>
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<tr>
<td>8</td>
<td>20</td>
<td>90.2</td>
<td>1.90</td>
<td>24.9</td>
<td>89</td>
<td>8</td>
<td>football 1 hr 2x/wk.; weights 3x/wk., basketball 2 x/wk.</td>
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<td>28</td>
<td>81.8</td>
<td>1.81</td>
<td>24.9</td>
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<td>31</td>
<td>66.3</td>
<td>1.80</td>
<td>20.4</td>
<td>72</td>
<td>1</td>
<td>cycle 1 hr 1-2 x/wk.</td>
</tr>
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<td>34</td>
<td>71.5</td>
<td>1.83</td>
<td>21.3</td>
<td>75</td>
<td>6</td>
<td>Pilates 1 hr 1-2x/wk.; weights 45 min 3x/wk.</td>
</tr>
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<td>37</td>
<td>67.4</td>
<td>1.64</td>
<td>25.0</td>
<td>82</td>
<td>10</td>
<td>no planned exercise</td>
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<td>25</td>
<td>71</td>
<td>1.89</td>
<td>19.8</td>
<td>75</td>
<td>2</td>
<td>cycling 1 hr 5x/week</td>
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<td>1.75</td>
<td>19.3</td>
<td>75</td>
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<td>19</td>
<td>69</td>
<td>1.75</td>
<td>22.5</td>
<td>70</td>
<td>2</td>
<td>gym 1 hr 3x/week</td>
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<tr>
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<td>26</td>
<td>62</td>
<td>1.73</td>
<td>20.7</td>
<td>82</td>
<td>8</td>
<td>no exercise</td>
</tr>
<tr>
<td>18</td>
<td>23</td>
<td>63</td>
<td>1.75</td>
<td>20.5</td>
<td>72</td>
<td>4</td>
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</tr>
<tr>
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<td>22</td>
<td>68.3</td>
<td>1.85</td>
<td>19.9</td>
<td>74</td>
<td>2</td>
<td>walk 1 hr daily; tennis 1.5 hr/wk.</td>
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<tr>
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<td>18</td>
<td>55</td>
<td>1.72</td>
<td>18.5</td>
<td>68</td>
<td>2</td>
<td>No reported</td>
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</table>
Appendix IV: Flow diagram of participants in Chapter 6

**Enrolment**
- Assessed for eligibility (n=24)
- Excluded (n=4)
  - Not meeting inclusion criteria (n=0)
  - Declined to participate (n=2)
  - Other reasons (n=2)

**Randomized** (n=20)

**Allocation**
- Allocated to interventions (n=20)
  - Received all allocated interventions (n=18)
  - Completed 2 interventions and withdrew due to time constraints (n=1)
  - Excluded for failing to adhere to study protocol (n=1)

**Analysis**
- Analysed (n=18)
Appendix V

Appendix V: Appetite ratings measured by VAS in Chapter 7

Figure legend: Hunger (A), fullness (B), nausea (C), bloating (D) during 60-min infusions of 0.5 kcal/min (P0.5), 1.5 kcal/min (P1.5), 3 kcal/min (P3) or saline control (C). Data are means ± SEM; n=16, treatment*time interaction analysed using repeated measures ANOVA; Significant effects determined by post-hoc comparisons using Bonferroni’s correction.
Appendix VI

Appendix VI: Flow diagram of participants in Chapter 8

1. **Enrolment**
   - Assessed for eligibility (n=28)
     - Excluded (n=4)
       - Not meeting inclusion criteria (n=2)
       - Declined to participate (n=2)
       - Other reasons (n=0)

2. **Randomized**
   - Randomized (n=24)

3. **Allocation**
   - Allocated to intervention (n=24)
     - Received allocated intervention (n=24)

4. **Analysis**
   - Analysed (n=24)
Appendix VII: Flow diagram of participants in Chapter 9

1. **Enrolment**
   - Assessed for eligibility (n=32)
   - Excluded (n=9)
     - Not meeting inclusion criteria (n=5)
     - Declined to participate (n=2)
     - Other reasons (n=2)

2. **Randomized (n=23)**

3. **Allocation**
   - Allocated to intervention (n=23)
     - Received all allocated interventions (n=18)
     - Received 4 full allocated interventions, and experienced side effects between t=75-90 min (n=2)
     - Discontinued participation due to adverse effects (n=3)

4. **Analysis**
   - Analysed (n=20)
Appendix VIII

Appendix VIII: Appetite ratings measured by VAS in Chapter 9

Figure legend: (A) Hunger, (B) prospective consumption, (C) fullness, (D) nausea and (E) bloating ratings measured using visual analog scales (VAS) during 90-minute, 3 kcal/min intraduodenal infusions of a saline control (C), whey protein hydrolysate (P3), Intralipid and whey protein hydrolysate in 1:2 (L1P2) and 2:1 (L2P1) ratios, or pure lipid (L3). Data are means ± SEM; n=20. Treatment effect by one-way ANOVA for AUCs of the VAS profiles, significant differences (P<0.05) determined by post-hoc comparisons using Bonferroni’s correction. (A) treatment effect (P=0.09); (B) treatment effect (P<0.05), no significant differences in post-hoc paired comparisons; (C) treatment effect (P=0.076); (D) treatment effect (P<0.01), (*) significantly different vs. C and L1P2 (P<0.05), (§) trend for significant difference vs. P3 (P=0.07); (E) treatment effect (P=0.07).
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