Role of dietary protein and carbohydrate in

acute appetite regulation in overweight subjects.

A thesis submitted by

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Abbreviations

AGRP	agouti related protein
AP	adequate protein
ARC	arcuate nucleus
AUC	area under the curve
BCAA	branched chain amino acids
CART	cocaine and amphetamine regulated transcript
DIT	dietary induced thermogenesis
DMH	dorsomedial hypothalamus
DPP-IV	dipeptidyl-peptidase IV
GHS	growth hormone secretagogue
GIP	glucose-dependent insulinotropic polypeptide
GIT	gastrointestinal tract
GLP-1	glucagon like peptide-1
GMP	Glycomacropeptide
HC	high carbohydrate
HF	high fat
HP	high protein
LHA	lateral hypothalamic area
MC4R	melanocortin–4 receptor
MCH	melanin-concentrating hormone
αMSH	α melanocyte stimulating hormone
NTS	nucleus tractus solitarius
NPY	neuropeptide Y

PFA	perifornical area
POMC	pro-opiomelanocortin
PVN	paraventicular nucleus
PYY	peptide YY
TEF	Thermic effect of feeding
VMH	ventromedial hypothalamus

Abstract

Context

Dietary protein induces greater satiety compared to carbohydrate. A mechanism to explain this has not been described, although gut hormones with a role in acute appetite regulation may be involved. The effect of macronutrients on appetite has been observed in lean subjects only and requires demonstration in the overweight population in whom appetite regulatory mechanisms may differ. Little is known about the influence of macronutrient source on this relationship, the duration of satiety after protein consumption and the impact of combining protein with carbohydrate. Therefore the overall objective of the studies that comprise this thesis is to compare the effect of various dietary proteins and carbohydrates on acute postprandial changes in appetite sensations, *ad libitum* energy intake and associated regulatory hormones in overweight/obese adults.

Study Design

Three randomised double-blind crossover studies examined the acute appetite response to preloads over 3-4h followed by a buffet meal. Studies were conducted at an outpatient research clinic. Overweight and obese, healthy men participated in all studies, with an additional group of lean men in Study 2. Interventions consisted of liquid preloads (1MJ, ~450mL) containing ~55g of dietary protein (whey, casein, soy, gluten), carbohydrate (glucose, lactose and fructose) or combined whey protein/fructose (Study 3). The primary outcome measures were fasting and postprandial plasma glucose, insulin, total ghrelin, glucagon like peptide 1 (GLP-1 7-36) (Study 2 and 3 only), cholecystokinin-8, subjective appetite ratings (visual analogue scales) and *ad libitum* energy intake.

Results

Ad libitum energy intake was approximately 10% higher 3h after ingestion of glucose preloads relative to protein-based treatments, although this was not observed at 4h. Protein ingestion was consistently associated with prolonged elevation of plasma cholecystokinin and GLP-1 for 120 min relative to glucose, independent of the protein type. Similarly, dietary proteins were associated with prolonged suppression of ghrelin that persisted 4h after preload consumption. The contrasting ghrelin profile after glucose consumption with an increase above fasting concentration at 3-4h coincided with a decrease in glucose below baseline, increased hunger ratings (Study 1) and higher *ad libitum* food intake. Replacing some whey with fructose attenuated the effect of protein on these gut hormones.

The effects of treatment on energy intake and appetite hormones were independent of body weight status, despite overall higher GLP-1 and lower ghrelin concentrations in overweight/obese subjects.

Conclusion

Liquid preloads rich in dietary proteins reduce *ad libitum* intake over 3h in overweight and obese subjects, relative to carbohydrate. This may be partly attributed to the 'satiating' profile of gut hormones (prolonged suppression of ghrelin and elevation of GLP-1 and cholecystokinin) that is independent of protein source and body weight status and which may relate to the amount of protein consumed.

Chapter 1   General Introduction

1.1   Context

Overconsumption of energy from food and beverages is an important precursor (1, 2) to the recent and rapid increase in obesity prevalence worldwide (3-5), alongside increased sedentary behaviour. While the strong association between obesity and risk of cardiovascular disease, type 2 diabetes and certain cancers (6) is reversed by a reduction in body weight (7, 8) few dieters achieve and maintain weight loss (9). Targeting appetite regulation has been identified as a possible approach to tackling obesity (10). As such, dietary factors that modulate acute appetite responses are of interest.

Dietary patterns with a moderately increased proportion of energy from protein are associated with lower *ad libitum* energy intake, higher satiety ratings and greater weight loss compared to higher carbohydrate intakes (11-13). Acute studies also show that dietary protein consumption lowers subsequent appetite and energy intake relative to carbohydrate and fat (14-17). To date there has been limited investigation of the contribution that gastrointestinal derived appetite hormones make to the effect of protein on satiety. Similarly, little is known about means to prolong this and whether protein source influences the strength of the relationship.

Carbohydrates that produce a gradual and prolonged increase in blood glucose concentration are also proposed to extend satiety, relative to carbohydrates that are rapidly absorbed and metabolised (18, 19). However there is substantial disagreement

about this relationship (20) and whether blood glucose (and/or insulin) *per se* affects appetite, or if other dietary factors associated with carbohydrates are involved.

Dietary factors that influence short-term appetite responses have potential applications in the overweight population. Despite this, most appetite studies have been performed in lean subjects. Such findings may not directly translate to the overweight population given differences in eating behaviour (21) which are associated with body weight. Similarly peripheral hormones involved in energy balance are affected by excess adiposity (22). Therefore ingestive behaviour and the associated appetite regulatory responses to dietary factors require demonstration in overweight subjects.

1.2 Objective

The objective of this thesis is to investigate the effect of various dietary proteins and carbohydrates on acute changes in gastrointestinal derived appetite hormones, subjective appetite ratings and *ad libitum* energy intake in overweight/obese subjects.

1.3 Hypothesis

The overall hypothesis of this thesis is that dietary proteins and carbohydrates have different effects on acute changes in gastrointestinal derived appetite hormones, and that these changes correspond to differences in subjective appetite ratings and *ad libitum* energy intake in overweight/obese subjects.

1.4 Aim

The specific aims of the studies contained in this thesis are:

Study 1: To compare the effects of two proteins which differ in their rate of gastric emptying (whey; fast, casein; slow) with two carbohydrates which differ in glycemic index (glucose; high, lactose; low), on subjective (visual analogue scale ratings) and objective (*ad libitum* energy intake) appetite markers, rate of gastric emptying, and postprandial plasma ghrelin, cholecystokinin, glucose, insulin and amino acids responses over 3h in overweight men.

Study 2: To compare the effect of soy, whey and gluten proteins on subjective (visual analogue scale ratings) and objective (*ad libitum* energy intake) appetite markers and postprandial changes in plasma ghrelin, GLP-1 and cholecystokinin over 3h, relative to a glucose control.

To investigate the effect of body weight on subjective (appetite ratings) and objective (*ad libitum* energy intake) appetite markers and postprandial changes in plasma ghrelin, GLP-1 and cholecystokinin.

Study 3: To describe the effect of whey protein on subjective (visual analogue scale ratings) and objective (*ad libitum* energy intake) indicators of appetite and appetite regulatory hormones (ghrelin, cholecystokinin and GLP-1) relative to an isocaloric glucose preload over 4h.

To investigate the effect of combining whey and fructose on postprandial appetite hormones and energy intake, relative to either dietary component consumed alone.

Chapter 2   Appetite

2.1   Appetite definition

"Appetite" collectively describes a range of sensations that encompass a number of distinct phases. Hunger is the sensation that results in initiation of an eating episode. Satiation is the feeling of satisfaction that develops during a meal and determines meal duration, termination and size when food availability is not limiting. Satiety is the absence of hunger during the inter-meal period (23). These definitions are widely accepted and form the basis of the 'satiety cascade' model (Figure 1) (24). 'Sensory specific satiety' refers to the development of early satiation after consumption of foods with a similar taste or other organoleptic property compared to the consumption of a variety of foods; after eating a food its pleasantness decreases more than uneaten foods (25). In this review, the term appetite will be used to refer generally to all components described above; hunger, satiation or satiety will be used when specificity is intended.

Figure 1: The satiety cascade conceptualises the phases of satiation and satiety that develop during and after consumption of foods, adapted from Blundell (24).
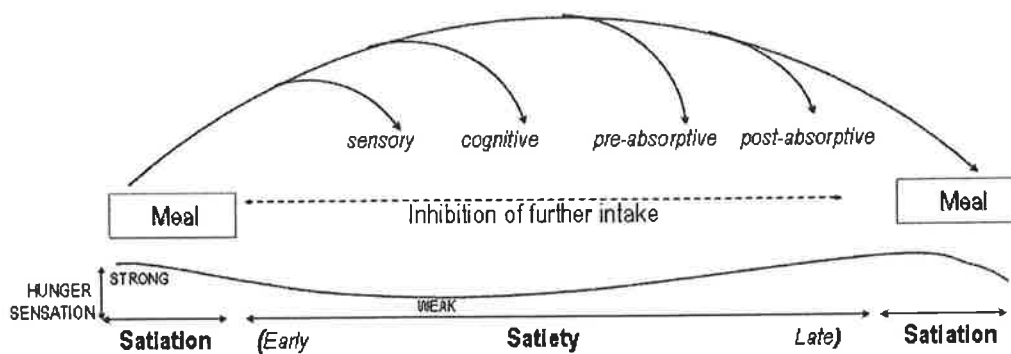
The satiety cascade model includes further subdivisions of the post ingestive phase. Oro-sensory effects of the consumed food influence sensory satiety. Beliefs relating to the food determine cognitive satiety. Pre-absorptive satiety refers to the effects of the food and nutrients whilst in the gastrointestinal lumen. The effects of the nutrients after absorption are proposed to influence post-absorptive satiety. Determinants of the latter two phases are of particular interest for this thesis; it is proposed that dietary manipulations may enable the pre- and post-absorptive phases to be extended and consequently decrease the size of the subsequent meal or delay its onset.

These sensations are frequently assessed in the research environment in isolation. However it is important to consider them in the context of the free living setting where multiple cognitive (e.g. time of day, learned eating habits, physical activity), psychological (e.g. stress, boredom, anxiety, energy level), environmental (e.g. weather, social setting, food availability) and sensory (e.g. smell, taste, texture of food) factors also alter eating behaviour (26).

Chapter 3   Physiological regulation of appetite

The physiological regulation of appetite is a complex, seemingly redundant and incompletely understood system of afferent signals and efferent effectors co-ordinated by the hypothalamus and brainstem (27). These areas receive neural and humoral inputs from the vagus nerve, gastrointestinal tract and adipocytes, providing information about food intake, metabolic activity and nutrient availability (Figure 2) (28). This chapter provides an outline of the central regulation of appetite and describes in more detail the role of appetite regulatory hormones arising from the gastrointestinal tract.

3.1   Central regulation of appetite

Within the hypothalamus there are several nuclei involved in appetite regulation although the arcuate nucleus (ARC) appears to be the most important (Figure 2). The ARC is located close to the median eminence, which has an incomplete blood brain barrier. As such the ARC is exposed to humoral inputs directly from the peripheral circulation, including some gastrointestinal derived appetite hormones. It also receives and integrates a multitude of signals from other nuclei, including the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial and ventromedial hypothalamus (DMH and VMH respectively) and perifornical area (PFA) (Figure 2).

Afferent fibres carry signals via the vagus nerve between the brainstem (specifically nucleus tractus solitarius (NTS) and area postrema) and the liver (relaying information on energy metabolism in particular glucose sensing), the stomach (providing mechanical signals of gastric volume and motility) and neuroendocrine cells of the

intestinal lumen. The NTS also receives inputs from the peripheral circulation because of its close vicinity to the area postrema which has an incomplete blood brain barrier (28).
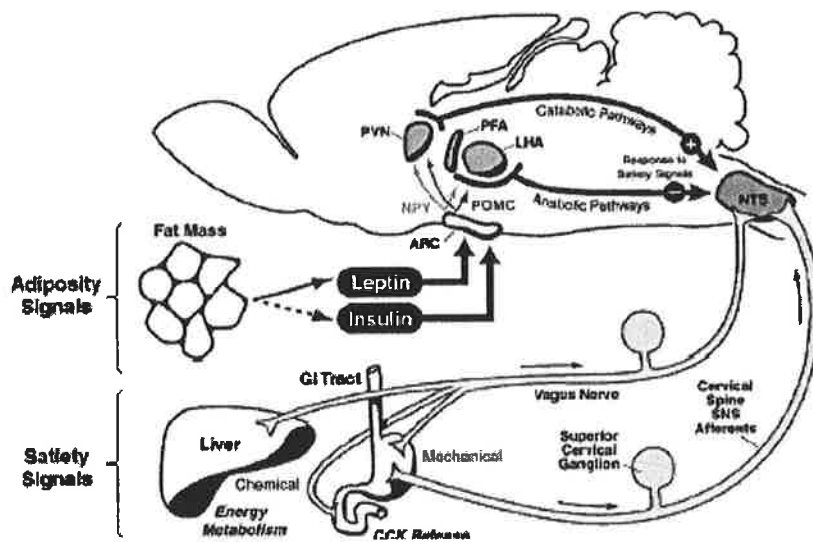
Figure 2 This diagram depicts the hormonal and mechanical signals arising from the periphery that interact with central receptors in the nucleus tractus solitarius (NTS) and arcuate nucleus (ARC) within the hypothalamus via the vagus nerve and circulation. Within the ARC, orexigenic and anorexigenic neurons project to other areas associated with appetite control (29).

The ARC contains "anabolic" neurons which express orexigenic neuropeptide Y (NPY) and agouti related protein (AGRP) and "catabolic" neurons which expresses anorexigenic pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (Figure 3) (30). NPY/AGRP and POMC/CART neurons receive inputs directly from the periphery (e.g. insulin and leptin) and have projections that innervate the PVN, LHA, DMH and PFA (31).
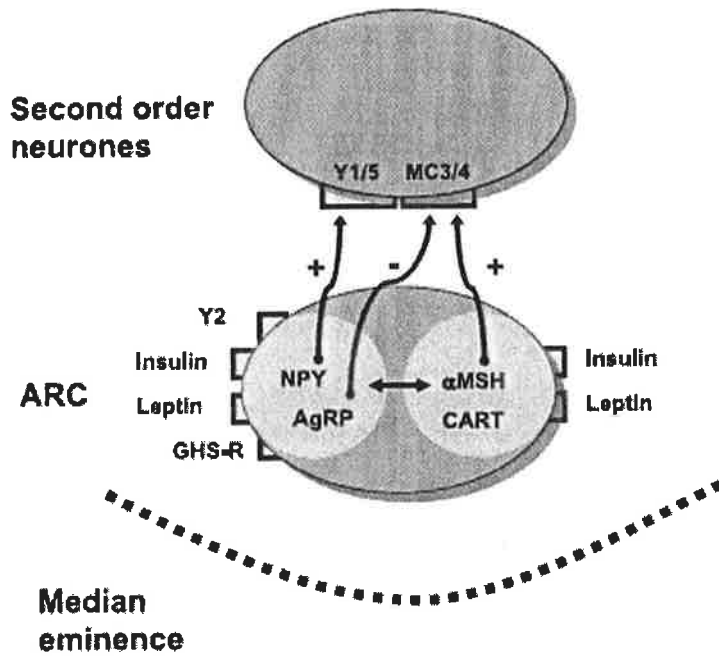
Figure 3 This figure shows the peripheral stimuli, including growth hormone secretagogue receptor (GHS-R), of orexigenic (neuropeptide Y; NPY and agouti related protein; AgRP) and anorexigenic (α melanocyte stimulating hormone; αMSH and cocaine and amphetamine regulated transcript; CART) neuropeptides involved in appetite regulation (32).

3.1.1 Anabolic Neuropeptides

NPY is primarily expressed in the hypothalamus and is the most potent known orexigenic neuropeptide (33). NPY is part of the pancreatic polypeptide-fold family of polypeptides that binds to 7 transmembrane domain G-protein coupled receptors named Y1 to Y6 (34). These receptors have differing levels of activity and Y5 seems particularly important in energy balance regulation (35). Activation of these neurons causes release of NPY into the PVN which stimulates food consumption and reduces energy expenditure by inhibiting thermogenesis (36), sympathetic nervous activity (37) and the thyroid axis (38). NPY neurons also project within the ARC to inhibit POMC activity (described below) (39).

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NPY neurons co-express AGRP, which has a similar effect as NPY although achieved by a different mechanism. AGRP is an endogenous antagonist of the melanocortin–4 receptor (MC4R) which binds the catabolic neurotransmitter, α melanocyte stimulating hormone (αMSH) (40). Therefore AGRP increases food intake by inhibiting the catabolic effect of another neuropeptide. AGRP also differs from NPY in the duration of effect; a single intracerebral injection of AGRP increases food intake for up to a week, whereas the effects of a similar NPY treatment last a few hours (41). There are many other identified neurotransmitters that are also associated with increased energy intake; melanin-concentrating hormone, galanin, orexin A and B, opioids, nitric oxide and cannabinoids (32).

3.1.2   Catabolic neuropeptides

POMC is a precursor molecule that is cleaved into a number of different peptides identified as melanocortins; the most important of these is αMSH. Activation of POMC/CART neurons stimulates the release of αMSH which binds to the melanocortin receptor MC4R in the PVN (42) and inhibits food intake. Defects in this receptor are a significant contributor to genetic obesity in humans (43).

CART is the third most abundant neurotransmitter in the hypothalamus and is co-expressed by POMC neurons primarily in the ARC, PVN and LHA (42). GLP-1 (44), serotonin and neurotensin also act in the hypothalamus to attenuate food intake (45).

3.2   Peripheral regulation of appetite

The homeostatic regulation of energy balance involves signals from the periphery that provide information on nutrient availability and the size of energy stores. Adiposity-derived hormones such as leptin indicate the size of energy stores. Acute changes in nutrient ingestion and metabolism are relayed by hormones derived from the gastrointestinal tract and include cholecystokinin, glucagon like peptide-1 (GLP-1), ghrelin, pancreatic polypeptide, amylin, enterostatin, oxyntomodulin, peptide YY (PYY) and glucose-dependent insulinotropic polypeptide (GIP). These hormones act in the hypothalamus and brainstem via the circulation and/or receptors on the vagus to provide interrelated and seemingly redundant meal initiation, meal termination and satiety signals (Figure 4).

Figure 4 Peripheral regulation of appetite.
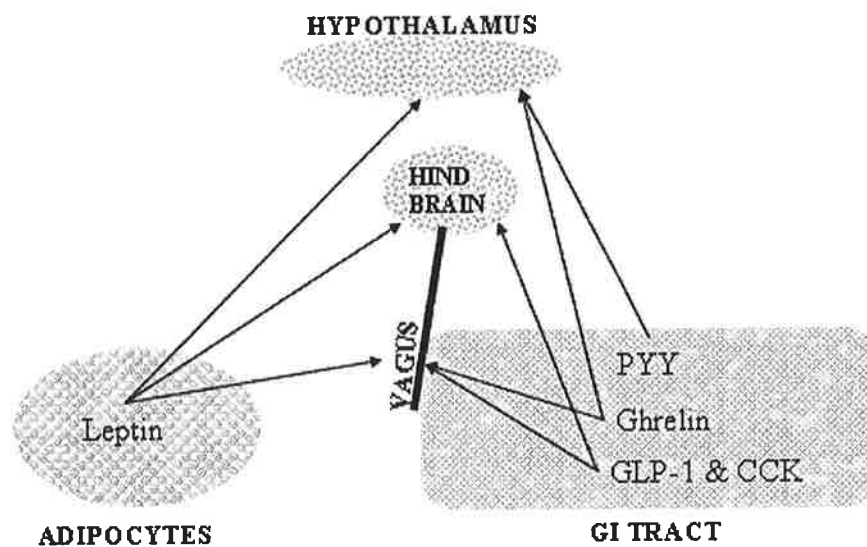
This diagram depicts the origin and site of action of gastrointestinal (GI) - derived (ghrelin, PYY; Peptide YY, GLP-1; glucoagon like peptide 1, CCK; cholecystokinin) and adipocyte-derived hormones involved in appetite regulation.

This chapter will describe current understanding of the role, mechanism of action and regulation of ghrelin, GLP-1 and cholecystokinin as these hormones were studied in this thesis. A discussion of the effect of macronutrient consumption (protein and carbohydrate) on these hormones is provided in Chapter 5.

3.2.1 Ghrelin

Ghrelin is the 28 amino acid endogenous ligand for the growth hormone secretagogue (GHS) receptor, discovered in 1999 by Kojima and Kangawa (46). In addition to stimulating growth hormone, ghrelin is the only identified peripheral orexigenic hormone (47) and accordingly has attracted intense research interest. Intravenous ghrelin infusion for 270min in healthy humans at a near physiological dose (5.0pmol/kg/min) increased hunger ratings (+46%, $P < 0.05$) and *ad libitum* energy intake (+28%, +1284kJ, $P < 0.001$) assessed 240min after start of infusion (48). Prolonged ghrelin infusion in animals (49) and cachexic humans (50) results in hyperphagia and weight gain, suggesting that the effects of this hormone also contribute to long-term energy balance.

Expression of ghrelin is highest from neuroendocrine oxyntic mucosal cells in the stomach (specifically from the fundus), which account for ~75% of circulating ghrelin (46, 51). It is also secreted from the pituitary gland (52), pancreas (53) and the small and large intestine in quantities that decrease distally (52, 54). Ghrelin secreting cells in the small intestine are closed (i.e. not exposed to nutrients in the lumen) although these cells become more "open" (i.e. exposed to luminal contents) distally (55), suggesting that nutrient contact further along the GIT may stimulate ghrelin release. The ARC and other brain regions involved in appetite regulation also secrete ghrelin in minute quantities (56).

Animal models show that human ghrelin crosses the blood brain barrier via a saturable transport mechanism (57). The GSH receptor is expressed in regions associated with energy homeostasis, including NPY/AGRP neurons in the ARC (58) and ghrelin stimulates this orexigenic pathway (47). Ghrelin also acts centrally via receptors on vagal afferents innervating the NTS; ghrelin infusion suppresses vagal afferent firing and deafferentation of the gastric vagal nerve reduces the effect of peripheral ghrelin on NPY neurons (59). Ghrelin receptors are present in multiple peripheral sites and stimulation increases gastrointestinal motility (60, 61), gastric acid secretion (62) and pancreatic exocrine function (63) – all of which seemingly prepare the GIT for digestion of food.

Ghrelin's role in appetite regulation appears to be as a meal initiator. Endogenous plasma concentration shows a predictable increase with fasting and this corresponds to spontaneous meal requests in the absence of time related cues in lean humans (64). The orexigenic effect of ghrelin seems to be executed by increasing the number of

meals, rather than by increasing meal size (65). The pre-prandial peak is followed by a rapid decline following food intake that reaches a trough after 60-120min (66, 67) proportional to the calories consumed (68). While hunger ratings decrease in parallel with ghrelin concentration (69), the importance of the magnitude of this nadir in determining meal termination or subsequent intake has not been demonstrated. Indeed, chronic ghrelin administration in animals increases overall energy intake (49) due to the consumption of an extra meal, rather than by increasing meal size (65). Therefore the onset of the next eating episode may be influenced by the timing, rate or magnitude of return to pre-prandial ghrelin concentration. Despite this, much of the research conducted to date has focused on the size and timing of the nadir and area under the curve (AUC). These parameters give little insight into temporal changes at critical times associated with subsequent meal initiation. It is an hypothesis of this thesis that the changes in ghrelin that occur after the nadir may partly determine the degree of hunger at the next meal when consumed at a fixed time, or spontaneous timing of the next eating episode.

The mechanism to explain the meal-related changes in ghrelin is not clearly defined. Ghrelin suppression is independent of gastric distension and chemo-sensitisation, indicating involvement of post-gastric feedback (70). Glucose infusion causes a reduction in plasma ghrelin that is insulin (71, 72) and dose-dependent (69). Insulin and glucose clamp techniques show that this occurs in the absence of changes in plasma glucose (73) although glucose has an additional suppressive effect (72, 74). However recent meal studies have found that insulin does not predict ghrelin (75), or only has a weak inverse correlation (76).

Dietary factors that affect the rate and magnitude of ghrelin returning to pre-prandial levels may influence ingestive behaviour. Postprandial ghrelin suppression is prolonged when gastric emptying is delayed, induced by GLP-1 infusion (76). This suggests that ghrelin may be influenced by the rate of delivery or presence of nutrients in the small intestine. Plasma ghrelin suppression is prolonged in lean males when a greater length of the small intestine (>60cm) is exposed to glucose (77). As such, foods that reach more distal regions of the small intestine due to slow digestion may prolong ghrelin suppression. This could have a functional relationship to the change in ghrelin secreting cells from closed to open type along the small intestine (55). This represents a new area of investigation to understand the stimuli for postprandial changes in ghrelin. Further discussion of the effect of dietary composition on ghrelin regulation is given in Chapter 5.4.4. There is also evidence to suggest that the premeal rise in plasma ghrelin is a cognitive anticipatory response and not associated with nutrient status (78).

Body weight status affects ghrelin concentration; fasting ghrelin concentration is ~155 ± 25 fmol/mL in lean subjects and ~106 ± 23 fmol/mL in obese subjects (79). The postprandial nadir is also compromised (80) or not observed (81) in obese subjects. Insulin resistance is associated with an additional reduction in ghrelin (82-84). Reduced ghrelin circulation (and therefore presumably a lower hunger signal) appears to be an appropriate response to a state of positive energy balance. Diet-induced weight loss is associated with increased ghrelin (85), which is suggestive of increased hunger that may compromise maintenance of a lower body weight. However this seems to be transient; ghrelin levels returned to pre-weight loss concentrations after six months of maintaining a reduced bodyweight (-8.5% bodyweight) (86). Other

states of negative energy balance, such as anorexia nervosa (87) and cancer cachexia (88), are associated with increased ghrelin. Interestingly, the impact of these adiposity and/or energy balance-dependent changes in ghrelin on eating behaviour is not defined. One study has shown that *ad libitum* food intake was equally increased in lean and obese subjects (+20% and +37% respectively) 45 min after commencing a ghrelin infusion (1.0 pmol/kg/min). However when the infusion rate was increased (5.0 pmol/kg/min) food consumption was significantly greater in the obese (+70%) compared to lean (+20%) subjects (89). These preliminary findings indicate that bodyweight status affects both ghrelin regulation and sensitivity.

Ghrelin exists in a number of forms. Intact ghrelin has a unique post-translational modification - an n-octanoyl ester on the third N-terminal amino acid serine residue. This is considered the active form and constitutes less than 10% of total circulating ghrelin as it is rapidly degraded (90). Des-acyl ghrelin (i.e. lacking the side chain) is considered to be an inactive form and does not have an identified receptor, although recently it has been shown to exert an opposite effect to active ghrelin in the hypothalamus (91). Total ghrelin is the sum of the intact, des-acyl and various C-terminal fractions of ghrelin. Commercial radioimmunoassay kits are available to quantify various fractions and total ghrelin. There is little consensus on the most appropriate form/s to assess for appetite research. Fasting concentration of total and active ghrelin correlate ($r = 0.62$, $P < 0.001$) (92). The postprandial (2h) changes in all ghrelin fractions after infusion of a 10% glucose solution (50g glucose) were similar when assessed in lean subjects (93). Another study reported that total, but not active, ghrelin showed a postprandial treatment effect (difference between high protein and high carbohydrate breakfasts) in lean males (n=15) (94). A drawback for assessing

active ghrelin is that the lower plasma concentration may make detection of treatment effects difficult.

In summary, ghrelin is the only identified meal initiation/hunger signal. Factors that influence the return of ghrelin to pre-prandial levels may affect subsequent meal initiation or *ad libitum* food intake; although to date investigation of such triggers has been limited. Preliminary findings indicate that bodyweight-dependent changes in ghrelin regulation or sensitivity may have an impact on appetite and eating behaviour.

3.2.2 GLP-1

Glucagon like peptide-1 (GLP-1) is 30 amino acid hormone that was first identified as an incretin (95) as it enhances postprandial glucose-dependent stimulation of insulin and inhibits glucagon secretion (96, 97). GLP-1 also contributes to the post ingestive ileal break by slowing gastric emptying (96) and reducing gastric acid secretion (98), in turn reducing postprandial glycemia. More recently this hormone has attracted attention for its effect on appetite, food intake and therapeutic applications as a weight loss drug. Infusion studies using GLP-1 and the GLP-1R antagonist, exendin 9-39, provide compelling evidence that this hormone is a potent satiation factor (44, 99). GLP-1 infusion at supraphysiological concentrations inhibits food intake in lean (100, 101), obese (102) and type 2 diabetic (103, 104) human subjects. A meta-analysis found *ad libitum* food intake was reduced by 12% (-727kJ) following GLP-1 infusion. This relationship is dose dependent and predicted by rate of infusion (105) but not by BMI, plasma concentration, duration of infusion or duration between infusion and *ad libitum* meal. Continuous subcutaneous administration of GLP-1 over 6 weeks in obese, type 2 diabetic subjects (n = 10) produced a sustained reduction in *ad libitum*

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energy intake and weight loss of 1.9kg compared to weight matched controls infused with saline (n = 9) (106). Interestingly, GLP-1R knockout mice show normal feeding patterns and body weight (107), indicating that other satiety signals may compensate for the appetite-specific effects of this hormone long-term.

GLP-1 is produced by L-cells throughout the small and large intestine (108, 109) and secretion is stimulated by nutrient intake (110). Peak postprandial plasma concentration (~25pmol/L) is observed within 5min of food intake (111) which suggests that GLP-1 contributes to satiation. The precise trigger for GLP-1 release is not known; however the speed of the postprandial increase and the observation that L-cells are not exposed to luminal contents (111, 112) suggests involvement of neural (i.e. vagus) mechanisms (113). GLP-1 concentration over 1h is increased by extending the length of the small intestine exposed to glucose (>60 cm post pylorus), compared to containing nutrient exposure from 0 to 60cm post pylorus (77). These data suggest that GLP-1 secretion is augmented when more of the small intestine is exposed to nutrients. As such it may be possible to prolong the secretion of this hormone and therefore its inhibitory effect on appetite by dietary manipulations that delay nutrient absorption and extend the length of the small intestine exposed to nutrients.

The active forms of the hormone (7-36 amide and 7-37) (114) constitute less than one third of total plasma GLP-1 and has a half life of ~2 min. GLP-1 is rapidly cleaved by dipeptidyl-peptidase IV (DPP-IV) into inactive metabolites (115). There is considerable interest in factors that inhibit DPP-IV, in order to maximise GLP-1 concentration and potentially serve as a treatment for type 2 diabetes (116). The

distribution of the GLP-1 receptor (GLP-1R; expressed in the NTS, LHA, DMH and VMH (117), β pancreatic cells and throughout the GIT) reflects a wide range of peripheral effects.

Fasting plasma concentration ranges from 5 to 10 pmol/L in lean subjects (114). It has previously been reported that GLP-1 is reduced in overweight and obese subjects (110); however evidence to support this is weak and conflicting. Incremental GLP-1 AUC (but not total AUC or fasting concentration) was lower in overweight compared to lean subjects (118). Fasting and postprandial responses to a breakfast plus guar gum solution were independent of body weight (119), although lean subjects had moderately higher GLP-1 concentration at 30 min compared to obese subjects when the same breakfast was consumed with water (119). These authors also reported that postprandial GLP-1 inversely correlated with percentage of body fat in women, but not in men (120). There was no difference in plasma concentration between lean and obese subjects after duodenal infusion of fat and carbohydrate (121). GLP-1 sensitivity appears to be independent of bodyweight; a meta-analysis of infusion studies found that the reduction in *ad libitum* energy intake was similar in obese and lean subjects after controlling for the overall lower intake in obese participants (105). Indeed, the relatively small differences in GLP-1 that are attributed to bodyweight may reflect variation in analytical methods between groups (110). Given the apparently powerful effect of this hormone on appetite, it is important to clarify how bodyweight status affects GLP-1 regulation, and whether this has an impact on appetite responses.

In summary, the rapid increase in GLP-1 after nutrient ingestion indicates a role for this hormone in satiation. Its role in satiety is not defined; however factors that prolong GLP-1 secretion may inturn prolong satiety. The impact of bodyweight on GLP-1 regulation also requires investigation.

3.2.3   Cholecystokinin

Cholecystokinin has multiple gastrointestinal functions that coordinate digestion following food intake. The rapid increase in cholecystokinin observed after nutrient ingestion slows gastric emptying, reduces gastric acid secretion, changes GIT motility (colon and small intestine) and stimulates gall bladder contraction and pancreatic secretion.

Fasting concentration of cholecystokinin is ~1pM, independent of age, gender and bodyweight. Cholecystokinin release into portal circulation is stimulated by the presence of fatty acids with a chain length of ≥12C (122, 123) and small peptides or amino acids (124) in the duodenum. Secretion is stimulated to a lesser extent by glucose (125). Plasma levels rapidly increase to ~5-8pM, after first after undergoing metabolism in the liver and decrease over 3-5h during the postprandial period.

There are multiple molecular forms of cholecystokinin derived from preprocholecystokinin expressed throughout the gastrointestinal tract and within the brain (126). Cholecystokinin –33 and –8 are secreted from enteroendocrine I-cells in the duodenum into the peripheral circulation (127). Cholecystokinin-8 is the most abundant form expressed in the hypothalamus and other the brain regions. There are also two main cholecystokinin receptors: cholecystokinin[1] (Type A) and cholecystokinin[2] (Type B). Expression of cholecystokinin[1] is highest in the periphery

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although it is also present in the NTS, area postrema and DMH (128). This receptor is associated with the effects of cholecystokinin-8 on satiation (129), whereas cholecystokinin[2] stimulation does not affect appetite responses (130). Cholecystokinin[1] has low and high affinity binding sites that require plasma cholecystokinin at pM and nM concentrations respectively (131). This, along with first pass hepatic clearance, has important implications for interpretation of infusion studies; seemingly supraphysiological plasma concentrations may be required to mimic local concentration at low affinity receptor sites.

There is considerable evidence from infusion studies to show that cholecystokinin stimulates meal termination. Early studies found that intraperitoneal injections of cholecystokinin-8 in rats dose dependently reduced intake energy (132). This was subsequently replicated in lean men (n = 12); intravenous infusion of cholecystokinin-8 at a supraphysiological concentration decreased hunger and *ad libitum* energy intake (-19%, -122g of food) relative to saline infusion (133). Similar effects are observed in obese subjects (134). Infusion at such concentrations also induces nausea (135) which may confound these data. More recent studies that achieve physiological plasma concentrations show mixed results; some studies confirm that cholecystokinin-8 and -33 shorten meal duration and limit meal size in lean and obese subjects by ~20% (136, 137), but not consistently (138, 139). Administration of the CCK[1] receptor antagonist, loxiglumide infused 1h before and during a mixed meal increased *ad libitum* intake by ~10% in healthy subjects (n=40) (140), although this is also not consistently observed (141). Despite this conflicting data, it is widely accepted that cholecystokinin plays a role in satiation (142).

The contribution of the slow postprandial decline in cholecystokinin, if any, to satiety has not been described in humans. Cholecystokinin-8 infusion in rats caused a reduction in *ad libitum* energy intake (i.e. increased satiation), however compensation for this occurred over 6 days by shortening the inter-meal interval and increasing meal frequency (i.e. reduced satiety) (143). By the end of the study energy intake had returned to pre-infusion amounts, suggesting that cholecystokinin does not have an independent role in regulating energy balance. Indeed CCK[1] knockout mice do not develop obesity (144).

3.3   Chapter summary

Understanding the regulation and effect of gastrointestinal derived appetite hormones has improved in recent times. Infusion studies using single hormones show subtle effects on appetite ratings and energy intake. Little is known about how these hormones interact with each other which is of greater physiological relevance. Co-administration of PYY and GLP-1 in lean subjects over 120 min resulted in a 27% reduction in energy intake relative to a saline infusion, assessed 90min after the start of infusion. This was larger than the effect of infusing PYY (-15%, P = 0.7) and GLP-1 (NS) alone (145). Similarly, total energy intake for the whole day (-13%) was lower after the combined infusion relative to all other treatments (P < 0.05) (145). Two studies performed in healthy lean men did not observe any synergistic effect on *ad libitum* energy intake after near physiological infusion of GLP-1 and cholecystokinin-8 (146) or cholesytokinin-33 (147) over 120 min and 60 min respectively. In animals, cholecystokinin injected intraperitoneally attenuated the stimulatory effect of ghrelin on feeding behaviour and neuronal activity in the ARC (148, 149) but this has not been described in humans.

Endogenous responses to nutrient loads show that appetite hormones appear to have a coordinated effect; the hunger signal increases prior to meals and decreases after food intake while satiety signals are low before meals and increase throughout the postprandial period. Additionally, peaks in satiety hormones seemingly occur sequentially throughout the postprandial period. Observing the post ingestive changes of a range of appetite-related hormones simultaneously is needed to improve the holistic understanding of acute appetite regulation.

Chapter 4   Appetite methodologies

The assessment of appetite is most commonly achieved by questionnaires and measuring *ad libitum* consumption of food. Together these approaches quantify the perceived motivation to eat (subjective) and actual consumption (objective).

4.1   Preload

The preloading method is commonly employed to assess the effect of various dietary manipulations on short-term appetite and associated regulatory mechanisms (150). This involves consumption of preloads that differ in a key variable (such as macronutrient composition, energy density (kJ/g) or physical form), followed by an observation period to record changes in appetite sensations (and other parameters) and an eating episode to measure *ad libitum* food intake. The physical form, nutrient composition and duration between preload and test meal have important effects on outcomes.

The impact of a single preload diminishes over a relatively short time, usually 1-4 hours. Assessment of *ad libitum* energy intake should coincide with the phase of appetite that the primary variable is hypothesised to affect. Therefore duration of the intermeal interval will partly determine the energy content and composition of the preload. For example, a 3MJ preload can theoretically be comprised of protein alone (176g protein), although this has limited relevance to food intake in the free-living setting. Alternatively the energy level can be increased by the addition of other macronutrients in smaller quantities; however this may affect other outcome variables, such as postprandial changes in appetite hormones.

Preload volume has an influence on subsequent energy intake. Increasing the volume of a preload by the addition of air or water without increasing caloric content reduces subsequent energy intakeby ~10% in lean subjects (151, 152). Energy intake was 17% lower when the volume of the preload was increased from 300 to 600mL (153). This effect is observed after 30 min and maintained for up to 4 h (153). It appears to be at least partially independent of sensory influences; increased volume (200-400ml) but not energy content (0.64-1.7MJ) infused directly into the stomach also lowered subsequent energy intake (154).

The physical form of the preload is another determinant of appetite responses. Liquid preloads are frequently employed to minimise variation in gastric emptying rate (155). Gastric emptying of liquids is faster than solids (156), which produces weaker satiation (14, 157). Additionally, the compensation (the adjustment of *ad libitum* energy intake in subsequent meals/eating episodes) for energy derived from liquids is less precise compared to solids (158). Current dietary patterns include a high proportion of energy from liquids (159) and beverage consumption is a predictor of obesity (160-162). Therefore results derived from liquids are relevant to the free-living setting; however such findings also require demonstration using solid foods.

4.2 Appetite Ratings

Visual analogue scales (VAS) questionnaires are the most commonly used tool to quantify subjective appetite sensations (163). Questions typically relate to different constructs of appetite, such as nausea, hunger, thirst, satiation, desire to eat and prospective food consumption (164, 165). Preference for particular tastes; "Would you like to each something savoury, fatty, sweet, salty?" are also commonly assessed (165). For each sensation, opposing extremes of the feeling are described at either end of a horizontal line (usually 100mm) and subjects draw a vertical line on the scale to indicate how they feel at that moment (Appendix 1). The questionnaire is commonly repeated throughout an observation period to generate a temporal pattern of change in response to a given intervention. Responses are quantified by measuring the distance from the left end of the scale to the mark.

The reproducibility (reliability) is a measure of the variability of responses assessed on two or more occasions (i.e. one immediately after another). When reproducibility is assessed in sequential tests there is a low level of variability in the free living setting (correlation coefficients $r = 0.89-0.96$, SEE = 5.81-8.97) (166). However lower repeatability (CR = 2SD) is observed when assessed on two different days (fasting; 2.86-5.24cm, peak; 3.16-6.44cm, mean; 1.36-1.88cm) (167). It is estimated that a sample size of 32 is needed to observe a 10% (10mm) difference in fasting, mean or peak/nadir values in a paired design study with power of 0.8 (165). Eighteen subjects would be required if peak/nadir responses were excluded. Lower reproducibility of assessments made on different days probably reflects true variation in the perception of appetite due to the numerous psychological, cognitive and environmental

influences which vary from day to day (168, 169). It is widely accepted that VAS ratings of appetite are sufficiently reproducible.

Validation studies examining whether VAS correlates with energy intake show mixed results. The mean VAS score over 4.5h for each question correlated more strongly with *ad libitum* intake (r = 0.50-0.53, P < 0.001) compared to the premeal VAS scores (r = 0.32-0.43, P < 0.05) (165). Certain questions also tend to show stronger correlations with energy intake (hunger, prospective food consumption, desire to eat and appetite for a meal) (157, 170-172) compared to other questions (satiety and fullness) (157, 170, 173). This may reflect a poor understanding of the term "satiated" and that fullness tends to be felt transiently in the early postprandial phase and may have little impact on subsequent energy intake. Nevertheless a correlation between VAS ratings and actual food intake is observed (174).

Sensitivity refers to the capacity of the VAS to show a treatment effect if one exists. Many studies show that VAS is sensitive to interventions where the dietary composition (175-177) or energy intake (178, 179) is manipulated. However it is important to note that interpretation of this is limited; the reason for a reduction in hunger may be due to the treatment, nausea, low palatability or influence of concomitant interventions (i.e. blood collection, duodenal infusion, energy expenditure measurement).

Categorical scales are less commonly used to assess appetite sensations (180-182). In this approach a line is equally divided into number of categories (typically 7) with specified differences in the sensation (Appendix 2). De Castro and Elmore found that

appetite ratings from a single full-hungry categorical scale correlated with self reported food intake (7d food diaries, n = 31, r = 0.27, P <0.05) (183). An assessment of the reliability and sensitivity of 5 commonly used appetite questions (including one 7-point, hunger-fullness categorical scale) was performed in lean, healthy subjects (n = 19) at 6 time points over 60 minutes (173). They found that the categorical scale produced the highest reliability (r = 0.64). A hunger-fullness VAS line produced similar reliability (r = 0.610) and the highest sensitivity to the satiating capacities of the 4 test foods. A criticism of categorical scales is that the difference in measured distance between categories does not necessarily translate to equal increments or decrements in the sensation.

A number of groups have recently transferred VAS questionnaires from "pen and paper" method to a hand held, electronic format (Apple Newton, Apple Computer, Inc., Cupertino, CA and Palm Pilot, PalmOne, Milpitas, CA) referred to as electronic appetite rating system (EARS) (166, 184-186). EARS is less labour intensive than the traditional method and reduces measurement and data entry error. Validation studies in the free living and laboratory settings show that the EARS and pen and paper methods produce similar results (166, 184, 186). However subjects tend to not use the extreme ends of the EARS scale and therefore data are less variable; maximum scores for EARS were less than those for the pen and paper method and minimum scores for EARS were higher (166). The lower variance in the data may reflect 2 key differences that probably discourage subjects from responding at extremes of the scale. The EARS scales is smaller (52 -66mm) compared to the VAS (100-150mm). Secondly the scale takes up a smaller proportion of the page width on paper, whereas its length is almost the whole screen width in electronic format. When assessing the reliability

of the two methods (completed immediately one after the other over two days in the free living setting) the lower variance did not compromise detection of a treatment effect; subjects (n = 16) reported lower hunger and higher fullness after a high protein snack compared to a high fat snack using EARS (187). The high protein snack also resulted in lower subsequent *ad libitum* energy intake.

In summary, the reproducibility of VAS scores is relatively low however this is probably an appropriate reflection of the highly variable nature of subjective appetite responses. Electronic versions of VAS produce reliable, valid and sensitive data, although variance is lower than the traditional pen and paper method. Data from VAS provides information on likelihood of consuming food, although this is not interchangeable with assessment of energy intake.

4.3   Food consumption

Buffet meals are commonly used to measure *ad libitum* energy intake in clinical studies. Subjects are provided with food in an excess quantity (varying in amount of choice and palatability) and are instructed to eat until comfortably satisfied. The foods are weighed by investigators before and after subjects eat, enabling the amount, energy content and macronutrient composition of the food consumed to be calculated. This method allows for a high level of control over data collection; data from self reported food diaries in the free living setting are less reliable due to errors in recording the food type, amount and preparation method, social and environmental influences and systematic underreporting by obese subjects (188-190).

The composition of the buffet meal has an important effect on *ad libitum* intake. Energy intake predictably increases in proportion to the level of food variety (191,

192), palatability (193) and amount of food provided (194). Data derived from buffet meals that provide a wide range of highly palatable foods may not reflect normal eating patterns; people do not typically eat meals comprised of multiple palatable foods (although they presumably select a palatable meal from a range of foods available in the home or the place of purchase). Conversely having no choice in the meal can lead to the development of sensory specific satiety and therefore reduce food intake before satiation would otherwise be reached. Additionally provision of one food may not allow for day-to-day variation in a subject's food preference in cross over studies.

Food with a smooth consistency assists in preventing subjects eating one component and not eating another (e.g. leaving the crust section of pizza) as this reduces the accuracy of nutritional analysis. Presentation of such a food in a large quantity also conceals the amount actually consumed (195) and minimises subjects eating according to their usual pattern rather than according to hunger level at that moment.

4.4   Chapter Summary

The assessment of appetite is challenging; it is difficult to maintain an acceptable level of external validity while controlling the complex array of environmental and physiological variables that influence the subjective and objective outcomes.

The methods described in this chapter address the reliability, validity and some practical considerations for quantifying *ad libitum* food consumption and subjective motivations in the clinical setting. The value of VAS and buffet meals is increased when collected together as they provide complementary information. However one is not interchangeable for the other; in the free-living setting food is eaten in the absence

of hunger because of planned meal times, social and cultural celebrations, emotional triggers or the presentation of a highly palatable food at the end of a meal. Conversely food is not eaten when hungry due to precluding circumstances such as a lack of time or food. Additionally fullness does not necessarily translate to satiation; intake of a large volume of a savoury food will not necessarily satisfy the desire for a sweet taste. This has an impact on the interpretation of research findings and their application to the free-living setting.

Chapter 5 Role of macronutrients in appetite regulation

Ad libitum energy intake is predictably increased by consumption of foods that are highly palatable, presented in large portion/serving sizes (196, 197) and energy dense (192, 194, 198). Intake is also increased by expanding the variety of foods offered (191, 192) and after previously consuming a low energy meal (174). The macronutrient composition of a meal is also a determinant of acute appetite responses; energy intake and hunger ratings are lower after consumption of high protein foods relative to other macronutrients. Indeed, dietary protein was one of three predictors, along with fibre and water content, to positively correlate with postprandial satiety (2h) in a study of 38 isoenergetic foods consumed by 41 male and female subjects (r = 0.37, P < 0.001) (199). The impact of dietary protein on satiety has been principally demonstrated in acute crossover studies of preloads or mixed meals rich in a particular macronutrient (Table 1).

5.1 Effect of dietary protein on ad libitum energy intake

Eight out of the 18 studies that have assessed energy intake observed lower *ad libitum* consumption after the high protein treatment compared to another macronutrient, with the reduction in intake ranging from ~150kJ (200) to >1MJ (201, 202). This relationship is more consistently observed when protein is consumed as a solid compared to liquid (15, 16, 171, 187, 200, 203, 204), suggesting that the rate of gastric emptying and/or rate of nutrient digestion and absorption may affect the impact of macronutrients on appetite.

Characteristics of studies that observed lower energy intake after solid, protein-rich test foods were an intermeal interval of 1.5 – 4h and consumption of at least 40g total protein. Conversely studies that did not observe lower energy intake had a long intermeal interval (7h) relative to the energy content of the preload (2.3MJ) (205), or a low absolute amount of protein (21g in HP and 7g in HF) (202). A further two studies simultaneously assessed energy expenditure using the ventilated hood method which requires subjects be resting supine (206) or in a metabolic chamber (176) and these conditions may affect the development of hunger. Finally a lack of treatment effect in one study may be partly due to the self-recorded food intake data (207) which is associated with a high level of error (188).

One study observed lower energy intake after a liquid HP preload (201) relative to carbohydrate; all other studies that tested liquid or semi solid (e.g. yoghurt) preloads found that energy consumption was independent of macronutrient composition, regardless of short (0.5-1h) (208, 209), intermediate (~3h) (207, 210) or long (24h) (177) intermeal intervals.

There has not been a clear demonstration of a dose dependent relationship between protein and appetite response. One study observed lower *ad libitum* energy intake at a buffet meal (1240kJ, 1034 kJ, 943kJ) 4.5h after consuming liquid preloads with increasing protein content of (0g, 40g, 80g respectively, P<0.05) (201).

Incorporating the protein into in a mixed meal (16, 171, 187, 200) does not appear to attenuate its effect on subsequent intake. An effect of macronutrient type was also observed despite differences in energy density between treatments (15, 201, 204). The

composition of the buffet meals in these studies ranged from one key food (e.g. casserole) (16, 171, 200) to a variety of highly palatable sweet and savoury foods and drinks (15, 201, 204). This suggests that the impact of protein may be adequately powerful to overcome the known effects of palatability, variety and energy density on intake. However a study performed by Raben and co-workers (206) compared 4 isoenergetic meals using whole mixed meals rich in protein, carbohydrate, fat or alcohol with similar energy density (4.8kJ/g), fibre content (1.8g/MJ) and palatability. There was no effect of macronutrient composition on subsequent *ad libitum* energy intake after the 5h inter-meal interval (206). The number of subjects (n = 19) may not have been adequate to detect differences between the 4 treatments and the simultaneous assessment of energy expenditure may have influenced the development of appetite.

5.2 Effect of dietary protein on subjective appetite ratings

Fourteen out of the 20 studies that included VAS reported reduced appetite after dietary protein ingestion, compared to carbohydrate and/or fat (Table 1). The higher satiety and/or lower hunger ratings after high protein (HP) preloads were observed during the early postprandial phase (15, 171, 202), late postprandial phase (16) and throughout the whole observation period (177, 204). Consistent with the effects of protein on energy intake, only three (201, 210, 211) out of the six studies that used liquid preloads observed lower appetite ratings after the high protein treatment. Two studies that recorded appetite over a 24h period (in metabolic chamber) found that the HP diet only produced higher satiety/lower hunger between breakfast and lunch (177, 212). The lack of treatment effect after this time may reflect fatigue in completing the questionnaire, reducing the accuracy of responses. Yet a similar study conducted over

36h in a metabolic chamber reported that a HP dietary pattern was associated with higher satiety and lower hunger ratings, compared to AP diet (213).

5.3 Effect of dietary protein on other indicators of appetite

High protein foods may also induce satiation earlier than similar HF foods. *Ad libitum* consumption of HP (54% of total energy from protein, 45% fat) and HF (15% protein, 80% fat) omelettes was compared. Subjects (n = 13) consumed 1726kJ (55g protein) of the HP omelette compared to 2995kJ (26g protein) of the HF omelette (P < 0.001) (202). In another experiment subjects (n = 10) consumed a controlled portion of the HP omelette (27g protein, 848kJ) and HF omelette (13g protein, 1512kJ) as an entrée and then ate *ad libitum* from a main meal (202). Total consumption of the entrée and main course was lower after the HP treatment (5964kJ) compared to the HF treatment (7098kJ; P < 0.001). The difference between treatments remained when the entrée serving was doubled (HP; 5922kJ, LP/HF 7599kJ) (202). Sensory specific satiety also develops earlier for HP compared to LP foods (214).

The effect of protein on spontaneous meal requests has been assessed as a marker of the latency of hunger. A 1MJ HP snack (omelette) with 77% energy from protein consumed in a non-hungry state (i.e. 4 h after *ad libitum* meal) delayed spontaneous meal requests by 60 min, compared to a delay of 25 min and 34 min for high fat (HF) and HC treatments respectively (relative to a "no snack" control; P < 0.01) (198). There was no effect of treatment on *ad libitum* energy intake at the requested meal (198); however this may have been influenced by differences in energy density (14, 152) between the omelette snacks (HP 5kJ/g, HC 10kJ/g, HF 15kJ/g). In a subsequent similar study that controlled energy density (5kJ/g), the HP snack (1MJ, 65% energy

from protein) delayed the spontaneous meal request by ~40 min compared to no delay following the HC snack (1MJ, 66% energy from carbohydrate; P < 0.01) although energy intake was independent of treatment (215).

One unique study demonstrated the effect of increased protein intake on satiety and subsequent *ad libitum* energy intake in the free-living setting (12). Nineteen subjects (BMI = 26.2 ± 2.1 kg/m^2) firstly followed an energy controlled, weight maintenance diet for 2 weeks (15% energy from protein, 50% carbohydrate, 35% fat). During this time subjects completed a daily food diary and VAS questionnaire for hunger and fullness. After this period subjects were instructed to follow an isocaloric (~8.4MJ) diet in which some energy from fat was replaced by protein, resulting in a doubling of the protein content (30% protein, 50% carbohydrate, 20% fat) for another 2 wk phase (weight stable). Subjects reported a significant and persistent reduction in hunger and increase in fullness after commencing the HP diet. In the third phase (12 wk) subjects followed the same HP dietary composition, but were allowed to self-select the amount of food consumed (i.e. *ad libitum*). Subjects spontaneously reduced the amount of food consumed by ~2MJ from the first day of this phase. Food records showed that this reduction was maintained for the next 12wk. VAS responses returned to phase-1 levels, indicating that their usual level of satisfaction was achieved when consuming significantly fewer calories. The change in self reported energy intake was validated by an overall reduction in bodyweight of 4.9kg by the end of the study. This weight loss over 12wk equates to ~0.4kg/wk which approximately corresponds to the reduction in energy intake of 2MJ/d. While this study suggests that HP intake increases satiety, reduces energy intake and results in weight loss, there was no control group, which diminishes the strength of these results. Spontaneously lower

energy intake has also been observed over 6d when consuming a HP diet (31% of energy from protein) compared to the American Heart Association diet (15% protein) (13).

Despite some inconsistent findings, it is generally accepted that increased dietary protein reduces subsequent *ad libitum* energy intake more than fat and carbohydrate (17, 216, 217). A number of aspects of this relationship require clarification:

To date, all acute studies investigating the effect of macronutrients on appetite have been performed with normal weight subjects (i.e. BMI 20 - 25 kg/m^2). Since obesity is associated with altered eating behaviour (218) and secretion of many hormones involved in appetite regulation (81, 219), the impact of macronutrients on appetite requires demonstration in overweight and obese subjects;

The duration of effect of dietary protein on satiety and the possibility of a dose-response relationship requires specific investigation (207). Similarly, it is not clear whether HP meals delay the onset of a meal (lengthen intermeal interval), in addition to reducing meal size;

Some studies have employed mixed meals to replicate how food is consumed in the free-living setting; however specific interactions between macronutrients have not been investigated.

The influence of the source or type of each macronutrient on appetite responses has received little attention;

There has been limited investigation of the impact of successive high protein meals on appetite over longer period of time;

The relative importance of dietary protein on appetite responses has not been compared to the influences of other dietary characteristics, such as energy density or physical form.

Table 1 Randomised crossover trials in adults of the effect of macronutrients on acute ad libitum energy intake and subjective appetite ratings

Reference	Sample, sex Weight status	Intervention			Time (preload - ad libitum intake) (h)	Ad libitum meal composition	Ad libitum energy intake	Satiety ratings
		Energy content (MJ); ratio of % percentage of total energy from protein:carbohydrate:fat	Amount of protein (g)	Form				
Barkeling 1990 (200)	n = 20F lean	2.6 HP (43:36:21) HC (10:69:21)	HP 64.5 HC 15.5	Solid HP (meat meal) HC (vegetable meal)	4	1 homogenous food (Swedish casserole)	HP 1266kJ < HC 1424kJ P < 0.05	NS
Booth 1970 (203)	n = 15F & M lean	-	HP 40 LP 6	Solid	2.5	-	HP 1063kJ < HC 1382kJ P < 0.05	NA
Crovetti 1998 (205)	n = 10F lean	2.3 HP (68:13:19;) HC (10:69:21) HF (9:21:70)	HP 93 HC 14 HF 12	Solid HP (meat +crackers) HC (pasta) HF (cheese+crackers)	7h	13 foods, 2 drinks	NS	HP>HC/HF P = 0.002
De Graaf 1992 (207)*	n = 29F lean	1.67 HP (70:27:3; milk protein) HC (1:99:0; maltodextrose) HF (3:5:92;cream)	HP 69 HC 1 HF 3	Liquid (550mL)	3.5	Self recorded intake, free living setting, rest of day	NS	NS**
Geliebter 1979 (209)	n = 12M lean	HP (100:0:0) HC (0:100:0)	-	Liquid	1h 10min	-	NS	NS
Fischer 2004 (211)	N = 15M lean	1.67 HP (100:0:0) HC (0:100:0) HF (0:0:100)	HP 98 HC 0 HF 0	Semi solid	3h (respiratory measurement)	Mixed foods	NA	HP>HC/HF f ratio = 2.62
Hill & Blundell 1986 (220)	n = 13	2.0 HP (31:21:29) HC (15:52:33)	HP 41 HC 17.8	Solid (sandwich + biscuit (HP) or + peanut (HC)	1	-	NA	HP>HC P< 0.05
Johnson 1993 (204)*	n = 8F, 6M lean	1.26 HP (81:0:19) HC (14:83:3) HC (3:10:87)*	HP 57 HC 10 HF2	Solid HP (chicken), HC (pasta) Liquid HF (cream)	1.5	20 savoury, 4 sweet foods	HP 2692kJ< HF 3419kJ P < 0.01	HP/HC > HF P < 0.05
Johnstone	n = 6M	15.2MJ/d;	HP 336/d	whole foods+ test	24	Ad libitum	NS	HP> HC/HF (24h

1996 (177)	lean	HP (37:34:28) HC (10:61:29) HF (10:33:57)	HC 92/d	milkshake	(calorimeter)	access to set menu for 24h		mean) $P < 0.001$
Latner & Schwartz 1999 (201)	n = 12F lean	1.76 HP (71.5:9.5:19.2) HC (0:99:0) Mixed (35.7:55.1:9.6)	HP 80.4 HC 0 Mixed 40	Liquid (840mL)	4.5	10 savoury foods2 sweet foods, 2 drinks	HP 3961kJ < HC 5204kJ Mixed 4343kJ $P < 0.05$	HP>HC/mix $P < 0.05$
Lejeune 2006 (213)	n = 12F lean	BMRx1.7 HP (30:40:30) AP (10:60:30)	HP 180/d AP 60/d	Solid (mixed foods)	24h	-	NA	HP>AP $P < 0.05$
Poppitt 1998 (16)	n = 12F lean	1MJ Control meal + 1MJ P,F,C,A HP (59:20:19) HC (11:68:19) HF (11:21:69) HA (11:21:20:A47)	HP 72 HC, HF& HA; 12	Solid (mixed meal)	1.5h	Chicken risotto and fruit bread	HP 2195kJ < HC 2502kJ/ HF 2558kJ/ HA 2772kJ $P < 0.05$	HP >HC/HF/HA $P < 0.05$
Porrini 1995 (171) *	n = 10M lean	HP 3.7 (56:19:25) HC 4.0 (17:56:27)	HP 122 HC 42	Solid HP meatballs HC pasta	2	Cheese, crackers, fruit tart	HP 1840kJ < HC 3406kJ $P< 0.05$	NS
Porrini 1997 (202)	n = 14M lean	HP 1.2 (54:1:45) HF 1.2 (15:6:79)	HP 21 HC 7	Solid (omelette)	2	7 savoury, 6 sweet foods, 2 drinks	NS	HP> HF (after preload only) $P < 0.001$
Raben 2003 (206)	n = 9F, 10M lean	2.5 (F), 3.0 (M); HP (32:37:31), HC (12:65:24), HF (12:24:65), HA (12:43:24:A23)	HP 47 (F) 57 (M); HC 18 (F) 21 (M)	Solid (mixed foods)	5 (ventilated hood)	Pasta, meat sauce and vegetables	NS	NS
Rolls 1988 (15)	n = 10F lean	1.3 HP (74:0:24) HStarch (9:66:8) HSucrose (2:86:9) HF (3:0:93) Mixed (5:57:36)	HP56 Hstarch 6.6 Hsucrose 1.8 HF2, Mixed 3.6	Solid HP chicken Hstarch pasta Hsucrose confectionary HF cream cheese Mixed chocolate	2	6 savoury foods, 4 sweet foods, 3 drinks	HP/HStarch > HSucrose /HF/ mixed (excluding "liquid" energy) $P < 0.05$	HP/HStarch > HF/HSucrose/mixed $P <0.05$
Stubbs 1996 (176)	n = 6M lean	5.2; HP (59:22:19) HC (19:61:21) HF (21:22:57)	HP 183 HC 57 HF 64	Solid mixed foods	5	Mixed foods	NS	5h: HP/HC>HF $P< 0.001$ 24h: HP>HF/HC $P = 0.049$
Stubbs 1999 (187)	n = 16M lean	5.8MJ/d; HP (60:20:20) HC (19:61:20) HF (20:20:60)	HP 203/d HC 65/d HF 63/d Mixed	Solid mixed foods	2h	HP (x10), HC (x10) and HF (x10) foods	Lunch: HP 3.58MJ, HC 3.87MJ, mixed 3.65MJ < HF	HP/mixed > HC/HF $P = 0.001$

		Mixed (33:33:34)	113/d				5.73MJ P = 0.028	
Teff 1989 (210)	n = 32M lean	HP (71:19:10) HC (0:100:0)		Semi solid (pudding)	3	?	NS	HP>HC
Vozzo 2003 (208)	n = 16M lean	3.0 HP (29:44:24) HC (14:60:24) HF (14:40:42) no preload (control)	HP 51 HC 25 HF 25	Semi solid (yoghurt; 500g)	0.5 (over 7h)	12 savoury foods, 6 sweet foods, 3 drinks	NS	NS
Westerterp 1999 (212)	n = 8F lean	8.9MJ/d HP (30:60:10) NP (10:30:60)	HP 173g/d NP 52g/d	Mixed foods	36h Respiration chamber	-	NA	HP>NP P < 0.001

F = female, M = male, P = protein, HP = high protein, LP = low protein, C = carbohydrate, HC = high carbohydrate, F = fat, HF = high fat, A = alcohol, HA = high alcohol, BF = breakfast, REE = resting energy expenditure, NS = not significant, NA = not assessed

* Also assessed all preloads at multiple energy levels. Results presented for highest energy level only

** Subjective rating of 'appetite for a meal' showed a dose response effect for protein treatment, but not for fat or carbohydrate treatments.

5.4 Proposed mechanisms for protein mediated satiety

An explanation for the higher satiety observed after consumption of dietary protein compared to other macronutrients is unknown, although a number of possibilities have been proposed. Understanding the mechanism may provide insight into strategies that enable the effect to be amplified.

5.4.1 Aminostatic theory

In 1956 Mellinkoff proposed the aminostatic hypothesis whereby a reduction in amino acids, detected by a 'satiety centre' in the brain, would trigger hunger. It was assumed that changes in peripheral plasma amino acid concentration reflected concentration within the brain and that this correlated with appetite (221). Studies investigating the timing of changes in peripheral and cerebral amino acid concentration after protein consumption are inconsistent (222, 223). Interestingly, the rate of change of plasma amino acid concentration (arising due to varying digestibility of proteins) affects the rate of protein synthesis and oxidation and this may partly contribute to mediating the satiating effect of dietary protein (224, 225).

5.4.2 Thermic effect of feeding

Thermic effect of feeding (TEF) or dietary induced thermogenesis (DIT) refers to the increase in energy expenditure above basal metabolic rate following food intake that represents the additional energy required to digest, absorb and metabolise nutrients. The digestive and metabolic processing of protein, fat, carbohydrate and alcohol differ and accordingly the TEF for each macronutrient also differs. Excess dietary protein is not efficiently stored and must undergo gluconeogenesis or be metabolised to urea. The energy cost of these processes is high; the TEF of a 2MJ pure protein load assessed over 7h was 529kJ, whereas equicaloric pure carbohydrate and fat loads were

66 and 99kJ respectively (n = 12 lean males, P < 0.01) (226). Similarly a HP mixed meal (2.3MJ; percentage energy from protein: carbohydrate: fat; 68:13:19) produced a TEF of 261 ± 59 kJ over 7 h, compared to equicaloric HC (10:69:21) and HF (9:21:70) meals (92 ± 67 and 97 ± 71 kJ respectively, P < 0.001) (205). Interestingly, the higher TEF of the HP treatment in this study correlated with fullness ratings (r = 0.41, P = 0.025) (205). Macronutrient specific differences in TEF are maintained over longer periods; the TEF measured over 24h in a respiration chamber (n = 8 lean females) was 1.3 MJ/d and 0.93 MJ/d for HP (29:61:10) and HF (9:30:61) dietary patterns, respectively (P < 0.01) (227). In this study, only the HP dietary pattern had a positive correlation with 24h satiety ratings (r = 0.6, P < 0.05) (227). Lejeune (213) reported that the TEF of a HP diet (30:40:30) differed from a HC diet (10:60:30) over 24 h (0.91 ± 0.25 and 0.69 ± 0.24 MJ/d respectively, P < 0.05), and again only the HP treatment correlated with appetite ratings.

It is proposed that the satiety associated with higher TEF may be due to greater oxygen consumption. Other states of oxygen 'deprivation' are associated with reduced appetite, such as chronic obstructive pulmonary disorder (228) and high altitude conditions (229). Additionally the higher TEF of protein increases body temperature (230) which may be interpreted as a satiety signal.

5.4.3 Protein-mediated gluconeogenesis

Mitheux (231) recently proposed that the higher satiety after protein consumption may be due to the stimulation of small intestinal gluconeogenesis which increases concentration of glucose in portal blood but does not noticeably change plasma concentration (232). These authors have demonstrated that an increase in portal blood

glucose concentration, (detected by glucose sensitive cells in the wall of the portal vein) (233) reduces feeding (234, 235) and stimulates the same hypothalamic nuclei that are stimulated after protein consumption in rodents (ARC, VMN, PVN, LHA) (231). These effects are not observed after portal vein denervation (231). However, the contribution of the small intestine to total gluconeogenesis remains controversial (236) and demonstrating such a relationship in humans will be challenging.

5.4.4 Gastrointestinal appetite hormones

The presence of nutrients in the gastrointestinal tract changes the secretion of appetite regulatory hormones, including ghrelin, glucagon like peptide-1 (GLP-1) and cholecystokinin. The stimulatory effect of duodenal fat on cholecystokinin secretion is clearly defined (123, 237); however less is known about the effects of other macronutrients on this and other gastrointestinal hormones. Further, there has been limited exploration of the possible relationship between macronutrient dependent changes in these hormones, gastric emptying and appetite responses.

Ghrelin

Two recent studies have shown ghrelin is suppressed for longer after consumption of dietary protein compared to carbohydrate and fat. Total ghrelin remained below baseline 3h after lean men consumed yoghurt preloads that were supplemented with whey protein (57g). In contrast, concentration returned to pre-prandial levels after the equicaloric HC preload (19g protein, 46g saccharose; Table 2) (94). The nadir after the HP treatment (-25%) occurred at 120 min, whereas the nadir after the HC treatment (-18%) occurred at 60 min (94). Appetite ratings and energy intake were independent of treatment (94).

Ghrelin also remained suppressed 3h after a high protein, mixed meal (Table 2) whereas HF and moderate carbohydrate isoenergetic meals resulted in a decrease below baseline for 1h (238). Appetite and energy intake were not assessed in this study (94, 238). Another study that used whole foods reported similar 24h ghrelin profiles after consuming either three NP or three HP meals (Table 2) (213). Although there was no overall time by diet interaction, a reduction in hunger after the breakfast meal was related to ghrelin for both treatments ($r^2 = 0.45$-0.52, $P < 0.05$) (213).

Erdmann and co-workers reported a small increase in total plasma ghrelin above fasting concentration 3h after consumption of a turkey meat preload, whereas ghrelin decreased after bread, butter and marmalade (HC) and cream (HF) intake (Table 2) (239). In a similar study by the same authors plasma ghrelin concentration remain unchanged 4h after consumption of pork (240). These conflicting findings may be due to the test food form; the influence of solid forms of muscle protein on postprandial ghrelin may require assessment over a period longer than 3 to 4h, given that slow gastric emptying delays the postprandial ghrelin nadir (76) and changes in ghrelin require post gastric feedback (70). Indeed, treatment effects for ghrelin are not observed until 120-180 min after semi-solid (yoghurt rich meal) preloads (94, 238). The preloads in the studies by Erdmann and colleagues also differed in caloric load, macronutrient composition and energy density (239, 240), all of which affect postprandial responses. One study has compared ghrelin responses to macronutrients in lean (n = 6) and morbidly obese (n = 10; BMI 43.4kg/m^2) subjects. Liquid preloads rich in either protein fat or carbohydrate reduced ghrelin by ~40% in lean and ~27% in obese subjects over 2h, independent of macronutrient type (92).

After 12 weeks of consuming an *ad libitum* HP diet, twenty-four h ghrelin AUC (determined by pooling individuals samples and measuring average hourly concentration) was increased (15,456 pg/24h/mL) relative to an energy controlled HC diet (13,979 pg/24h/mL) (P< 0.05) (12). However subjects were in a negative energy balance during the HP phase, which is also associated with increased ghrelin.

Table 2: Effect of macronutrients on postprandial ghrelin, appetite and energy intake in adults.

Author	Sample; sex & weight status	Intervention*	Duration (h) **	Ghrelin	GLP-1	Cholecystokinin	Ad libitum energy intake	Satiety rating
Al Awar 2005 (238)	n = 11; women lean	1.7MJ HP (35:45:20) HF (10:45:45) HC (20:50:30)	3	Total: HP<HF, MC 180min after preload.	NA	NA	NA	NA
Blom 2006 (94)	n = 15; men lean	1.65MJ yoghurt HP (58:14:28; whey) HC (19:47:34; saccharose)	3	Total: HP<HC 180min after preload. Nadir: HP 120min, HC 60min.	HP > HC P < 0.0005	HP > HC P < 0.01. HP produced biphasic response: peak at 15 and 60 min postprandial.	NS	NS
Elliot 1993 (241).	n = 8 lean	1.5MJ HP turkey meat HF cream HC glucose beverage	3	NA	HP remained elevates at180 min relative to HC	NA	NA	NA
Erdmann 2003 (239)	n = 10 lean	HP (1260kJ, turkey) HC (1090kJ, bread, butter, marmalade) HF (2520kJ cream)	3	Ghrelin increased after HP treatment.	NA	NA	NA	NA
Erdmann[1] 2004 (240)	n = 14	HP 2.3MJ lean pork HF 2.3MJ high fat homogenised meat HC 2.8MJ bread Fruit 1.8MJ Vegetables 0.6MJ	4	No change after HP	NA	NA	Fruit & vegetable> HP, HF, HC	NA
Feinle 2002 (242)	n = 10 lean	Duodenal infusion HP (98:2:0) HF (100:0:0) Mixed 18:60:22	1.5	NA	NA	HF peak at 5min after infusion, HP/mixed peak at 70 min after infusion. (P < 0.001)	NA	NS

Lejeune 2006 (213)	n = 12 women: lean	NP (10:60:30) HP (30:40:30)	24	NS	HP>AP for dinner meal (P < 0.05)	NA	NA	HP>AP
Marzullo 2006 (92)	n = 10 obese + 6 lean	Liquid preload: 2.5MJ HP (30:45:25) HC (17:53:30) HF (16.5:28:55.5)	2	Treatment NS	NA	NA	NA	NA
Raben 2003 (206)	n = 9F, 10M lean	2.5 (F), 3.0 (M); mixed foods HP (32:37:31), HC (12:65:24), HF (12:24:65), HA (12:43:24:A23)	5	NA	HP>HC, HF, HA	NA	NS	NS
Weigle 2005 (12)		HP ad libitum HC energy controlled	24 (AUC)	HP 15,456 pg/24h/mL HC 13,979 pg/24h/mL	NA	NA	HP<NP	HP>NP

GLP-1 = glucagon like peptide-1; HP = high protein; HF = high fat; MC = moderate carbohydrate; HC = high; carbohydrate; NP = normal protein; HA, high alcohol; BF = breakfast; AUC = area under the curve; NS = Not significant; NA = Not assessed; AP = adequate protein

*ratio of % energy from protein:carbohydrate:fat

**time between the preload and ad libitum intake

[1]Preload provided ad libitum.

<u>Glucagon like peptide-1</u>

GLP-1 was measured after healthy subjects (n = 19) consumed mixed meals high in each of the 4 macronutrients (Table 2) (206). Peak GLP-1 concentration occurred 120 min after the HP treatment and remained above baseline at the end of the 5h-sampling period, whereas concentration returned to baseline after the other treatments (P < 0.001). GLP-1 AUC was also highest after the HP meal (P < 0.01) (206). These differences did not relate to subsequent energy intake or appetite ratings (206). A similar acute study comparing HP and HC yoghurt preloads over 3h found that the HP treatment tended to produce higher postprandial GLP-1 concentration (P = 0.07) with the largest difference occurring in the late postprandial phase (120-180 min) (94). GLP-1 AUC for the HP yoghurt was ~66% higher compared to the HC preload, although this did not reach significance (P = 0.10). Finally an acute study compared GLP-1 responses to equicaloric (1.5MJ) portions of test foods (HP; cooked turkey meat, HF; double cream and HC; glucose-rich beverage). GLP-1 remained elevated 3h after the turkey relative to the HC treatment, although these results are difficult to interpret given the different physical form and energy density of the test foods (241).

Twenty-four hour GLP-1 profile measured in healthy subjects (n = 12) was similar after consuming either three AP meals (10% energy from protein, 60% carbohydrate, 30% fat) and after three HP meals (30% energy from protein, 40% carbohydrate, 30% fat) (213). The authors reported a non-significant trend (P = 0.10) for GLP-1 to be higher after the HP breakfast and a significant increase after the HP dinner, relative to the AP meal (213). The higher GLP-1 after the dinner meal correlated with an increase in satiety for the HP treatment (r^2 = 0.41, P < 0.05).

Cholecystokinin

High protein, HF and "mixed nutrient" solutions were infused into the duodenum of healthy subjects and plasma cholecystokinin was measured over the next 90 min (242). The rise in plasma cholecystokinin after the protein and mixed nutrient treatments were delayed relative to the high fat treatment; peak concentration occurred after 70min and remained elevated at the end of the study (242). Peak concentration after the HF infusion occurred after 10min (242). Cholecystokinin elevation was prolonged after consumption of a HP yoghurt compared to a similar HC treatment (Table 2) (94). Peak concentration for the HP treatment occurred later than the HF treatment (120 vs. 80 min respectively) (94). Cholecystokinin after the HP preload remained above baseline at the end of the sampling period (180 min), whereas concentration for the fat preload returned to baseline (94). Interestingly the HC preload produced a similar cholecystokinin profile, albeit lower in concentration which may have been due to the protein content of this preload (18g) (94). Both preloads contained relatively high and different amounts of fat (HP; 28% of total energy, HC; 34%). Given the contribution of fat to cholecystokinin release, this variation may confound these findings.

In summary current findings indicate that gastrointestinal hormones contribute to macronutrient specific differences in acute appetite responses. This has been observed in lean subjects and requires demonstration in the overweight population. Preliminary findings suggest that protein may prolong the suppression of ghrelin and elevation of cholecystokinin and GLP-1. However this has not been associated with a reduction in food consumption. The impact of dietary protein on the timing and duration of these effects requires exploration.

5.5   Impact of protein type on appetite responses

Discrepancies in the hierarchy of satiety associated with different macronutrients are reported (176, 177, 202, 205-208, 210). This may be partly explained by differences in the satiating capacity of foods within each macronutrient group. Hall and co-workers reported that *ad libitum* energy intake was 19% lower 90 min after consuming a liquid preload containing 48g (1.7 MJ) of whey protein isolate compared to a similar casein-based preload (243). Appetite ratings on that day were independent of treatment, however on another test day the same whey treatment produced lower desire to eat and higher fullness compared to casein.

Energy intake of healthy males (n = 13) 1 h after consuming a whey based liquid preload was marginally lower (3599 ± 234 kJ) compared to egg albumin (4649 ± 272 kJ), but was not different to the soy treatment (3887 ± 293 kJ) (244). Lang and co-workers investigated the effect of a range of proteins consumed at lunch on appetite ratings and *ad libitum* energy intake (245, 246). Healthy males (n = 12) consumed isocaloric meals (5.2MJ, 67g protein) that included covertly manipulated test foods (mousse and soup) rich in plant (soy, pea or gluten) or animal (egg albumin, casein, gelatine) proteins. Energy intake and appetite ratings over 8 h were independent of protein type (246). Intake and appetite ratings were also similar when the energy and protein levels were reduced (3.6MJ, 50g of casein, gelatin or soy protein; 1.8MJ, 25g of protein) (n = 9 healthy males) (245, 246). A comparison of different muscle proteins found that a hunger-fullness rating was higher 120-180min after consuming cooked fish compared to chicken and beef (50g protein) although energy intake was not assessed (182). The peak in plasma amino acids was delayed for fish compared to beef and chicken, which the authors attributed to lower digestibility. These authors

did not measure palatability which may have influenced the satiety ratings (182). A similar study comparing minced beef or fish served in a mixed rice dish (2.5MJ, 70.6g protein, 13.4g fat, 49.7g carbohydrate) reported similar satiety and *ad libitum* intake at the subsequent evening meal 4h later (fish 2802 ± 1095kJ; beef 3133 ± 904 kJ, P = 0.07).

Dietary protein type may also affect other responses. Postprandial insulin AUC (2h) was highest for a whey based test meal (18g protein, 25g carbohydrate), relative to similar test meals containing milk, cheese, whey, cod and gluten (247). This difference occurred even though the change in glucose was lowest for whey and GLP-1 was independent of treatment (247). Earlier studies have also observed differences in postprandial plasma insulin after different proteins (248, 249), although this is not consistently observed (250). The variation in insulinemia may reflect a stronger insulinotropic effect of certain amino acids such as leucine, phenylalanine and tyrosine (251) and the content of these amino acids varies between proteins.

A whey preload produced higher plasma concentration of GIP (0- 90min), GLP-1, cholecystokinin concentration (90-180 min; after an additional control meal had been consumed) and total and branched chain amino acid concentration (243). The authors proposed that the differences in appetite and *ad libitum* energy intake were related to faster gastric emptying and plasma amino acid appearance observed after whey (224). Faster gastric emptying would shorten the time for gastric digestion and therefore the chyme that enters the small intestine may require more extensive intestinal digestion (252). Consequently luminal contents may move further distally in the small intestine and therefore prolong the stimuli for changes in satiety hormones, as previously

proposed. The influence of gastric emptying and digestion rates on hormonal responses requires exploration.

Dietary proteins may also differ in their thermic effect. One study has reported 2% higher energy expenditure (24h) when subjects consumed a diet rich in animal protein (pork) compared to a similar vegetable protein (soy) diet (253). A possible impact of this on appetite has not been investigated, although is likely to be small.

The composition of whey protein may further enhance its satiating capacity. Whey contains high levels of branched chain amino acids (BCAA; leucine, isoleucine and valine) compared to other animal and plant proteins. These amino acids have a unique extra-hepatic metabolism and interact with the insulin signalling pathways (254) which may enhance satiety (255). Glycomacropeptide (GMP; a C-terminal fragment of kappa casein) is a by-product of rennet digestion formed in the human gut and is in high concentrations in whey (256). GMP stimulates cholecystokinin secretion (257), suggesting it may contribute to appetite suppression; however this has not yet been explored.

In summary, the role of proteins in appetite regulation may be affected by the source of protein, although data to describe this is limited. Observation of gastrointestinal responses to various plant and animal derived proteins may give further insight into the mechanism by which proteins differentially affect appetite relative to carbohydrates.

5.6 Impact of carbohydrate type on appetite

Increasing obesity prevalence has coincided with the replacement of some dietary fat with carbohydrate rich foods (258). This has lead to speculation that carbohydrates may promote excess energy intake and lead to weight gain (258).

Chemical structure has historically been used to classify carbohydrates; simple sugars (mono- and disaccharides) and complex carbohydrates. However it has become apparent that this has little to do with metabolic effects. In 1981 Jenkins proposed the concept of glycemic index (GI) as a means of grouping carbohydrate-containing foods (259). High GI foods are those that produce a rapid early increase in postprandial glycemia. Low GI foods produce a lower, prolonged blood glucose response. GI was initially intended as a tool for limiting postprandial hyperglycemia to aid in the dietary management of type-1 diabetes, although it has also been applied to broader dietary issues. Epidemiological studies have shown a positive association between consumption of high GI foods and obesity, insulin resistance and type 2 diabetes (260). These correlations may also serve as a marker for diet quality, where high GI foods (e.g. carbonated beverages, white bread, potato and confectionary) are typically consumed as part of a high calorie, high fat, energy dense diet which is low in fibre and micronutrients (261). This topic remains the source of intense debate (262, 263) which is unlikely to be resolved whilst long-term studies of adequate power remain scarce. The remainder of this chapter will focus on the parallel debate of the contribution of glycemic index to acute appetite responses.

Associated with GI is the glucostatic theory proposed in 1953, (264) whereby an increase in postprandial glycemia attenuates hunger sensations and a reduction in

concentration stimulates meal initiation. Clamp studies have demonstrated that spontaneous meal requests are frequently preceded by a transient reduction in blood glucose (~-10%) (265, 266) and appetite is reduced when postprandial glucose is elevated, although not consistently (267).

Assessing the contribution the GI of whole foods and individual carbohydrates on appetite is complicated. Both appetite and glycemic response are affected by the dose, form (solid or liquid) (18) and volume (174) of carbohydrate containing foods. The GI of a mixed meal is also influenced by the presence of other macronutrients, pH, cooking and storage processes, plant variety and ripeness and mastication (particle size). Low GI foods are often associated with dietary fibre, which slows gastric emptying and transit time (268), which influence both glycemic and appetite responses. Carbohydrate ingestion stimulates other appetite regulatory hormones, including GLP-1, ghrelin and cholecystokinin (269) – making it difficult to separate the effects of glycemic and insulinemic responses from other appetite hormones. Therefore it is difficult to distinguish the role of glycemic response, *per se*, from other carbohydrate-induced effects.

The timing of *ad libitum* food intake assessment also influences interpretation of results. High GI foods are associated with reduced hunger in the sort term (+60min) but seem to make a small contribution to satiety (199, 270), whereas low glycemic profiles produce satiety for up to 6h (271), perhaps due to the prolonged availability of glucose in the post absorptive state and absence of overshoot to below fasting level.

Finally, participant characteristics influence outcomes. Overweight and obese subjects have compromised insulin sensitivity (118) relative to lean subjects therefore findings need to be demonstrated in both subject groups.

A meta-analysis of 31 studies investigating the effect of high and low GI foods (and meals) on appetite and *ad libitum* energy intake (20) reported low GI treatments produced greater satiety compared to high GI foods in half of the studies (15). Seven out of the 15 studies that assessed *ad libitum* food consumption reported a positive correlation between GI and subsequent energy intake; energy intake was ~0.4 - 2.2MJ lower after consumption of the low GI test foods compared to the high GI treatments. Three of these studies used pure fructose preloads, which may have caused malaise (see Chapter 5.6.1). Half of the 12 studies that controlled energy content and macronutrient composition of the preloads reported that the low GI treatment reduced subsequent energy intake. A review of literature published at the same time as this meta-analysis concluded that short-term studies "generally" presented an inverse association between GI and satiety (272).

The effects of glycemic responses on appetite related hormones are relatively unexplored. One study reported lower ghrelin AUC after a "complex" carbohydrate preload (complex; 102 g maltodextrin + 12 g exopolysaccharide) relative to a "simple" carbohydrate preload (121g maltodextrin) and this correlated with lower appetite AUC (69). However the glycemic response to both preloads was similar. Extending the length of the small intestine exposed to glucose was associated with prolonged ghrelin suppression (77), indicating that ghrelin may be involved in lowering appetite after consumption of carbohydrates that require extensive digestion.

Understanding the relationship between the characteristics of carbohydrate containing foods and postprandial insulin, glucose and appetite related hormones might clarify the role of GI in satiety.

5.6.1 Fructose

The use of fructose (and high fructose corn syrup) as a sweetener in processed food and beverages is increasing, although this is occurring in parallel with a reduction in the use of sucrose (273). In the United States average per capita per annum "disappearance" (data are unadjusted for spoilage, plate waste and other losses) has increased from 0.68kg to 28.4kg (274). This is attracting attention due to the potential consequences of its unique metabolic handling (275, 276) and the parallel increase in obesity prevalence (275-277).

Fructose empties from the stomach more rapidly than glucose (278). Entrecotes in the jejunum express the glucose specific hexose transporter GLUT-5 that facilitates fructose uptake. When consumed alone fructose absorption is incomplete and slow. The resultant hyperosmolar luminal contents causes a fluid shift, diarrhoea and malaise (279). The presence of some glucose increases the rate of fructose absorption and prevents these side effects (279). Absorbed fructose is transported to the liver via the portal vein. Hepatic metabolism of fructose is unique because it avoids the rate-limiting step in glycolysis controlled by phosphofructokinase and therefore allowing unregulated de novo lipogenesis. Consequently fructose consumption is associated with a prolonged postprandial elevation in plasma triglycerides (280, 281) and this may have a detrimental effect on lipid profiles. Fructose does not directly stimulate insulin secretion because pancreatic β-cells do not express the GLUT-5 transporter (282). There is a small increase in insulin observed after fructose consumption (281,

283) which is probably due to the release of the incretin GLP-1 (284). Additionally some fructose is metabolised to glucose in the liver and this produces a marginal increase in blood glucose concentration. The glycemic index of fructose is low (GI: 50).

Early studies of pure fructose loads reported lower acute *ad libitum* energy 0.5 – 2.25h after consumption of fructose beverages (50g) relative to glucose (285, 286), and when assessed as a semi-solid after 2.25h (287). However more recent evidence indicates that fructose-rich and glucose-rich liquids equally affect appetite ratings and food intake in lean subjects after 1h (18), 2h (284, 288, 289) and 3h (demonstrated in lean and overweight) (290) and when incorporated with mixed meals (281, 286, 291) and starch (286). These findings differ from what may be predicted by the GI of fructose.

The impact of fructose relative to other sugars on energy balance and body weight is also inconsistent. A small number of long term studies have reported that prolonged consumption of fructose beverages (3-12 wk) promotes weight gain relative to a non caloric, sweetened control beverage (234, 292). Yet similar studies have also demonstrated that sucrose sweetened beverages result in excess energy intake and weight gain, relative to a higher starch diet (293). There has not been a study to investigate whether sugars in beverages differ in their propensity for excess energy intake and weight gain. The findings described above may indicate poor compensation for energy consumed in liquid form (161, 294). *Ad libitum* energy intake and bodyweight (+0.5kg) over 4wk was lower when subjects consumed

1880kJ/d of sugar as a confectionary food compared to the crossover treatment of a similar carbohydrate amount consumed as a liquid (295).

To date, there is limited information on the effect of fructose on gastrointestinal appetite hormones. Lean weight women (n = 12) consumed test beverages (30% of total energy from fructose or glucose) along with standardised breakfast (0900h), lunch (1300h) and dinner (1800h) meals and blood samples were collected over 24h (30 min – 1 h intervals) (281). The fructose treatment produced a smaller ghrelin nadir (after breakfast only), prolonged GLP-1 elevation (lunch and dinner meals only) and lower insulin, leptin and GIP relative to the glucose treatment (281). Oral fructose and glucose loads (100g) caused a reduction in ghrelin relative to a saline infusion (n = 6 lean males) although repeated measures statistics were not reported to determine if there was a significant difference between these sugars (296).

A study in lean subjects found that GLP-1 concentration was lower 0-60 min after liquid preloads containing 75g fructose compared to glucose, but this difference resolved 60-180min after the treatments (284). Beverages containing 75g of glucose or fructose produced similar postprandial GLP-1 in overweight and type 2 diabetic subjects (290). Despite variation in the effect of fructose on GLP-1, these differences were not related to subsequent *ad libitum* food intake (281, 284, 290).

The limited data available to date suggests that *ad libitum* energy intake after fructose consumption is similar to glucose, despite the lower glycemic and insulinemic response to fructose. The impact of this sugar on gastrointestinal satiety hormones requires further investigation.

Progression of research program

The objective of this thesis is to investigate the effect of various dietary proteins and carbohydrates on acute changes in gastrointestinal derived appetite hormones, subjective appetite ratings and *ad libitum* energy intake in overweight/obese subjects.

The first study compared the effects of high protein (whey and casein) and high carbohydrate (glucose and lactose) liquid preloads on postprandial changes in appetite hormones, glycemia, insulinemia, aminoacidemia and gastric emptying. Both proteins similarly prolonged suppression of ghrelin and elevation of cholecystokinin compared to the glucose treatment, which corresponded with lower appetite and gastric emptying respectively. Whey and casein also produced similar total plasma amino acid profiles. This contrasted an earlier report indicated that whey was associated with lower energy intake and higher aminoacidemia, GLP-1 and cholecystokinin (243).

The second study compared different sources of dietary protein (whey, soy, gluten) relative to glucose to further explore the contribution of protein source to appetite responses. We confirmed the observation from study one that protein prolonged ghrelin suppression and cholecystokinin elevation, and that this was independent of protein source. Additionally, it replicated our observation that a reduction in plasma glucose below fasting levels 3h after glucose ingestion coincided with an increase in ghrelin above baseline which was associated with the highest ad libitum consumption. The postprandial elevation in plasma GLP-1 concentration was also prolonged after all protein treatments, relative to glucose. It was shown for the first time that fasting and postprandial concentration of GLP-1 is increased in overweight subjects compared to their lean counterparts. Macronutrient specific differences in these

appetite hormones and overall energy intake were similar in lean and overweight/obese individuals, despite lower ghrelin and higher GLP-1 responses to the preloads in those with high BMI.

Since the first and second studies did not show any differences between the protein sources, the third study aimed to provide preliminary data on the interaction between dietary protein and carbohydrate and to extend the previous observations to 4 hours post preload. The objective of the combined protein/carbohydrate treatment was to explore dietary approaches that may prolong satiety. The carbohydrate (fructose) was chosen to avoid the reduction in plasma glucose below baseline, which we had previously observe coincide with an increased ghrelin concentration. Additionally, fructose has recently received considerable attention due to increased consumption, particularly in the form of high-fructose corn syrup in carbonated beverages.

The studies contained in this thesis provide new insights into the effects of dietary proteins and carbohydrates on acute appetite responses and associated regulatory hormones in overweight men.

Chapter 6   Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men.

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Developed protocol, prepared ethics application, conducted meal studies, performed laboratory analysis, statistical analysis, interpreted data, wrote manuscript and acted as corresponding author.

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Manny Noakes

My contribution to this paper involved:

>   Contribution to protocol design, assistance with data interpretation and manuscript evaluation.

I give consent for Jane Bowen to present this paper for examination towards the Doctor of Philosophy

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>   Supervisior for the analysis of plasma amino acids.

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Assistance with data interpretation and manuscript evaluation.

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Energy Intake, Ghrelin, and Cholecystokinin after Different Carbohydrate and Protein Preloads in Overweight Men

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Context: Dietary proteins appear to be more satiating than carbohydrate. The mechanism and effect of protein and carbohydrate type are unclear.

Objective: The objective of the study is to compare the acute effect of different proteins and carbohydrates on indicators of appetite and appetite regulatory hormones.

Design: This is a randomized cross-over study of four orally consumed preloads followed by blood sampling (+15, 30, 45, 60, 90, 120, 180 min), then a buffet meal.

Setting: The study was carried out in an outpatient clinic.

Patients and Other Participants: Nineteen overweight (body mass index 32.1 ± 0.9 kg/m^2) men participated.

Interventions: Liquid preloads (1 MJ) contained whey (55 g), casein (55 g), lactose (56 g), or glucose (56 g).

Main Outcome Measures: Plasma ghrelin, cholecystokinin (CCK), insulin, glucose and amino acids, gastric emptying rate (plasma paracetamol), appetite rating (visual analog scale), and *ad libitum* energy intake were the main outcome measures.

Results: Energy intake was $10 \pm 3\%$ higher after the glucose preload compared with lactose and protein preloads ($P < 0.05$), which were predicted by ghrelin at 120 min ($P < 0.05$). CCK was $71 \pm 6\%$ higher 90 min after the protein preloads compared with glucose and lactose ($P < 0.05$), which predicted appetite at 180 min ($P < 0.05$). There was a small increase in branched chain amino acids after the whey preload compared with casein ($P < 0.01$), but this was independent of appetite and energy intake.

Conclusion: Acute appetite and energy intake are equally reduced after consumption of lactose, casein, or whey compared with glucose, which was consistent with differences in plasma ghrelin. Higher CCK responses after proteins correlated with satiety but did not affect energy intake. (*J Clin Endocrinol Metab* **91:** 1477–1483, 2006)

CONSUMPTION OF DIETARY protein generally produces greater satiety (absence of hunger during the inter-meal period) and reduces *ad libitum* energy intake compared with carbohydrate or fat (1–3). Discrepancies in this ranking of macronutrients are reported (4), and this may be explained by further differences in the satiating capacity of foods within each macronutrient group.

Both carbohydrate and protein type appear to have different effects on satiety. Glycemic index (GI) ranks carbohydrate-containing foods based on postprandial glycemia. Low GI foods are thought to increase satiety by prolonging the availability of glucose in the postabsorptive state and by producing a lower insulin response; however, this association remains controversial (5). Higher satiety and lower *ad libitum* energy intake was observed after whey protein consumption compared with casein in lean subjects (6). The authors proposed this was a consequence of faster gastric emptying of whey (7).

Gastrointestinal hormones involved in appetite regulation could mediate the differences in satiety between proteins and carbohydrates. Ghrelin is an orexigen that is mainly released from the stomach (8) in response to feedback from either the intestine or a postabsorptive site (9). Plasma concentration rises before meals and decreases within 15–20 min of food consumption (10). Administration of ghrelin is associated with increased food intake in humans (11). The dynamics of ghrelin during the postabsorptive phase (*i.e.* return to preprandial concentration) and effects on subsequent meal size are poorly understood. Few studies have investigated the effect of macronutrients on postprandial ghrelin (12–14).

Cholecystokinin (CCK) regulates the rate of nutrient delivery from the stomach to the small intestine, and secretion is stimulated by presence of duodenal protein and fat (15). The secretion of gastrointestinal hormones such as ghrelin may be affected by gastric emptying rate. For example, a rapid emptying rate reduces time for gastric digestion and releases chyme that requires extensive small intestinal digestion (16). Consequently luminal contents will move further distally in the small intestine, and this may prolong the stimuli for changes in satiety hormones. In addition to regulating gastric emptying, CCK is also associated with satiation in animals (17) and humans (18).

The aim of this study was to compare the effects of two proteins, which differ in their rate of gastric emptying (whey, fast; casein, slow), with two carbohydrates, which differ in GI

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(glucose, high; lactose, low), on appetite and food intake in overweight men. Additionally, we investigated whether differences in satiety were related to postprandial plasma ghrelin, CCK, glucose, insulin, amino acids, and the rate of gastric emptying. The study was performed in men to avoid the effects of menstrual cycle on appetite (19). Additionally, eating behavior (20) and circulation of satiety hormones (21) are different in lean and obese subjects. Because dietary strategies that may optimize satiety are most applicable to overweight subjects, we selected overweight subjects for this study.

Subjects and Methods

Subjects

Twenty men were recruited by public advertisement and one withdrew before the study commenced. All subjects were weight stable, nonsmokers, unrestrained eaters (assessed by a validated three-factor eating questionnaire) (22), and did not have medical conditions that affect gastrointestinal motility or appetite. The study was approved by the Commonwealth Scientific Industrial Research Organisation Human Ethics Committee. All subjects gave informed, written consent to participate.

Experimental protocol

Subjects attended the clinic on four occasions with 7 d between visits. On each occasion, subjects arrived after fasting overnight and refraining from exercise, paracetamol, and alcohol for 24 h. Subjects' weight and height were measured (Mettler scales, model AMZ14; A&D Mercury, Kinomoto, Japan) in light clothing. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters^2). A cannula was inserted into a lower arm vein, and a fasting blood sample was taken (−15 min). Subjects then completed a validated visual analog scale (VAS) questionnaire to assesses nausea, hunger, thirst, satiation, desire to eat, and amount of food that could be eaten (23). Opposing extremes of each feeling were described at either end of 100-mm horizontal lines and subjects drew a vertical line on the scale to indicate how they felt. The liquid preload was consumed at 0900 h (time 0). Subjects were asked to finish the beverage in 5 min, and the beverage was followed by 1500 mg paracetamol (GlaxoSmithKline, Ermington, Australia) dissolved in 100 ml water. Postprandial plasma paracetamol concentration is a validated, indirect marker for the rate of liquid and semisolid gastric emptying (24). Subsequent blood samples and VAS measurements were collected 15, 30, 45, 60, 90, 120, and 180 min after time 0. Subjects were then given a buffet-style lunch. Each subject served their own meals from designated portions of the buffet foods and ate until satisfied. After 30 min, subjects departed the clinic.

Dietary protocol

Preloads (1 MJ) were made from water (100 g), milk (1% fat; 200 g), artificially sweetened chocolate syrup (50 g), and either whey protein isolate (55 g; Murray Goulburn, Brunswick, Australia), calcium caseinate (55 g, Murray Goulburn), glucose (60 g; GI = 100; Glucodin, Boots Healthcare, North Ryde, Australia) or lactose (56 g; GI = 43; Ace Chemical Company, Adelaide, Australia) (Table 1). The preloads did not contain dietary fiber and were controlled for energy, energy density, palatability, and consistency.

The buffet lunch included 600 g meat sauce, 500 g pasta, 600 g veal casserole, 500 g white rice, 180 g white bread, 50 g margarine, 200 g shortbread biscuits, and 210 g cake (20 MJ, 18% of total energy from protein, 31% from fat, and 51% from carbohydrate). The hot foods (meat sauce, pasta, casserole, and rice) were prepared in one batch, frozen until required, and reheated according to a standard protocol. Each food was weighed to the nearest gram before and after eating using digital scales. Energy and nutrient composition was calculated by one dietitian using Food Works Analysis Package 3.02 (Xyris Software, Highgate Hill, Australia) based on the Australian Food Composition Tables (25).

TABLE 1. Dietary composition of the four different preloads[a]

Preload	Whey	Casein	Glucose	Lactose
Energy (kJ)	1069	1090	1025	1025
Protein (g) (% of energy)	52.2 (83)	52.4 (83)	7.2 (12)	7.2 (11)
Fat (g) (% of energy)	0.5 (2)	1 (3)	0.2 (1)	0.2 (1)
Carbohydrate (g) (% of energy)	10.3 (15)	10.2 (15)	56.0 (87)	56.0 (88)
Glycemic load[b]	4.4	4.4	51.1	25.7
Energy density (kJ/g)	2.7	2.7	2.5	2.5

[a] Dietary composition based on data from Australian Food Composition Tables (24) and ingredient manufacturers (whey, casein, lactose).

[b] Glycemic load [carbohydrate (grams) × glycemic index] is a measure of the total glycemic response to a food containing carbohydrate.

Biochemistry

Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 min. Blood for plasma was collected in sodium fluoride/EDTA (1 g/liter) tubes containing aprotinin (500 KIU/ml blood; Roche, Basel, Switzerland) and stored on ice. Blood for plasma amino acid analysis was collected in tubes containing EDTA and heparin at 0, 30, 60, 90, and 120 min. Serum and plasma were isolated by centrifugation for 10 min at 2000 × g (5 C) (Beckman GS-6R centrifuge; Beckman Coulter, Fullerton, CA) within 1 h of collection and aliquots were stored at −80 C.

Total ghrelin in unextracted plasma was measured by competitive RIA (Phoenix Pharmaceuticals, Belmont, CA); the detection limit was 70 pg/ml and interassay variation was 5.5%. CCK was analyzed by competitive RIA (Euro-diagnostica, Malmo, Sweden) using an antiserum raised against CCK-8 with cross-reactivity to CCK-33; the detection limit of the assay was 0.3 pmol/liter and interassay variation was 14%. CCK was extracted using ethanol; plasma was mixed with an equal part of 96% ethanol, vortexed for 10 sec, left to stand at room temperature for 10 min, and then centrifuged at 1700 × g for 15 min. The decanted supernatant was evaporated to dryness using a speed-vac concentrator (Savant, Farmingdale, NY) at 37 C, then reconstituted to original plasma volume with assay buffer. Serum paracetamol (acetaminophen) was measured using an enzymatic kit (Cambridge Life Sciences, Cambridgeshire, UK) adapted for a Cobas Bio centrifugal analyser (Roche Diagnostics, Basel, Switzerland). Serum insulin was measured using a enzyme immunoassay kit (Mercodia, Uppsala, Sweden). Plasma glucose was determined using an enzymatic kit (Hoffmann-La Roche Diagnostics, Basel, Switzerland) and control sera on a Hitachi 902 Automatic Analyzer (Roche Diagnostics).

Plasma amino acid analysis was performed for samples after the whey and casein treatments. Plasma was diluted with internal standard solution (nor-leucine) and placed in a Centricon YM-10 ultra filtration device, vortexed, and centrifuged for 25 min at 5200 rpm (10 C). The filtrate was diluted with HPLC buffer, and the amino acids were separated using a cation exchange HPLC column in the lithium form (Shodex CXpak amino acids column) and quantified after post column derivatization with ninhydrin (26).

Biochemical analyses were performed after study completion, and all samples for individuals were analyzed in the same assay.

Statistical analysis

Results are presented for 19 subjects and are expressed as means ± SE (SEM) except for subject characteristics (SD). No differential effects between whey and casein were identified for any parameters, so data for both preloads are presented as a mean, called "protein" (except amino acid data).

The distance between the left end of the VAS scale and each mark was measured (millimeters). The change in rating from baseline was calculated. Reliability analysis was performed (Cronbach's alpha) to assess inter-item correlation of all VAS questions. By factor analysis the reliability of all questions (α = 0.59) was improved by excluding nausea and thirst (α = 0.73), confirmed by Pearson correlation (two-tailed, $P < 0.01$). Accordingly, an overall "appetite" measure was calculated as the mean

response to "amount that could be eaten", "desire to eat", "hunger", and "satiation".

Total area under the curve (AUC) for glucose, insulin, ghrelin, CCK, paracetamol, and amino acids was calculated using the trapezoidal equation (27). AUCs were compared using one-way ANOVA.

ANOVA with repeated measures was used to determine the effects of the treatment and time (minutes) with the treatment order, BMI, and fasting insulin and glucose included as covariates. Where ANOVA showed a statistically significant main effect, Tukey's *post hoc* tests were performed to compare group differences. Relationships between appetite and energy with other variables (BMI, weight, AUC, and fasting, peak, and nadir concentrations) were examined using multiple linear regressions. Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). Differences are considered significant if $P < 0.05$.

Results

Subjects

Nineteen subjects completed this study with a mean BMI of 32.1 ± 3.7 kg/m^2 (range, 26.8–40.4), fasting glucose concentration of 110 ± 1 mg/dl (range, 93–141 mg/dl), and aged 53.3 ± 6.1 yr (range, 41–63 y). Inclusion of one subject with elevated fasting blood glucose concentration (141 mg/dl) did not affect outcomes or significance and, therefore, was included in data analysis. There were no differences in the fasting values of any parameters between treatments.

Energy intake, appetite, and ghrelin

Ad libitum energy intake after the glucose treatment was 493 ± 141 kJ and 541 ± 190 kJ greater than after the protein and lactose preloads, respectively ($P < 0.05$; Table 2). There was no treatment effect on macronutrient composition of the intake (data not shown).

There was no time ($P = 0.11$) or treatment ($P = 0.43$) effect for nausea or thirst ratings. Hunger, satiation, desire to eat, and amount of food that could be eaten showed an effect of time ($P < 0.0001$) but not treatment. There was a time by treatment interaction for the overall appetite rating ($P < 0.05$); appetite was higher after the glucose preload compared with the protein and lactose preloads ($P < 0.05$; Fig. 1).

The ghrelin nadir ($-25 \pm 2\%$) occurred at 60 min (time effect, $P < 0.01$) and returned to baseline levels at different rates (time by treatment interaction, $P < 0.05$; Fig. 1). After the protein and lactose preloads, the nadir concentration remained stable for 1 h before returning to the preprandial concentration. After the glucose preload, ghrelin returned to baseline levels within 1 h of the nadir and then exceeded this level in the next hour.

There was no treatment effect for appetite or ghrelin AUC (Table 2).

Paracetamol and CCK

Paracetamol increased after consumption of all preloads (Fig. 2). There was a significant time by treatment effect ($P < 0.01$); plasma levels were lower 90 min after the protein preloads compared with both carbohydrate preloads (Fig. 2). Paracetamol AUC was also lower for the protein treatments compared with both carbohydrates ($P < 0.01$; Table 1)

CCK concentration increased 6-fold within 15 min of consuming the preloads (time effect, $P < 0.0001$) and remained elevated between 60–120 min after the protein preloads (time by treatment interaction, $P < 0.01$; Fig. 2). AUC for the protein treatments was also greater than AUC for the carbohydrate treatments ($P < 0.01$; Table 2).

Glucose, insulin, and amino acids

There was a time ($P < 0.0001$) and time by treatment effect for plasma glucose ($P < 0.01$; Fig. 3). The glucose preload produced a higher peak glucose concentration ($P < 0.01$) and AUC ($P < 0.01$; Table 2) compared with the lactose treatment, confirming the difference in their glycemic indices. Postprandial insulin peaked at 45 min and returned to baseline concentration by 120 min (time effect; $P < 0.01$; Fig. 3) independent of treatment. There was a time by treatment effect between whey and casein preloads for branched chain ($P < 0.01$), but not for total plasma amino acids (Fig. 3).

Multiple regression analysis

The strongest predictor of energy intake was ghrelin at 120 min (adjusted $r^2 = 0.055$, $P = 0.024$). Inclusion of peak insulin concentration in the regression model decreases the adjusted r^2 value from 0.055 to 0.037 ($P = 0.130$). Fasting glucose was the strongest predictor of ghrelin at 0 min (adjusted $r^2 = 0.141$, $P = 0.001$), 120 min (adjusted $r^2 = 0.134$, $P = 0.001$), and 180 min (adjusted $r^2 = 0.127$, $P = 0.001$). The strongest predictor of appetite at 180 min was CCK at 90 min (adjusted $r^2 = 0.055$, $P = 0.029$), and the strongest predictor of appetite AUC was CCK AUC (adjusted $r^2 = 0.047$, $P = 0.048$).

TABLE 2. *Ad libitum* energy intake and postprandial responses after consuming preloads[a]

	Protein[b]	Glucose	Lactose
Energy intake (kJ)[c]	$4,279 \pm 207$	$4,772 \pm 264$[d]	$4,231 \pm 247$
AUC			
Appetite (mm/180 min)	-591 ± 456	446 ± 663	-218 ± 497
Ghrelin (pg/180 min/ml)	$30,378 \pm 3,592$	$33,067 \pm 4,262$	$30,533 \pm 3,589$
Paracetamol (mg/180 min/dl)	$1,183 \pm 61$[e]	$1,336 \pm 83$	$1,387 \pm 76$
CCK (pmol/180 min/liter)	388 ± 43[e]	208 ± 39	242 ± 37
Glucose (mg/180 min/dl)	37 ± 7	468 ± 92[f]	220 ± 42
Insulin (mU/180 min/liter)	$10,594 \pm 1,409$	$13,530 \pm 1,297$	$10,183 \pm 1,716$

[a] Mean ± SEM; n = 19.
[b] Mean of whey and casein treatments.
[c] Consumed 3 h after preload at a buffet lunch.
[d] Greater than protein and lactose ($P < 0.05$, one-way ANOVA).
[e] Different to glucose and lactose ($P < 0.01$, one-way ANOVA).
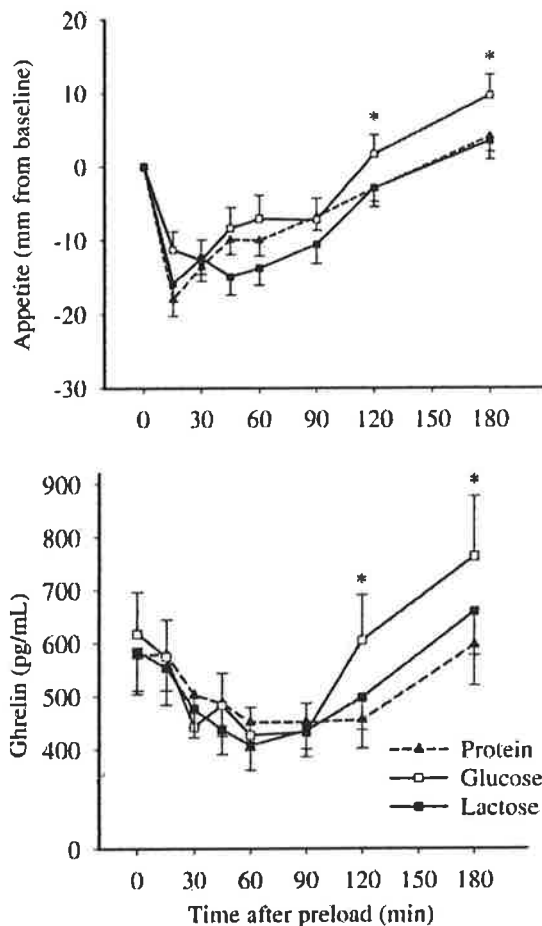[f] Greater than protein and lactose ($P < 0.01$, one-way ANOVA).

FIG. 1. Mean (±SEM) change in appetite (mean subjective ratings for hunger, satiety, desire to eat, and amount of food that could be eaten; *top*) and plasma ghrelin concentration (*bottom*) in overweight males (n = 19) after ingestion of 1 MJ preloads containing approximately 80% of energy from protein (mean response to the whey and casein treatments; ▲), glucose (□), or lactose (■). For conversion from picograms per milliliter to picomoles per liter for ghrelin, multiply by 0.296. *, Significantly greater than protein and lactose treatments (*P* < 0.05; time by treatment effect, repeated measures ANOVA with Tukey's *post hoc* test).

FIG. 2. Mean (±SEM) plasma concentration of postprandial serum paracetamol (*top*; an indirect marker of liquid gastric emptying) and CCK (*bottom*) in 19 overweight male subjects after ingestion of 1 MJ preloads containing approximately 80% of energy from protein (mean response to the whey and casein treatments; ▲), glucose (□), or lactose (■). For conversion from milligrams per deciliter to millimoles per liter for paracetamol, multiply by 66.1. *, Significantly different to glucose and lactose (*P* < 0.01; time by treatment effect, repeated measures ANOVA with Tukey's *post hoc* test).

Discussion

This study has shown that appetite and *ad libitum* energy intake were higher after consumption of a glucose-based liquid preload compared with the lactose and both protein preloads in overweight males. The glucose treatment was also associated with an earlier return of ghrelin to fasting levels. Plasma CCK concentration remained elevated after consumption of dietary proteins, and this correlated with appetite.

The glucose preload resulted in an earlier return of ghrelin to the preprandial concentration and higher *ad libitum* energy intake (+500 kJ) compared with all other treatments. This suggests that ghrelin may be involved in mediating the variable satiety responses observed after consumption of dietary carbohydrates with differing GI (28). Consistent with this, earlier findings suggest that the rapid postprandial decrease in ghrelin after oral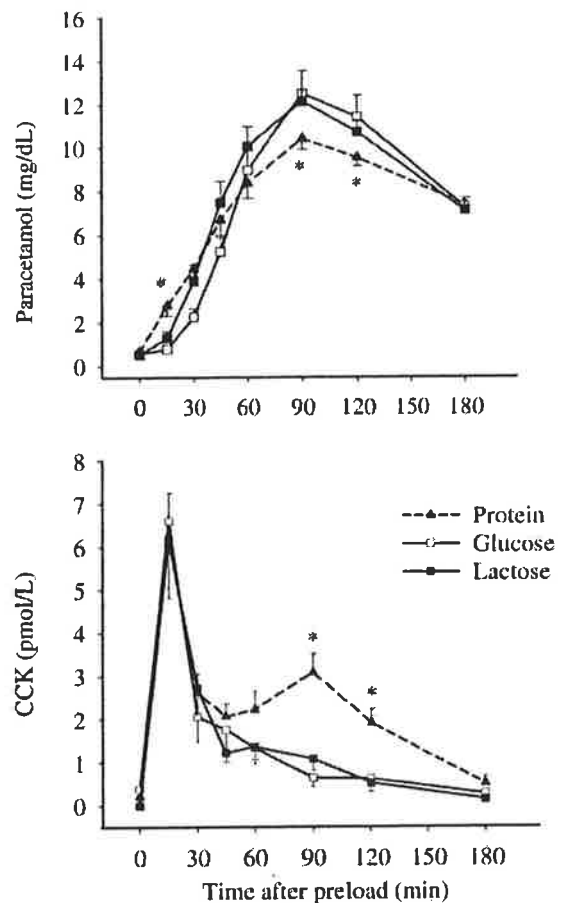 glucose loads (29) is dependent on sub-sequent changes in plasma insulin (30), which would vary depending on GI. Only one other study has explored the effect of different carbohydrates ("simple", 121 g maltodextrin; "complex", 102 g maltodextrin and 12 g exopolysaccharide) on postprandial plasma ghrelin (31). In that study, ghrelin AUC was lower after the complex carbohydrate preload, and this correlated with lower appetite AUC (31), although the glycemic response to both preloads was similar. We also observed that ghrelin and appetite ratings remained suppressed after the lactose preload despite a similar insulin response to the glucose treatment. Indeed, ghrelin remained low even after insulin returned to baseline concentration. Our results, and others (31, 32), indicate that there are additional factors that are involved in regulating the ghrelin response to ingestion of different carbohydrates. Our assessment of gastric emptying and CCK do not give further insight into possible candidates.

The prolonged postprandial suppression of ghrelin after consumption of both dietary proteins may partly explain the
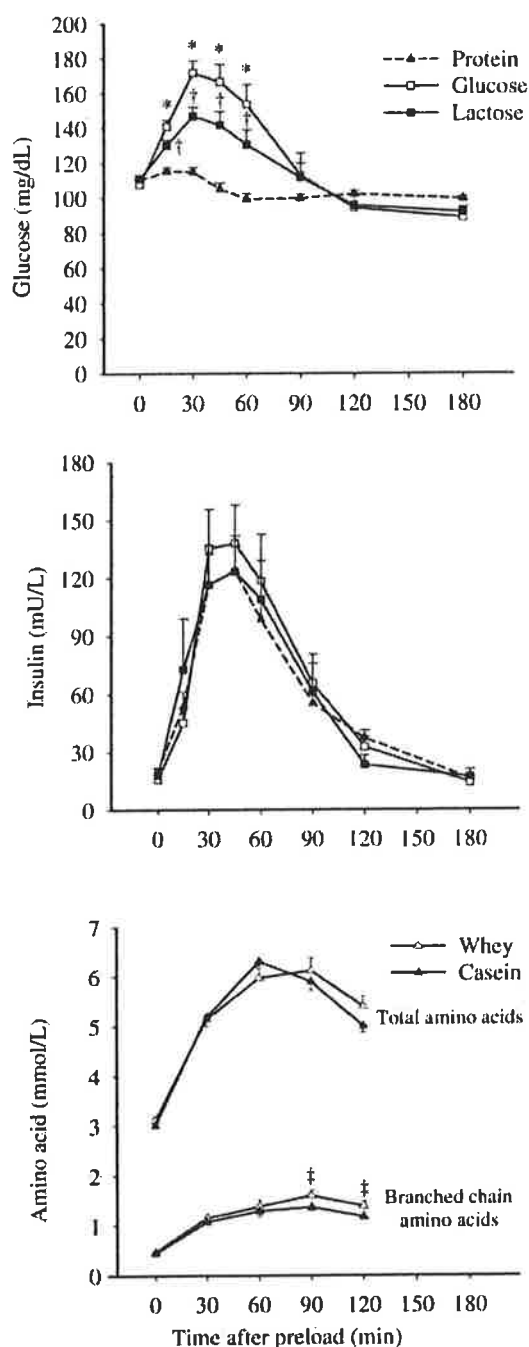
FIG. 3. Mean (±SEM) plasma concentration of glucose (*top*) and insulin (*middle*) in overweight males (n = 19) after ingestion of 1 MJ preloads containing approximately 80% of energy from protein (mean response to the whey and casein treatments; ▲), glucose (□), or lactose (■). Mean (±SEM) plasma total and BCAA (*bottom*) concentration in overweight males (n = 19) after ingestion of 1 MJ preloads containing approximately 80% of energy from whey (△) or casein (▲). For conversion from milligrams per deciliter to millimoles per liter for glucose, multiply by 0.056. For conversion from milliunits per liter to picomoles per liter for insulin, multiply by 7.175. *, Significantly greater than protein and lactose treatments (P < 0.01; time by treatment effect, repeated measures ANOVA with Tukey's *post hoc* test). †, Significantly greater than protein (P < 0.01; time by treatment effect, repeated measures ANOVA with Tukey's *post hoc* test). ‡, Significantly different than casein (P < 0.05; time by treatment effect, repeated measures ANOVA with Tukey's *post hoc* test).

association between dietary protein and higher satiety, compared with carbohydrates (1–3). Recently, another study also found that ghrelin remained suppressed for 3 h after consumption of a high protein, mixed meal, whereas it remained below baseline for 1 h after high fat and moderate carbohydrate isoenergetic meals (33). Previous studies investigating the effect of protein on ghrelin regulation in humans provide conflicting results. Erdmann *et al.* (12) found that plasma ghrelin increased after consumption of a high protein test food (1260 kJ; turkey meat), yet concentration decreased after carbohydrate (1090 kJ; bread, butter, marmalade) and fat (2520 kJ kcal; cream). Similarly, plasma ghrelin concentration did not change after consumption of pork and chicken (13, 14). Differences in the caloric load, macronutrient composition, form, and energy density between test foods in these studies may confound the results. The reduction in ghrelin we observed after all treatments coincided with an elevation in insulin. Again, however, insulin does not explain the prolonged suppression of ghrelin between 120–180 min when insulin had returned to baseline concentration. The regulation of ghrelin during this late postprandial phase appears to be important, as it was associated with effects on appetite and *ad libitum* energy intake.

Animal studies have recently shown that infusion of CCK blocks the orexigenic effect of ghrelin in the arcuate nucleus and reduces food intake (34, 35), but this relationship has not been described in humans. We observed a prolonged elevation in plasma CCK after consuming proteins, but not after either carbohydrate in overweight men. This confirms a similar effect of duodenal infusion (36) of protein and carbohydrate on CCK in lean subjects. We suggest an inverse interaction between CCK and ghrelin, which diminishes the orexigenic effect of ghrelin, may contribute to the higher satiety associated with proteins compared with carbohydrates. Indeed, we found that CCK at 90 min correlated with lower appetite ratings. What is not consistent with this proposition is the equally low ghrelin after the lactose preload despite a low CCK. Nevertheless, an interaction between CCK and ghrelin may influence satiety and the inter-meal interval.

There is some evidence to suggest that an increase in plasma free fatty acid (FFA) concentration decreases plasma ghrelin (37) although not consistently observed (38). The preloads in the present study contained a very small amount of fat (<1.0 g), which is unlikely to have substantially increased plasma FFA concentration. Furthermore, an increase in insulin, as was observed after all preloads, suppresses FFA in both lean and obese normoinsulinemic subjects (39). Therefore, independent of the actual effect of increased plasma FFA on ghrelin, it is unlikely to play an important role in postprandial responses in the present study.

The expected difference in energy intake between casein and whey was based on an earlier finding that *ad libitum* energy intake was 19% lower 90 min after consuming a liquid breakfast preload containing 1.7 MJ and 48 g of whey compared with a similar casein-based preload (6). Postprandial gastric emptying rate was faster and total amino acid concentrations were higher after the whey treatment, although these differences occurred after a subsequent meal (6). We did not observe any differences in appetite or satiety hor-

mones between protein types, perhaps because the interval between the preload and the buffet lunch was longer and not influenced by the consumption of other foods. Thus the postulated faster gastric emptying of whey does not appear to be an important factor in the satiety attributed to some dietary proteins (40). Whey contains high levels of branched chain amino acids (BCAA; leucine, isoleucine, and valine). It has been suggested that these amino acids have a unique metabolic role that may enhance satiety due to extra-hepatic metabolism and interactions with insulin signaling pathways (41). Our findings do not support a role for BCAA in satiety regulation.

Liquid preloads were chosen to avoid the confounding effects of differences in food form and, therefore, facilitate exploration of the effect of macronutrients on postprandial changes in hormones. The disadvantage of liquid preloads is that they do not represent the typical form in which food is generally consumed. Despite this, we did observe a reduction in energy intake that is similar in magnitude to an earlier study comparing proteins and carbohydrates in solid form (−12% *ad libitum* energy intake) (42). The similar insulin profile after all preloads may have been due to the liquid nature of the preloads and, therefore, explain the lack of an association between ghrelin and insulin in this study. Consumption of high-protein solid meals produces a lower insulin response compared with high-carbohydrate solid meals in healthy subjects (4). Accordingly, the macronutrient effects on satiety hormones that were observed in this study should be confirmed using solid foods.

Although the regression analysis in this study showed significant relationships, the strength of these relationships is weak. This highlights the difficulty of studying appetite given the capacity for behavioral and environmental factors to override physiological regulators of appetite. Further, acute satiety is regulated by other gastrointestinal hormones not assessed in this study, such as peptide YY, glucagon like peptide 1, glucose-dependent inhibitory polypeptide, and oxyntomodulin (43). It should also be noted that we measured total plasma ghrelin, although there are a number of fractions of ghrelin with differing levels of activity and plasma concentration (44). Intact and degraded forms of ghrelin show similar postprandial changes after glucose infusion (45), although this has not been demonstrated in obese subjects. Our group (46) and others (29) have previously found that ghrelin and appetite regulation differ in lean and overweight subjects (21). Therefore, we performed this study in overweight subjects to further explore appetite regulatory mechanisms in this group.

In summary, this study has observed differences in the gastrointestinal hormonal response (CCK and ghrelin) to liquid preloads containing protein (whey or casein), lactose, and glucose in overweight men. We have shown that appetite and energy intake are higher after glucose consumption and this may be mediated by ghrelin. Higher postprandial CCK concentrations after dietary protein provides a possible explanation for the difference in satiety that is established between protein and carbohydrates. Gastric emptying and amino acid absorption do not appear to contribute to acute appetite regulation.

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Chapter 7 Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in *ad libitum* energy intake.

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Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. *The Journal of Clinical Endocrinology & Metabolism 2006, 91(8):2913-2919.*

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Developed protocol, prepared ethics application, conducted meal studies, performed laboratory analysis, statistical analysis, interpreted data, wrote manuscript and acted as corresponding author.

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My contribution to this paper involved:

Contribution to protocol design, assistance with data interpretation and manuscript evaluation.

I give consent for Jane Bowen to present this paper for examination towards the Doctor of Philosophy

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Assistance with data interpretation and manuscript evaluation.

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Appetite Regulatory Hormone Responses to Various Dietary Proteins Differ by Body Mass Index Status Despite Similar Reductions in *ad Libitum* Energy Intake

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Context: Although dietary protein produces higher acute satiety relative to carbohydrate, the influence of protein source and body mass index (BMI) has not been clearly described.

Objective: The objective of the study was to assess postprandial responses to different protein sources, compared with glucose, in males with normal and high BMI.

Design: This was a randomized, crossover study of four preloads followed by blood sampling (+15, 30, 45, 60, 90, 120, 180 min) and buffet meal.

Setting: The study was conducted at an outpatient clinic.

Participants: The study population included 72 men, with a BMI range 20.6–39.9 kg/m².

Interventions: Interventions consisted of liquid preloads (1.1 MJ, 450ml) containing 50 g whey, soy, gluten, or glucose.

Main Outcome Measures: Fasting and postprandial plasma glucose, insulin, ghrelin, glucagon-like peptide-1 (GLP-1) and cholecystokinin (n = 38), *ad libitum* energy intake, and appetite ratings were measured.

Results: Energy intake was 10% lower after all protein preloads, compared with the glucose treatment ($P < 0.05$), independent of BMI status and protein type. All protein loads prolonged the postprandial suppression of ghrelin ($P < 0.01$) and elevation of GLP-1 ($P < 0.01$) and cholecystokinin ($P < 0.05$). Fasting GLP-1 concentrations [overweight, 17.5 ± 1.3; lean, 14.7 ± 0.1 pg/ml (5.2 ± 0.4 and 4.4 ± 0.1 pmol/liter, respectively); $P < 0.001$] and postprandial responses ($P = 0.038$) were higher in overweight subjects.

Conclusions: Whey, soy, and gluten similarly tend to reduce *ad libitum* food intake 3 h later in lean and overweight males relative to glucose. Postprandial ghrelin, GLP-1, insulin, and cholecystokinin may contribute to this higher satiety after protein consumption. GLP-1 concentrations are increased in overweight subjects, which may affect satiety responses in this group. (*J Clin Endocrinol Metab* **91:** 2913–2919, 2006)

CONSUMPTION OF DIETARY protein seems to decrease postprandial appetite (1–3) and subsequent energy intake (EI) (1, 2, 4, 5) more than fat and carbohydrate. A number of mechanisms have been proposed to explain this apparent satiety hierarchy of macronutrients, including higher thermogenic effect of dietary protein (6) and postabsorptive small intestinal gluconeogenesis (which is associated with decreased EI in rats (7). The gastrointestinal tract also produces hormones with roles in central appetite regulation. Glucagon-like peptide (GLP)-1 is secreted from L cells of the distal small intestine in response to EI. GLP-1 infusion dose and rate dependently reduces EI (8). Cholecystokinin is released from the duodenum and may contribute to meal termination (9), although this remains controversial (10). Ghrelin stimulates appetite, and its release from gastric oxyntic cells is inhibited by food intake (11). Postprandial changes in these hormones appear to be partially macronutrient specific (12–14) and therefore may contribute to macronutrient-specific differences in satiety.

The type of protein ingested may also affect postprandial responses. EI was lower 1–1.5 h after consuming whey-based preloads, compared with casein (15) and egg albumin (16) treatments. However, in a similar study, we found no difference in EI 3 h after the two dairy proteins (13). Consumption of mixed meals (5 MJ) high in plant (soy, pea, or gluten) or animal (egg albumin, casein, gelatin) proteins did not affect EI, although this was assessed 8 h after the test meal (17). Gastrointestinal responses to plant- and animal-derived proteins may give further insight into the mechanism by which proteins differentially affect appetite, compared with carbohydrates.

Dietary manipulations that maximize satiety have obvious applications for the overweight population as a means of improving compliance with energy-restricted diets. However, appetite studies are frequently performed in lean samples (1–5, 9, 11, 14–18). Outcomes derived from lean subjects may not be applicable to overweight subjects due to differences in glucose metabolism, ghrelin regulation (19), and eating behavior (20). Macronutrient-specific effects on appetite regulation should also be compared across weight groups.

The aims of this study were to compare the effect of soy, whey, and gluten proteins in liquid preloads on subjective (appetite ratings) and objective (*ad libitum* EI) appetite markers and postprandial changes in plasma ghrelin, GLP-1, and cholecystokinin relative to a glucose control. We also investigated the effect of body weight on these outcomes. This

study was performed in males because the menstrual cycle is known to influence EI (21).

Subjects and Methods

Subjects

Healthy men with a body mass index (BMI) greater than 20 kg/m², aged 20–65 yr and a stable body weight were recruited by public advertisement. Exclusion criteria were hypersensitivity to foods used in the study, a score of greater than 10 on the eating restraint section of the validated Three Factor Eating questionnaire (22), and illnesses or use of medications that affect glucose metabolism or appetite. Eighty-two participants were eligible for participation. Ten subjects withdrew before study commencement (n = 3 illness, n = 3 personal reasons, n = 4 lost to contact). The study was approved by the Commonwealth Scientific and Industrial Research Organisation's Human Nutrition Ethics Committee, and all participants gave informed, written consent to participate.

Dietary protocol

Standardized evening meals (3.5 MJ, 26% of total energy from protein, 31% from fat, and 43% from carbohydrate), consisting of frozen meals, cheese (20 g), chocolate (15 g), and fruit (140 g) were consumed by participants before each visit to control intake.

The preloads were beef-flavored soups, which contained water, beef flavoring, vegetable oil (2 g; whey and glucose treatments only), and protein (whey, soy, gluten) or glucose (Table 1). Energy, energy density (Table 1), palatability, and consistency were matched and preloads did not contain dietary fiber.

The buffet lunch (20 MJ; 18% of total energy from protein, 31% from fat, and 51% from carbohydrate) provided to each subject consisted of 600 g meat sauce, 500 g pasta, 600 g veal casserole, 500 g white rice, and 150 g shortbread biscuits. Each food was weighed to the nearest gram before and after eating using digital scales (23). Energy and nutrient composition were calculated by one dietitian using Food Works 3.02 (Xyris Software, Highgate Hill, Australia) based on the Australian food composition tables (24).

Experimental protocol

Subjects attended the outpatient clinic on four occasions with a 7-d interval between treatments. Subjects refrained from alcohol and strenuous exercise for 24 h before visits, consumed the standardized meal on the evening before, and fasted thereafter (water permitted).

Subjects' weight and height were measured (Mettler scales, model AMZ14; A&D Mercury, Kinomoto, Japan) in light clothing. BMI was calculated as weight (kilograms) divided by height (square meters). Total fat mass [coefficient of variation (CV) 2.3 ± 8.7%] and total fat-free mass (CV 2.1 ± 0.4%) were assessed by tetrapolar, single-frequency (50 Hz) bioelectrical impedance analysis (BIA; IMP5TM; Impedimed Pty. Ltd., Brisbane, Australia) using the offline general algorithm. Duplicate

measurements were made while subjects were supine (legs apart, arms not touching the body) and after emptying the bladder. BIA shows good correlation with the gold standard method for assessing body composition, dual-energy x-ray absorptiometry, although in absolute values BIA tends to underestimate percentage fat mass in obese males with a percentage fat mass greater than 25% (25).

A randomly selected subgroup of 18 lean (BMI < 25.0 kg/m²) and 20 overweight (BMI > 25.1 kg/m²) participants had blood samples collected during the 3-h test period at all visits. An indwelling cannula was inserted into a lower arm vein of these participants upon arrival at the unit. The remaining subjects (n = 34) had single fasting blood samples collected by venipuncture at each visit for analysis of insulin and glucose.

All subjects then completed a visual analog scale questionnaire asking "how hungry do you feel" and "how much food would you like to eat now?" Opposing extremes of each feeling were described at either end of a 100-mm horizontal line, and subjects marked the line to indicate how they felt at that moment (26).

The preloads were served in a randomized order and consumed within 7 min. All subjects completed the appetite questionnaire at 15, 30, 45, 60, 90, 120, and 180 min after commencing the preload. Blood samples were also collected from the subgroup of cannulated subjects at these times for analysis of plasma cholecystokinin, ghrelin, GLP-1, glucose, and insulin. Cannulae were removed after the final blood sample. All subjects were then offered a buffet-style lunch, at which each subject was provided with large servings of all four foods. Instructions were given to eat until comfortably satisfied, and the food was removed after 30 min.

Biochemistry

Blood was collected into prechilled sodium fluoride/EDTA (1 g/liter) tubes for plasma insulin and glucose analysis. Aprotinin (500 KIU/ml of blood; Trasylol; Bayer, Leverkusen, Germany) was added to tubes for plasma cholecystokinin and ghrelin analysis and dipeptidyl peptidase-IV inhibitor (10 μl/ml blood; Linco, St. Charles, MO) was added to tubes for plasma GLP-1 analysis. Blood samples were stored on ice, and the plasma was isolated within 30 min of collection by centrifugation (10 min, 2000 × g, 5 C) (Beckman GS-6R Centrifuge; Fullerton, CA). Aliquots were stored at −80 C.

Commercially available RIA kits were used to measure total ghrelin (Phoenix Pharmaceuticals, Belmont, CA; CV 5.5%) and cholecystokinin-8 (Euria-Diagnostica, Malmo, Sweden; CV 14%). Ethanol extraction was performed on plasma for cholecystokinin analysis according to the manufacturer's instructions. Active GLP-1(7–36 and 7–37) was measured by fluorescence immunoassay (Linco; CV 8.0%). Plasma insulin was measured using an ELISA immunoassay kit (Mercodia, Uppsala, Sweden). Plasma glucose was determined using an enzymatic kit (Hoffmann-La Roche Diagnostics, Basel, Switzerland) and control sera on a Hitachi 902 automatic analyzer (Roche Diagnostics, Basel, Switzerland).

TABLE 1. Nutrient composition and content of preloads

	Glucose	Soy	Whey	Gluten
Nutritional analysis				
Energy (kJ)	1158	1199	1216	1227
Protein [g (% total energy)]	1 (1.5)	50 (71)	51 (71)	51 (71)
Fat [g (% total energy)]	3.6 (11)	3.6 (11)	3.6 (11)	3.9 (12)
Carbohydrate [g (% total energy)]	63.0 (87)	13.5 (18)	13.5 (18)	13.5 (17)
Energy density (kJ/g)	2.7	2.8	2.9	2.9
Content (g)				
Beef flavoring	20	20	20	20
Test food	65[a]	57[b]	55[c]	57[d]
Vegetable oil	2.5	0	2	0
Water	340	350	350	350

[a] Glucodin (Boots Healthcare, North Ryde, Australia).
[b] Isolated soy protein (The Solae Co., West Chatswood, Australia).
[c] Whey protein isolate (Murray Goulburn, Brunswick, Australia).
[d] Gemtec (Manildra Group, Auburn, Australia).

Statistics

Results are expressed as means ± SEM and are presented for 72 subjects for all baseline characteristics, appetite, and *ad libitum* EI. BIA data are presented for 70 subjects; two subjects with internal metal pins were not measured. Postprandial blood parameters are presented for 38 subjects. For analysis of the appetite questionnaire, the baseline value was subtracted from postprandial responses to normalize between-subject differences, and total area under the curve (AUC) was calculated using a trapezoidal equation.

ANOVA with repeated measures was used to determine the effect of time (minutes) and treatment. BMI status (lean; BMI < 25 kg/m^2, overweight; BMI > 25.1 kg/m^2) was included as a between-subject factor, age was a covariate, and Bonferroni adjustments were used for multiple comparisons. Where ANOVA showed a significant main effect, Tukey's *post hoc* tests were performed to compare group differences. Appetite AUC was compared using two-way ANOVA with treatment and BMI as factors. Relationships between EI and glucose, insulin, ghrelin, GLP-1, and cholecystokinin at all time points were examined using multiple linear regressions, which adjusted for the repeated nature of the data. Percentage variance is derived from the r^2 value. The satiety hormone that might be most closely related to the impact of meal type on EI was examined in an analysis of covariance (ANCOVA) with EI as the dependent variable using general linear model. Differences between treatments at the 3-h time point were examined first. All statistical analysis was performed using SPSS 11.5 for WINDOWS (SPSS Inc., Chicago, IL) except the ANCOVA (SAS-STAT; SAS Institute, Cary, NC). Differences are considered significant if $P < 0.05$.

Results

Subjects

Seventy-two men completed the study (Table 2). There were no differences in baseline characteristics or responses to preloads between subjects allocated to the group in which fasting blood samples were drawn compared with multiple blood samples (data not shown; $P > 0.05$). All preloads were well tolerated by participants.

EI and appetite

Ad libitum EI was significantly higher after the glucose treatment than gluten (+560 ± 136 kJ; $P < 0.05$; Table 3). A similar, nonsignificant trend was observed with lower intake after the soy and whey treatments.

Using all time points in a regression model with all biochemical parameters entered, EI was predicted by overall

TABLE 2. Baseline characteristics

	Lean (n = 25)	Overweight (n = 47)
Age (yr)	50.5 ± 2.4[a]	56.8 ± 1.1
Height (m)	1.8 ± 0.1	1.8 ± 0.1
Weight (kg)	73.0 ± 1.2	94.8 ± 1.8
BMI (kg/m^2)	23.3 ± 0.2	30.1 ± 0.5
Lean mass (kg)	57.0 ± 1.2[b]	64.8 ± 1.1
Fat mass (kg)	15.6 ± 1.0[b]	29.8 ± 1.0
Fasting glucose (mg/dl)	95.2 ± 0.9[a]	104.6 ± 1.1
Fasting insulin (μU/ml)	4.3 ± 0.5[a]	8.4 ± 0.8
Fasting ghrelin (pg/ml)	684 ± 34[a,c]	493 ± 13[d]
Fasting GLP-1 (pg/ml)	14.7 ± 0.4[a,c]	17.5 ± 1.3[d]
Fasting CCK (pmol/liter)	0.8 ± 0.1[c]	0.4 ± 0.1[d]

$\bar{x}$ ± SEM. CCK, Cholecystokinin.

[a] Significantly different to overweight subjects (Independent samples *t* test, $P < 0.001$).
[b] n = 23.
[c] n = 18.
[d] n = 20.

ghrelin ($P = 0.039$) and glucose ($P = 0.012$) and inversely by cholecystokinin ($P = 0.056$) and insulin ($P = 0.001$). This accounted for 5.2% of the variance in EI. Inclusion of preload type in the analysis rendered all predictors nonsignificant except insulin, which became a stronger inverse predictor of EI ($P = 0.0005$). This model (preload type and insulin) accounted for 9.8% of the variation in EI. Inclusion of all covariates at 180 min and preload type in the ANCOVA revealed that both a low glucose ($P < 0.05$) and a low GLP-1 ($P < 0.03$) explained the differences in EI. Examination of other time points did not reveal any other significant covariates.

Appetite ratings ($P > 0.05$, data not shown) and change (Δ) in desired amount of food AUC or Δ hunger AUC ($P > 0.05$; Table 3) were independent of treatment.

Glucose and insulin

Postprandial plasma glucose increased after the glucose treatment (time-by-treatment effect; $P < 0.0005$; Fig. 1A). Peak postprandial insulin occurred at 30–45 min and was highest after the glucose treatment (time-by-treatment interaction; $P < 0.01$; Fig. 1B).

Appetite hormones

There was a time-by-treatment interaction such that the ghrelin nadir after all protein treatments occurred later (90–120 min) and remained below baseline at 180 min, compared with the glucose treatment (time by treatment interaction, $P < 0.01$; Fig. 1C).

The highest GLP-1 concentration occurred later (30–90 min) and remained elevated for longer after all protein treatments, compared with the glucose preload (time by treatment interaction; $P < 0.01$; Fig. 1D).

Cholecystokinin remained elevated until 120 min after the protein preloads, whereas concentration declined after the initial peak at 15 min for the glucose treatment (time by treatment interaction; $P < 0.05$; Fig. 1E).

Body weight

Age was significantly higher in overweight subjects, compared with lean subjects ($P < 0.01$; Table 2). Inclusion of age as a covariate did not influence outcomes. Postprandial plasma glucose and appetite hormones were assessed in 18 lean (mean BMI 23.2 ± 0.3, range 21.3–24.9 kg/m^2) and 20 overweight (mean BMI 31.4 ± 0.8, range 25.2–39.9 kg/m^2) subjects.

There was no effect of BMI status on mean overall EI (lean, 3371 ± 141 kJ; overweight, 3310 ± 105 kJ; $P > 0.05$). Baseline and Δ AUC for both appetite questions were also independent of body weight ($P > 0.05$).

Fasting ($P < 0.001$; Table 2) and postprandial response ($P < 0.001$; Fig. 2) for glucose and insulin were higher in overweight subjects. There was a time by treatment by BMI status interaction such that the postprandial change in plasma glucose after the glucose preload was smaller in lean subjects, compared with overweight subjects ($P < 0.05$; Fig. 2A).

Fasting ghrelin ($P < 0.001$; Table 2), postprandial response

TABLE 3. Postprandial total AUC and nutritional analysis of *ad libitum* food intake at a buffet lunch after equicaloric preloads containing glucose, soy, whey, or gluten[a,b]

	Glucose	Soy	Whey	Gluten
Ad libitum EI (kJ)	3546 ± 168[c]	3209 ± 160	3219 ± 147	3006 ± 147
Protein (g)	63.4 ± 3.0[c]	57.2 ± 2.9	57.5 ± 2.9	54.0 ± 2.9
Fat (g)	19.3 ± 1.2	17.8 ± 1.6	17.5 ± 1.0	16.8 ± 1.1
Carbohydrate (g)	102.5 ± 5.3[c]	92.3 ± 4.9	93.2 ± 4.6	85.7 ± 4.37
AUC (mm per 180 min)				
Δ Desired amount of food	228 ± 19	192 ± 18	228 ± 21	202 ± 21
Δ Hunger	163 ± 14	152 ± 13	155 ± 14	165 ± 16

[a] Dietary composition based on data from Australian food composition tables (24).
[b] $\bar{x}$ ± SEM; n = 72.
[c] Different to gluten ($P < 0.05$, one-way ANOVA).

($P = 0.003$; Fig. 2C), and change from fasting to nadir concentrations were lower in overweight compared with lean subjects [-97.7 ± 7.5 *vs.* -151.8 ± 16.2 pg/ml, respectively (-28.9 ± 2.2 *vs.* -44.9 ± 4.8 pmol/liter); $P < 0.01$]. Fasting GLP-1 concentrations ($P < 0.001$; Table 2), postprandial responses ($P = 0.038$; Fig. 2D), and change from fasting to peak [21.5 ± 2.8 *vs.* 13.1 ± 1.8 pg/ml, respectively (6.4 ± 0.8 *vs.* 3.9 ± 0.5 pmol/liter); $P < 0.01$], were higher in overweight subjects. The macronutrient-specific changes in ghrelin and GLP-1 described above were observed in both lean and overweight subjects, despite overall differences in concentration. There was no significant effect of body weight on cholecystokinin.

Regression analysis

The relationship among satiety hormones, glucose, and insulin was explored in a regression model using all time points. Glucose ($P = 0.0005$), insulin ($P = 0.002$), and GLP-1 ($P = 0.0005$) predicted 9.7% of the variance of ghrelin. Cholecystokinin was the only predictor of GLP-1 and explained 25.2% of the variance ($P = 0.0005$). Insulin and GLP-1 explained 15.3% of the variation in cholecystokinin ($P = 0.0005$).

Power calculations

With 72 subjects completing the study, we had 80% power ($P < 0.05$) to detect a difference between treatments of 200 kJ in EI (~7%). Thus, the differences observed between glucose and gluten treatment were at the threshold of detection.

Discussion

We compared appetite ratings, *ad libitum* EI, and gastrointestinal appetite hormones after consumption of different dietary proteins and glucose in lean and overweight males. Postprandial changes in ghrelin, cholecystokinin, insulin, and GLP-1 and EI were similar after consumption of the soy-, whey-, and gluten protein-based liquid preloads and differed overall to the glucose treatment, which suggests that these factors may partly contribute to the higher satiety widely associated with dietary proteins. We have also shown that fasting and postprandial GLP-1 concentrations are increased in overweight subjects, but this did not affect appetite ratings and EI in this setting. Finally, the macronutrient-specific differences in EI and postprandial appetite hormone

responses were observed independent of body weight and overall differences in hormone concentrations.

Differences in EI previously observed 1–1.5 h after consumption of various dietary proteins in liquid preloads (15, 16) were not replicated after 3 h in the present study, which reflects a more typical intermeal interval. However, an effect of protein type on appetite may arise when the proteins are consumed in solid form due to variation in the rate of digestion and nutrient absorption. This may be important because it is the main form of protein consumed in the free living setting. Indeed a hunger-fullness rating was higher 2–3 h after consuming fish, compared with chicken and beef; however, palatability and EI were not assessed (27). EI was not affected after mixed meals (1.8–5.0 MJ) in which the protein source in test foods (mousse and soup) was covertly manipulated (17, 28), although EI was recorded after 8 h and the consistency of the test foods was similar (17, 28). The use of liquid preloads in the present study enabled a comparison of gastrointestinal responses with plant- and animal-derived proteins and controlling for potential differences in gastric emptying rate. The influence of varying gastric emptying and digestion rates of solid proteins on hormonal responses remains unclear.

Our findings suggest that the higher satiety associated with consumption of protein, compared with carbohydrate, may be at least partially mediated by prolonged ghrelin suppression. Such a reduction in the orexigenic signal may delay the initiation of a subsequent feeding episode or lower hunger and EI. Ghrelin remained below baseline for longer after high protein mixed meals with yogurt as the primary protein source (29, 30). However, appetite and EI either were not assessed (29) or did not correlate with ghrelin (30) in these studies. Two studies reported that plasma ghrelin concentration remain unchanged 3 h after consumption of cooked turkey (31) and 4 h after pork (32). These conflicting findings may be due to the test food form; the influence of solid forms of protein on postprandial ghrelin may require assessment over a period longer than 3–4 h. This is because slow gastric emptying delays the postprandial ghrelin nadir (33) and changes in ghrelin require postgastric feedback (34). Indeed, treatment effects for ghrelin are not observed until 120–180 min after liquid (13, 33, 35) and semisolid (yogurt-rich meal) (29) preloads.

We report that postprandial GLP-1 secretion was prolonged after dietary proteins, compared with the glucose
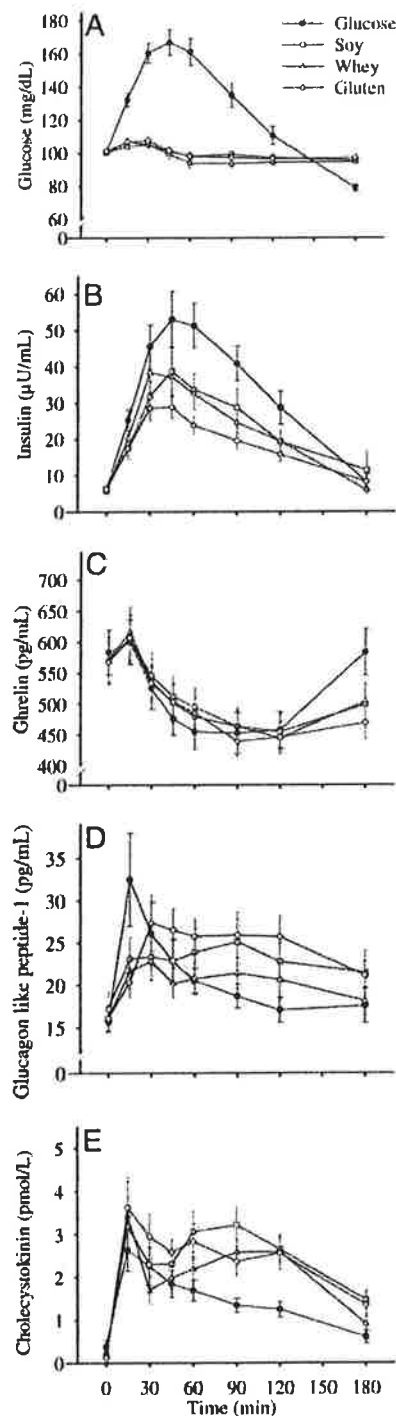
FIG. 1. The effect of four liquid preloads (1 MJ) containing approximately 70% of energy from soy (□), whey (△), gluten (◇), or glucose (●) on postprandial plasma glucose (A), insulin (B), ghrelin (C), GLP-1 (D), and cholecystokinin (E) in males (n = 38). Data are expressed as mean ± SEM. For conversion of milligrams per deciliter to millimoles per liter for glucose, multiply by 0.056. For conversion of milliunits per liter to picomoles per liter for insulin, multiply by 6.95. For conversion of picomoles per milliliter to picomoles per liter for glucose, multiply by 0.296. For conversion of picograms per milliliter to picomoles per liter for GLP-1, multiply by 0.298. There are significant time-by-treatment effects for glucose ($P < 0.0005$), insulin ($P < 0.01$), ghrelin ($P < 0.01$), GLP-1 ($P < 0.01$), and cholecystokinin ($P < 0.05$) (repeated measures ANOVA with Tukey's *post hoc* test).

treatment. Earlier studies are suggestive of a pattern of longer GLP-1 elevation after protein-rich mixed meals, relative to carbohydrate and fat (14, 36). Our findings show a clear distinction in the pattern of secretion between proteins and glucose treatments, and higher GLP-1 at 180 min was related to lower EI. It remains unclear whether GLP-1 reduces appetite centrally and/or as a consequence of slowing gastric emptying (37). If the latter is important, the liquid preloads used in this study (which bypass gastric distension-induced satiety signals) may have compromised the influence of GLP-1 on appetite regulation. Nevertheless, our observation of prolonged GLP-1 stimulation after protein consumption suggests that it may contribute to the satiety associated with dietary proteins.

The postprandial cholecystokinin response was temporally similar to GLP-1; cholecystokinin remained elevated for 1.5 h longer after the protein preloads, compared with glucose. This confirms previously reported macronutrient-related differences after duodenal infusion (38) and oral consumption (12, 13) of protein, carbohydrate, and fats. Whereas cholecystokinin is typically associated with meal termination (*i.e.* satiation), our findings suggest it may also contribute to greater satiety (*i.e.* delay the return of hunger), supported by the inverse association between cholecystokinin and EI that almost reached significance ($P = 0.056$).

The macronutrient-specific effects on gastrointestinal hormones reported in this study are small and relationships are relatively weak; however, we show that a range of hormones respond similarly after consumption of different macronutrients and in a coordinated way that is likely to influence acute appetite regulation. The ANCOVA results indicate that the low glucose and low GLP-1 at the end of the sampling period had an influence on EI. The present study also shows that ghrelin has an inverse secretory pattern to GLP-1 and cholecystokinin, confirming two similar recent observations (13, 33). Interestingly cholecystokinin inhibits the central effects of ghrelin in animals (39).

We believe this is the first demonstration that fasting and postprandial GLP-1 concentrations are significantly increased in overweight subjects, compared with lean counterparts, independent of the macronutrient consumed. It has previously been reported that GLP-1 is reduced in overweight and obese subjects (8), although the evidence to support this is limited; incremental GLP-1 AUC (but not total AUC or fasting concentration) was lower in overweight compared with lean subjects (40). Fasting and postprandial responses to a breakfast plus guar gum solution were independent of body weight (41); however, lean subjects had slightly higher GLP-1 concentration at 30 min, compared with obese subjects, when the breakfast was consumed with water (41). Duodenal infusion of fat and carbohydrate produced a similar GLP-1 response in lean and obese subjects (42). The higher GLP-1 we observed in overweight subjects appears to be an appropriate response to a positive energy balance and would presumably lower appetite. Yet there was no effect of weight status on EI or appetite ratings. This implies that overweight subjects may have diminished sensitivity to this hormone, which is analogous to the established reduced sensitivity to insulin and leptin in this population. A metaanalysis found that GLP-1 infusion had a
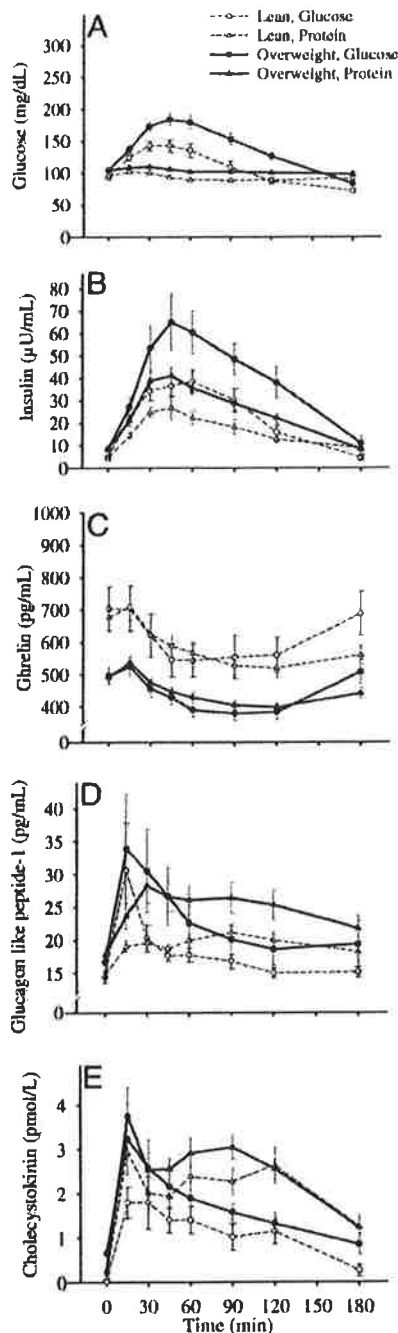
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FIG. 2. The combined postprandial response to three protein-based liquid preloads (1 MJ; containing ~70% of energy from soy, whey, or gluten), compared with a similar glucose preload, consumed by lean (BMI < 25.0 kg/m^2; n = 18; glucose, -O-; protein, -△ -) and overweight (BMI > 25.1 kg/m^2; n = 20; glucose, -●-; protein, -▲-) males for plasma glucose (A), insulin (B), ghrelin (C), GLP-1 (D), and cholecystokinin (E). Data are expressed as mean ± SEM. For conversion of milligrams per deciliter to millimoles per liter for glucose, multiply by 0.056. For conversion of milliunits per liter to picomoles per liter for insulin, multiply by 6.95. For conversion of picograms per milliliter to picomoles per liter for glucose, multiply by 0.296. For conversion of picograms per milliliter to picomoles per liter for GLP-1, multiply by 0.298. There are significant time by BMI status effects for glucose (P < 0.0005), insulin (P = 0.001), ghrelin (P = 0.003), and GLP-1 (P = 0.038) (repeated measures ANOVA with Tukey's *post hoc* test). There was a time by treatment by BMI status effect for plasma glucose (P < 0.05).

weaker effect on reducing *ad libitum* EI in obese compared with lean subjects, although this did not remain significant after controlling for the overall lower intake in obese participants (8). The similarity in EI in our study may also reflect a behavioral effect of randomly mixing lean and overweight subjects in groups and eating the buffet lunch together.

In summary, we show that dietary protein consumed in liquid preloads prolongs the postprandial suppression of ghrelin, elevation of cholecystokinin and GLP-1, and maintenance of glucose levels, compared with glucose ingestion. These responses are not affected by the type of protein consumed (soy, whey, or gluten) and are similarly observed in lean and overweight subjects, regardless of the overall differences in hormone levels. Preload type influenced EI through ghrelin, cholecystokinin, and glucose, whereas insulin was a predictor of EI that was independent of treatment, although overall these relationships were relatively weak. We have also observed increased fasting and postprandial GLP-1 concentration in overweight males, although this does not appear to affect appetite or EI.

Acknowledgments

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Chapter 8   Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages.

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STATEMENT OF AUTHORSHIP

Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages.

International Journal of Obesity 2007, in review.

Jane Bowen (Candidate)

Developed protocol, prepared ethics application, conducted meal studies, performed laboratory analysis, statistical analysis, interpreted data, wrote manuscript and acted as corresponding author.

Signed ....Date.11./4./0.7.......

Manny Noakes

My contribution to this paper involved:

Contribution to protocol design, assistance with data interpretation and manuscript evaluation.

I give consent for Jane Bowen to present this paper for examination towards the Doctor of Philosophy

Signed ....Date......18/7/07

Peter M Clifton

My contribution to this paper involved:

Assistance with data interpretation and manuscript evaluation.

I give consent for Jane Bowen to present this paper for examination towards the Doctor of Philosophy

Signed ..../... ....Date......12/4/2007

ORIGINAL ARTICLE

Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages

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Objective: To investigate appetite responses over 4 h to fructose beverages in obese men, relative to glucose and whey protein. Second, to investigate the effect of combining whey and fructose on postprandial appetite hormones.
Design: Randomized, double-blind crossover study of four beverages (1.1 MJ) containing 50 g of whey, fructose, glucose or 25 g whey + 25 g fructose. Blood samples and appetite ratings were collected for 4 h then a buffet meal was offered.
Subjects: Twenty-eight obese men (age: 57.0 ± 1.6 years, body mass index: $32.5 \pm 0.6 \, \text{kg/m}^2$)
Measurements: Plasma ghrelin (total), glucagon-like peptide-1 (GLP-1 7–36), cholecystokinin-8, glucose, insulin and appetite ratings were assessed at baseline and 30, 45, 60, 90, 120, 180, 240 min after beverages, followed by measurement of *ad libitum* energy intake.
Results: Fructose produced lower glycaemia and insulinaemia compared to the glucose treatment ($P < 0.0001$); whereas postprandial ghrelin, GLP-1 and cholecystokinin responses were similar after both treatments. Whey protein produced a prolonged (2–4 h) suppression of ghrelin ($P = 0.001$) and elevation of GLP-1 ($P = 0.002$) and cholecystokinin ($P = 0.003$) that were reduced when combined with fructose, while glucose and insulin responses were similar. Energy intake after 4 h was independent of beverage type (glucose 4.7 ± 0.2 MJ; fructose 4.9 ± 0.3 MJ; whey 4.6 ± 0.3 MJ; whey/fructose 4.8 ± 0.3 MJ; $P > 0.05$).
Conclusion: In obese men, fructose- and glucose-based beverages had similar effects on appetite and associated regulatory hormones, independent of the differing glycaemic and insulinaemic responses. The contrasting profile of plasma ghrelin, GLP-1 and cholecystokinin after whey protein consumption did not impact on *ad libitum* intake 4 h later and was attenuated when 50% of whey was replaced with fructose.
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Background

Dietary manipulations that promote satiety (that is the absence of hunger during the inter-meal interval) have potential applications for the overweight population, such as improving adherence to restricted energy intake. Many appetite studies are performed in subjects with a normal body mass index (BMI) ($20-25 \, \text{kg/m}^2$); however, results derived from healthy weight subjects may not apply to the overweight population. For example, fasting and postprandial concentration of gut hormones involved in appetite regulation differ in overweight subjects compared to lean

counterparts.[1–3] The impact of adiposity-dependent differences in appetite regulatory hormones on eating behaviour has not been well studied.

Studies in lean subjects have shown that hunger ratings and subsequent food intake are reduced following consumption of dietary protein, compared to carbohydrate and fat.[4,5] This may be explained by higher postprandial plasma concentration of the 'satiety' hormones glucagon-like peptide-1 (GLP-1) and cholecystokinin, observed in lean and obese men 1–3 h after ingestion of whey protein compared to glucose.[3,6,7] Additionally, ghrelin remains suppressed 3 h after dietary proteins relative to glucose[3,6] and this may contribute to a reduction in hunger. Any impact of protein on these hormones over a period longer than 3 h has not been investigated.

Carbohydrates that produce low glycaemic and insulinaemic responses also seem to prolong satiety.[8] Fructose

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ingestion produces a small increase in blood glucose concentration, which reflects low-level hepatic metabolism of some fructose to glucose. It does not stimulate insulin secretion directly, although a small postprandial increase is observed due to a fructose-stimulated rise in the incretin GLP-1.[9] Despite this low glycaemic response, studies in lean subjects show that fructose and glucose consumption similarly affect subsequent energy intake.[10–12] Understanding the role of fructose on appetite regulation is important, particularly in overweight subjects, given the possible correlation between obesity prevalence and consumption of beverages sweetened with high-fructose corn syrup.[9,13]

Ghrelin has an inverse secretory pattern to insulin[14–16] and glucose,[17] suggesting that it may contribute to the apparent effects of glycaemic response on appetite.[8] We previously found that ghrelin remained moderately reduced 3 h after lactose (low glycaemic index) and whey-based liquid preloads relative to glucose and this predicted lower *ad libitum* energy intake in overweight men.[6] The duration of this ghrelin suppression and impact on hunger has not been investigated. We hypothesize that the effect of protein on ghrelin may be prolonged by combining protein with a carbohydrate that also produces a low glycaemic and insulinaemic response. Extending ghrelin suppression, and perhaps minimizing hunger ratings, for 4 h is relevant to inter-meal intervals in the free-living setting.

The aim of this study was to compare postprandial glycaemia, appetite regulatory hormones (ghrelin, GLP-1 and cholecystokinin) and *ad libitum* energy intake after consumption of fructose- and glucose-based beverages in obese subjects. We also observed the effect of whey on these outcomes over 4 h when consumed alone and in combination with fructose.

Methods

This study was conducted at the Clinical Research Unit, Human Nutrition, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Adelaide, Australia, and was approved by the CSIRO Ethics Committee. All participants gave informed, written consent to participate.

Subjects
Healthy men aged 20–65 years were recruited by public advertisement. Inclusion criteria were a BMI > 25 kg/m² and a stable body weight for 3 months before the study. Exclusion criteria were illnesses or use of medications that affect glucose metabolism or appetite, a score of > 10 on the eating restraint section of the validated Three Factor Eating questionnaire[18] and hypersensitivity to foods used in the study. Females were excluded to avoid the influence of menstrual cycle on eating behaviour.[19] Thirty-three participants were eligible for participation. Five

subjects withdrew before commencement ($n = 3$ illness; $n = 1$ personal reason, $n = 1$ lost to contact). Twenty-eight subjects completed the study.

Experimental protocol
Subjects attended the Clinical Research Unit on four occasions with a 7-day interval between treatments. Subjects refrained from alcohol and strenuous exercise for 24 h before each visit and instructions were given to eat the standardized evening meal at the same time before each visit and fast thereafter (water permitted).

The weight and height of subjects were measured upon arrival at the clinic (Mettler scales, AMZ14, A&D Mercury, Kinomoto, Japan). BMI was calculated as weight (kg) divided by height (m)². An indwelling antecubital venous cannula was inserted and fasting blood samples were collected for measurement of gut hormones, insulin and glucose. After 15 min rest, subjects rated their appetite using a validated visual analogue scale (VAS) questionnaire[20] adapted to a sliding scale, computerized format (Northeast Data Corp. Slider ActiveX Custom Control (1.0) Charlotte, USA).[21] The questionnaire asked 'How hungry do you feel?', 'How satisfied do you feel', 'How full do you feel' and 'How much food would you like to eat now?' A 100 mm horizontal line was displayed below each question and opposing extremes of each feeling described at either end of the line (for example, 'I am not hungry at all' – 'I have never been hungrier'). Subjects moved the cursor along the line using the mouse to indicate how they felt at that moment. In addition, palatability, taste, aftertaste and smell were assessed after consuming the beverages using a similar VAS.

The beverages were served chilled (4°C) 15 min after the baseline measurements in an opaque, closed container and consumed through a straw within 7 min. Treatments were given in a randomized, double-blind order. Subsequent blood samples and VAS responses were collected 30, 45, 60, 90, 120, 180 and 240 min after commencing the test drink. Subjects were allowed to read, watch television and talk with other participants (avoiding food-related conversation) during this time. Subjects could drink water and the volume of this was kept constant between study days. Cannulae were removed after the final blood sample and the buffet lunch was provided. Instructions were given to eat until satisfied and the foods were available for 30 min.

Dietary protocol
A standardized evening meal was given to subjects for consumption before study days (3.1 MJ; 38% of total energy from protein, 21% fat, 41% carbohydrate).

The beverages contained 50 g of the test ingredient (whey protein, fructose, glucose or a combination of 25 g of whey and 25 g of fructose), water and milk (1% fat) (Table 1). The milk contributed a small, equal amount of protein, fat and carbohydrate to all treatments. Beverages were matched for

Table 1 Nutrient and food composition[a] of the four treatments

Treatment	Glucose	Fructose	Whey	Whey/fructose
Carbohydrate (g) (% of energy)	60 (88)	60 (88)	10 (14)	35 (50)
Protein (g) (% of energy)	7 (11)	7 (11)	57 (84.5)	32 (48.5)
Fat (g) (% of energy)	0.5 (1.0)	0.5 (1.0)	0.5 (1.5)	0.5 (1.5)
Energy (kJ)	1097	1097	1147	1122
Weight (g)	415	415	415	415
Volume (ml)	390	390	410	405
Energy density (kJ/g)	2.64	2.64	2.76	2.70
Ingredients				
Test food (amount, g)[b]	Glucose[c] (65)	Fructose[d] (65)	Whey protein isolate (55)[e]	Whey (27) fructose (33)
Skim milk (0.1% fat) (g)	200	200	200	200
Chocolate flavour (g)	20	20	40	30
Water (g)	130	130	120	125

[a]Dietary composition based on data from Australian Food Composition Tables[22] and ingredient manufacturer. [b]Weight of ingredient that contains 50 g of test protein/carbohydrate (or equivalent amount for the whey/fructose treatment). [c]Glucose; Ace Chemicals, Adelaide, Australia. [d]Whey; Murray Goulburn Nutritionals, Melbourne, Australia. [e]Fructose; Ace Chemical, Adelaide, Australia.

palatability and sweetness using a non-caloric, chocolate-flavoured sweetener.

The *ad libitum* 'buffet' style lunch included meat sauce, tuna casserole, pasta and rice. Each subject was allocated large servings (600 g) of each food and this provided total of ~12 MJ with 28% of total energy from protein, 29% from fat and 43% from carbohydrate. Foods were weighed to the nearest gram before and after lunch using digital scales. Subjects ate in a group of six to replicate the free-living setting. Energy and nutrient composition were calculated using Food Works 3.02 (Xyris Software, Highgate Hill, Australia) based on the Australian Food Composition Tables.[22]

Biochemistry
Blood was collected into chilled sodium fluoride/ethylene-diamine tetraacetic acid (1 g/l) tubes for plasma insulin and glucose analysis. Aprotinin (500 KIU/1 ml blood; Trasylol, Bayer, Leverkusen Germany) was preloaded into chilled tubes for cholecystokinin and ghrelin analysis. Dipeptidyl peptidase-IV (DPP-IV) inhibitor (10 μl/ml blood; Linco, MO, USA) was added to chilled tubes for GLP-1 analysis. Blood samples were stored on ice and plasma was isolated within 30 min by centrifugation (10 min, 2000 g, 5°C) (Beckman GS-6R Centrifuge, Fullerton, CA, USA). Aliquots were stored at −80°C.

Commercially available radioimmunoassay kits were used to measure total ghrelin (Phoenix Pharmaceuticals, Belmont, CA, USA; CV 5.5%) and cholecystokinin-8 (Euria-diagnostica, Malmo, Sweden; CV 14%). Ethanol extraction was performed on plasma for cholecystokinin analysis according to the manufacturer's instructions. Active GLP-1 (7–36) was measured by fluorescence immunoassay (Linco; CV 8.0%). Plasma insulin was measured using an ELISA immunoassay kit (Mercodia, Uppsala, Sweden). Plasma glucose was determined using an enzymatic kit (Hoffman-LaRoche Diagnostics, Basel, Switzerland) and control sera on a Hitachi

902 Automatic Analyser (Roche Diagnostics, Basel, Switzerland). The homeostasis model assessment (HOMA) was used as a surrogate measure of insulin sensitivity (fasting insulin(mU/l) × fasting glucose (mmol/l)/22.5).[23]

Statistics
Results are expressed as means±s.e.m. for 28 subjects. For analysis of the appetite questionnaire, the distance between the left end of the scale and each mark was measured (mm). Baseline values were subtracted from postprandial responses to normalize between-subject differences. Reliability analysis was performed (Cronbach's alpha) for the questions 'How hungry do you feel?', 'How satisfied do you feel', 'How full do you feel', 'How much food would you like to eat now?' to assess inter item correlation; that is, are the questions measuring the same construct. The reliability ($\alpha = 0.047$) improved by combining the questions 'How satisfied do you feel/How full do you feel?' ($\alpha = 1.789$), and separately combining 'How hungry do you feel/How much food would you like to eat now?' ($\alpha = 0.554$), confirmed by Pearson correlations (two-tailed, $P < 0.01$). The mean response to 'How hungry do you feel/How much food would you like to eat now?' and 'How satisfied do you feel/How full do you feel?' was calculated for each subject and used for subsequent analysis.

Analysis of variance (ANOVA) with repeated measures was used to determine the effect of time (min) and treatment and Bonferroni adjustments for multiple comparisons. HOMA was included as a covariate for ghrelin,[24] GLP-1,[25] insulin and glucose analysis. Tukey's *post hoc* tests were performed to compare group differences where ANOVA showed a significant main effect. Relationships between variables were examined using multiple linear regressions that adjusted for the repeated nature of the data. Statistical analysis was performed using SPSS 14.0 for WINDOWS (SPSS Inc., Chicago, USA). The study had 80% power ($\alpha = 0.05$) to detect a 20% change in energy intake at the buffet meal. Statistical significance was set at an α-level of $P < 0.05$.

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Results

Subjects

Twenty-eight subjects completed the study (age 57.0 ± 1.6 years; height 1.77 ± 0.02 m; weight 101.5 ± 2.0 kg; BMI 32.5 ± 0.6 kg/m^2). Subjective ratings of palatability, taste, aftertaste and smell did not differ between treatments (data not shown) and all treatments were well tolerated.
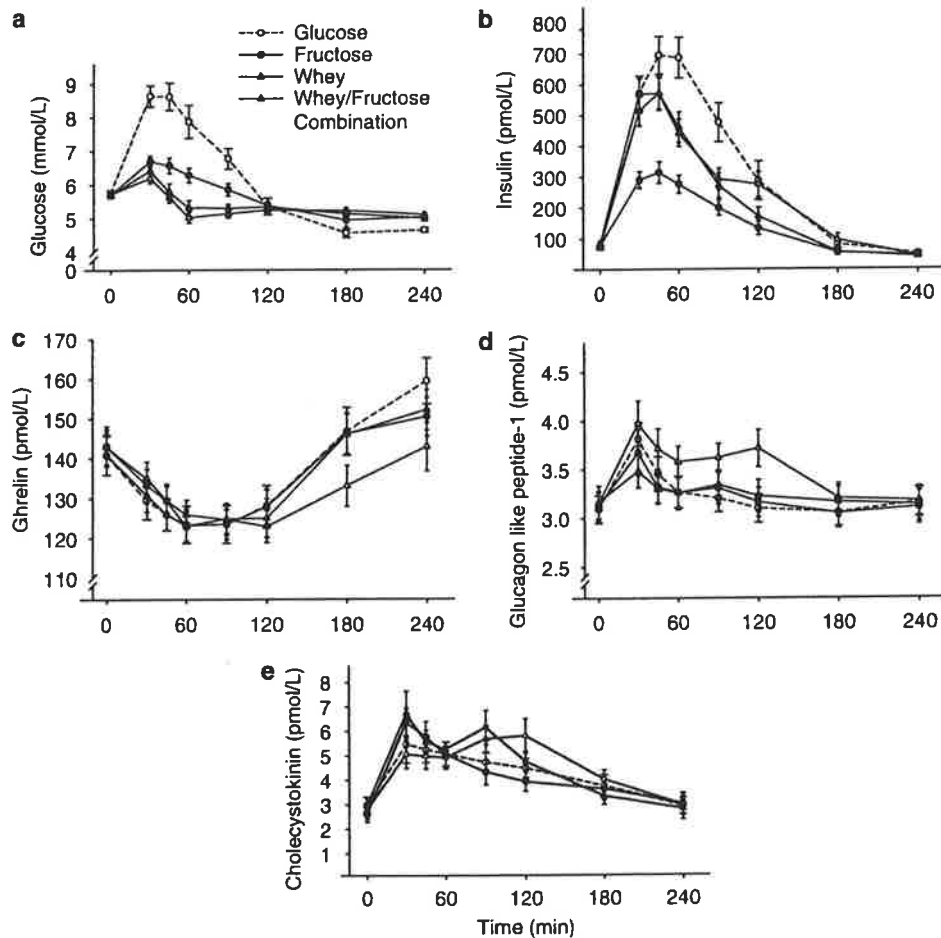
Glucose and insulin

Plasma glucose response was highest after the glucose treatment compared to fructose, which was in turn higher than the whey and whey/fructose treatments (time by treatment interaction; $P < 0.0005$; Figure 1a). By the end of the sampling period (240 min), glucose concentration was lower after the glucose beverage (4.56 ± 0.07 mmol/l) compared to all other treatments (fructose; 4.93 ± 0.08, whey; 5.01 ± 0.06, whey/fructose 4.91 ± 0.09 mmol/l; $P < 0.01$). Fructose produced an insulin response that was significantly lower than all other treatments (time by treatment interactions; $P < 0.0005$; Figure 1b) and these differences resolved by 180 min.

Appetite hormones

The mean ghrelin nadir (that is lowest value of the mean) was similar after all treatments, however, it occurred later

Figure 1 The effect of four beverages (1.1 MJ) containing $\sim 85\%$ of energy from glucose (--O--), fructose (--●--), whey protein (--Δ--) or a combination of whey and fructose (--▲--) on postprandial plasma concentration of glucose (a), insulin (b), ghrelin (c), glucagon-like peptide-1 (GLP-1) (d) and cholecystokinin (e) in obese men ($n = 28$). Data are expressed as mean $\pm$ s.e.m. and compared using repeated measures ANOVA with Tukey's *post hoc* test. Plasma glucose is significantly higher after glucose treatment compared to fructose, and the fructose response is greater than whey and whey/fructose (time by treatment interactions; $P < 0.0005$). Plasma insulin is significantly lower after fructose compared to all other treatments (time by treatment interactions; $P < 0.0005$). Plasma ghrelin after the whey treatment is different to the glucose treatment (time by treatment interactions; $P < 0.0005$). Plasma GLP-1 is significantly higher after whey compared to all other treatments (time by treatment interactions; $P = 0.002$). Cholecystokinin response to whey is greater than whey/fructose, which is also greater than the response to fructose and glucose ($P = 0.009$). ANOVA, analysis of variance.

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after the whey and whey/fructose treatments (120 min) compared to the fructose and glucose beverages (60 min). At 180 min, ghrelin remained below baseline after whey and at 240 min it was significantly lower (142 ± 6 pmol/l) than the glucose treatment (160 ± 6 pmol/l) (time by treatment interaction, $P < 0.001$; Figure 1c).

All beverages produced an early increase in GLP-1, after which concentration rapidly declined following the glucose, fructose and whey/fructose treatments. The response to the whey treatment was significantly different to all other treatments; GLP-1 remained 18% above baseline at 120 min (time by treatment interaction, $P = 0.002$; Figure 1d).

There was a similar peak in cholecystokinin 30 min after all treatments. This was followed by a gradual decline for the glucose and fructose treatments. Whey produced a second increase such that concentration at 120 min was 16% greater compared to 90 min concentration (time by treatment interaction; $P = 0.009$; Figure 1e). Similarly, concentration 90 min after the whey/fructose treatment was 17% greater compared to the concentration at 60 min, (time by treatment interaction; $P = 0.009$).

Energy intake and appetite ratings
There was no effect of treatment on macronutrient composition (data not shown) or total energy intake of foods consumed at the buffet lunch meal (glucose 4704 ± 274 kJ; fructose 4942 ± 280 kJ; whey 4623 ± 290 kJ; whey/fructose 4761 ± 277 kJ; $P = 0.121$). Similarly the VAS questions ($P = 0.311–0.703$; data not shown) were independent of treatment. The satisfied/full response showed a time by treatment interaction ($P = 0.046$; Figure 2a) whereby satisfaction/fullness was lower after fructose compared to whey. The hungry/how much food response remained independent of treatment ($P = 0.755$; Figure 2b).

There were significant correlations in energy intake between treatments (correlation coefficients varying from 0.56 to 0.76; $P < 0.01$). In a linear regression, nadir ghrelin and insulin at 30 min were weakly related to energy intake ($P < 0.01$ and $P = 0.06$, respectively); together, they accounted for 10% of the variance in energy intake ($P < 0.01$). Inclusion of HOMA as a covariate did not affect the statistical significance of time by treatment interactions.

Discussion

The first aim of this study was to compare the effects of fructose and glucose on appetite regulatory hormones and food intake in obese men. We found that both sugars produced similar plasma ghrelin, GLP-1 and cholecystokinin responses and *ad libitum* energy intake, despite marked differences in postprandial glycaemia and insulinaemia. Second, we have shown that ghrelin remains suppressed 4 h after whey protein relative to carbohydrate, although this (and the elevation of GLP-1 and cholecystokinin) was
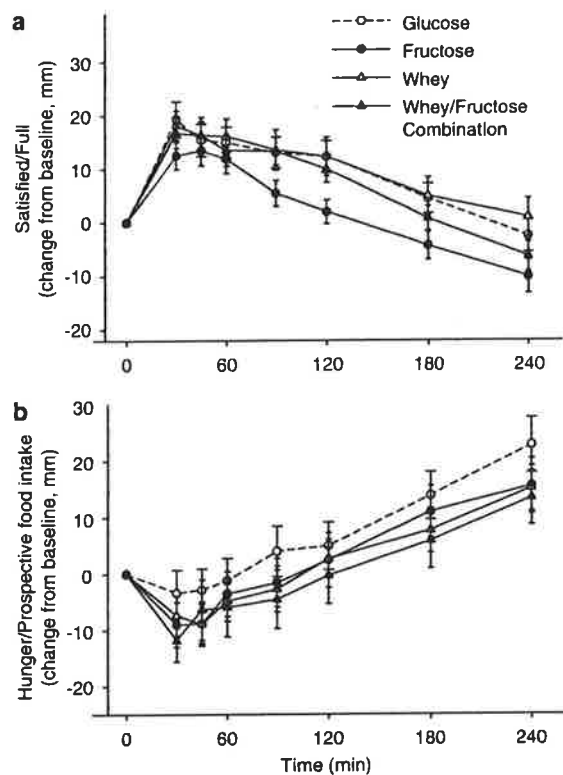
Figure 2 The effect of four beverages (1.1 MJ) containing ~85% of energy from glucose (--O--), fructose (-●-), whey protein (-△-) and whey/fructose (-▲-) on subjective appetite ratings of the visual analogue scale questions 'How satisfied do you feel?'/'How full do you feel?' (a) and 'How hungry do you feel?'/'How much food would you like to eat now?' (b). Data are presented as change from baseline and expressed as mean ± s.e.m. for obese men ($n = 28$). Satisfied/full response to the fructose treatment is lower than glucose (time by treatment interaction; $P = 0.046$; repeated measures ANOVA with Tukey's *post hoc* test). ANOVA, analysis of variance.

attenuated when combined with fructose. Despite these macronutrient-specific differences in appetite hormones, *ad libitum* food intake 4 h later was independent of treatment.

Fructose and glucose
Early studies in lean and overweight subjects reported lower energy intake after ingestion of fructose compared to glucose,[26–28] consistent with the proposed association between the glycaemic responses and appetite.[8] However, the present study in obese subjects, and others in lean subjects,[10–12,29] show that energy intake was comparable 2–4 h after both sugars. The modest reduction in satisfaction/fullness ratings observed after the fructose treatment may have been influenced by the faster gastric emptying of fructose,[30] although this did not affect subsequent intake.

The postprandial nadir and return to baseline concentration for the ghrelin was similar after consumption of fructose and glucose, reflecting previous findings after pure (100 g)

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fructose and glucose loads ($n = 6$).[31] Yet, when a fructose-rich beverage was consumed with a mixed breakfast meal the ghrelin nadir was smaller, relative to a glucose treatment.[32] The ghrelin response was not predicted by insulin, which contrasts the previously described inverse correlation between insulin and ghrelin.[15,16] Interestingly, the ghrelin nadir and insulin at 30 min were weak predictors of energy intake. This suggests a possible relationship between greater insulin response to glucose, subsequent reduction in glucose below baseline concentration and increased energy intake.

The moderate increase in ghrelin observed 240 min after the glucose treatment corresponded to a small reduction in plasma glucose concentration below fasting levels (180–240 min). Both increased ghrelin and low blood glucose concentrations are independently associated with hunger and meal initiation.[14,33] Our results suggest these may be related. Future studies investigating the contribution of blood glucose to acute appetite responses may consider assessing postprandial changes in capillary, rather than venous, glucose concentration. This is recommended for assessment of glycaemic responses[34] because capillary glucose concentration is higher and less variable compared to venous samples.[35] The mean capillary concentration was $+1.6$ and $+1.7$ mmol/l higher than venous plasma at 30 and 45 min, respectively, after consumption of solid and liquid carbohydrate-rich preloads by lean adults.[34] This difference represents tissue uptake of glucose in the forearm and is most pronounced in insulin-sensitive, lean subjects.[36] Consequently, the peak concentration in plasma glucose at the tissues is also likely to be somewhat higher in these obese subjects. Capillary measurements (that is, fingerpick measurements) were not performed in the present study to minimize the impact of sample collection on subjective appetite ratings and energy intake. Further, previous findings indicated that this possible relationship between low glucose and an increase in ghrelin[3,6] occurs at a concentration range (that is fasting) were there is a negligible difference between the two sampling sites.

The rapid and similar increase in GLP-1, 0–60 min after both sugars suggests involvement of neural mechanisms to trigger an early GLP-1 release. Indeed, GLP-1 secreting L-cells in the proximal small intestine are not exposed to luminal contents.[37,38] Interestingly, fructose does not stimulate another incretin, gastric inhibitory peptide as strongly as glucose.[39] While increased GLP-1 may promote satiation (that is, meal termination), it is unlikely to contribute to satiety given the rapid decline after carbohydrate ingestion and short half-life of the hormone.[40] Indeed, GLP-1 did not predict food intake.

Whey, glucose and fructose
Dietary proteins seem to have a coordinated effect on appetite hormones; GLP-1 and cholecystokinin are elevated in the early-mid postprandial phase and ghrelin remains suppressed during the late postprandial phase following liquid protein preloads relative to glucose.[3,6] The present study extends these findings to show that the suppression of ghrelin persists 4 h after whey protein consumption. It would be valuable to determine if spontaneous meal requests/initiation are also delayed after consumption of protein and if this relates to the reduced ghrelin. Others have reported a similar pattern of increase in GLP-1 and cholecystokinin after a semisolid, high-protein mixed nutrient food relative to high-carbohydrate, and that difference persisted 180 min after the treatment.[7] The prolonged elevation in that study may have been due to the physical form of that preload. This effect of dietary protein on GLP-1 may be associated with a recent finding in rodents; the augmented active GLP-1 response after dietary protein ingestion was associated with a concomitant reduction in the activity of DPP-IV that cleaves and inactivates GLP-1.[41]

GLP-1 and cholecystokinin reduce gastric emptying[42,43] and in turn, this influences postprandial appetite hormones[42,44] and appetite responses.[45] Therefore, the effect of these hormones on gastric emptying after protein ingestion may contribute to the previously observed effect of protein on satiety, relative to carbohydrate.

The length of the small intestine exposed to glucose affects the secretion of ghrelin and GLP-1 in human subjects;[46] ghrelin suppression and GLP-1 elevation were prolonged when >60 cm of small intestine was exposed to glucose. This may influence the hormone responses we have observed after dietary protein consumption. Protein requires longer than glucose to digest and absorb and therefore presumably reaches more distal regions of the small intestine. Whey protein is considered to be a 'rapidly' digested and absorbed protein[47] relative to other proteins, suggesting that the time course for postprandial changes in these hormones after ingestion of whole animal tissue proteins may be even longer than whey. Similarly, this relationship may influence ghrelin and GLP-1 responses after consumption of carbohydrates that require extensive digestion, compared to sugars that are rapidly absorbed in the proximal small intestine. Future studies should compare the effects of animal proteins, slowly digested carbohydrates and their combination on the time course of postprandial changes in appetite hormones, relationship to gastric empting and spontaneous meal requests.

Whey is a comparatively potent insulinotropic dietary protein[48] and also stimulates incretin release,[48] necessary for insulin-mediated amino-acid uptake into cells. The substitution of 50% of the fructose with whey was associated with an increase in postprandial insulin to a level comparable with the whey treatment, but did not display the synergistic effects previously observed when a combined fructose and whey protein (50 g) treatment was consumed by type 2 diabetics, relative to fructose (25 g) and whey alone (25 g).[49]

The amount of protein required to prolong satiety remains unknown and has potential implications for the contribution of dietary composition on appetite sensations in the

free-living setting. The diminished cholecystokinin response after the combined whey/fructose beverage (32 g total protein) compared to the whey beverage (57 g total protein) suggests that it is the amount of protein ingested that may influence the magnitude of the postprandial response. Conversely, the GLP-1 response to the whey/fructose beverage indicates a possible threshold effect, such that a given amount of protein in absolute terms or possibly relative to fructose is required to prolonged GLP-1 concentration.

The similar energy intake after all treatments may reflect a sensation of emptiness owing to the liquid form of the preloads or the low energy content relative to the timing of the buffet meal (+4 h). Both factors may have overwhelmed any influence of the preloads or subsequent appetite hormones. However, it is not known if subjects had reached 'maximum hunger' or whether preload consumption (independent of treatment) had an impact at the *ad libitum* meal. Inclusion of a non-caloric preload would indicate if ingestion of a 1 MJ preload affects consumption 4 h later. However, we were limited to the number of treatments that are feasible to implement in this crossover design before affecting participant burden and responsiveness.

The physical form of the preloads was also selected to minimize the influence of gastric emptying rate on appetite hormone responses[44] and because fructose is commonly consumed in beverages in the free-living setting. Given the early and prolonged increase in GLP-1 and CCK after the whey treatment, it is plausible that food intake may have been affected by treatment before the buffet lunch. Relating spontaneous meal requests to postprandial changes in these appetite hormones would provide further insight. Similarly, a relationship between dietary protein, postprandial changes in these appetite hormones and *ad libitum* intake may be observed after 4 h when preloads are higher in energy or solid.

This study investigated responses in obese, healthy men. While there was no effect of insulin sensitivity on these outcomes, this may be due to the selection of non-diabetic subjects and therefore narrow range of variation in HOMA. Similar studies are also required in diabetic subjects and women.

In conclusion, fructose and glucose beverages have similar effects on appetite and associated regulatory hormones, independent of differing glycaemic and insulinaemic responses in obese men. The seemingly satiating profile of ghrelin, GLP-1 and cholecystokinin after whey protein consumption is attenuated by the substitution of some whey with fructose, although such macronutrient-specific differences in appetite hormones did not influence *ad libitum* energy intake after 4 h. The effect of combining other dietary proteins (that is animal muscle) with slowly digested/absorbed carbohydrates on postprandial changes in appetite-related hormones and *ad libitum* energy intake warrants further investigation.

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Chapter 9   General Discussion

Protein consumption produces lower appetite ratings and reduces subsequent energy intake relative to carbohydrate and fat in lean subjects (Table 1). It was hypothesised that gastrointestinal derived hormones involved in appetite control may partly mediate this effect. As such the objective of this thesis was to investigate the effect of dietary proteins and carbohydrates on satiety and regulatory hormones. This was achieved by two approaches; assessing different sources of dietary proteins and by contrasting protein with carbohydrates (sugars) that produce differing postprandial glycemia and insulinemia. Gastric emptying rate (297), duration of effect (298)and interactions between protein and carbohydrate were also observed (298). The impact of macronutrients on appetite and postprandial responses were observed in overweight/obese (297-299) and compared with lean subjects (299). These studies provide new insight into a possible explanation for the impact of dietary protein ingestion on acute appetite regulation, summarised below.

Principal findings

It was demonstrated for the first time in overweight subjects that appetite ratings and *ad libitum* energy intake are reduced for 2-3h after protein-rich liquid preloads (50g) relative to glucose (297, 299) although not after 4h (298). These findings confirm previous results in lean subjects (Table 1) and are among few studies to demonstrate a difference between macronutrients following liquid preload consumption.

These studies also show a consistent effect of dietary proteins on some of the gut hormones involved in acute appetite regulation. The return of ghrelin to pre-prandial

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levels after protein ingestion was delayed relative to carbohydrates, independent of the type of protein (297-299). In contrast, a spike in ghrelin above baseline concentration after glucose preloads coincided with higher appetite ratings and *ad libitum* intake (297-299), supporting a likely role for ghrelin in meal initiation (64). This increase in ghrelin occurred in parallel with a small decrease in plasma glucose below fasting concentration (297-299), suggesting that the two events may be related. Factors that precipitate a decrease in glucose below fasting concentration warrant investigation (e.g. insulin response, rate of decline in plasma glucose).

GLP-1 and cholecystokinin also remained elevated for longer after all protein preloads relative to the carbohydrates (297-299). While these hormones are typically associated with satiation (111, 136, 137), the prolonged secretory patterns suggest a possible contribution to satiety as well. Both hormones slow gastric emptying (14, 157) which is also associated with reducing appetite.

The significant variation in insulinemic and glycemic response after fructose, lactose and glucose based preloads did not produce a consistent effect on early (0-120min) postprandial hormone profiles, with the exception of a high insulin response possibly promoting a decrease in glucose below baseline.

It was shown for the first time that overweight/obese subjects have significantly higher fasting and postprandial GLP-1 responses compared to lean subjects (297). Macronutrient specific differences in GLP-1 (and ghrelin and cholecystokinin) were observed independent of bodyweight (297). *Ad libitum* energy intake was also similar in lean and obese subjects (Study 2), which suggest that adiposity may confer a

reduced sensitivity to these hormones in overweight subjects. The energy intake data may have been influenced by the difference in the amount of protein consumed relative to body weight between the lean and obese subjects. The protein containing preloads provided 0.69g protein/kg body weight for lean subjects and 53g protein/kg body weight for obese subjects.

Energy intake

Approximately 50% of the energy consumed in the high protein preloads was compensated for at the buffet lunch 3h later, relative to glucose; *ad libitum* energy intake was ~0.5MJ lower after the 1MJ protein preloads (297, 299). This timeframe represents the typical duration between a mid-meal snack and a main meal in the free-living setting (e.g. mid-morning to lunch or mid-afternoon to dinner). Therefore manipulating the composition of a 'between meals' snack could help reduce subsequent intake. However it remains unclear whether the energy consumed in a 'high protein snack + *ad libitum* meal' results in lower overall intake compared to '*ad libitum* meal only'. Inclusion of a 'no preload' treatment arm would address this. One study included a 'no preload' treatment and found that the total energy of food consumed over a 7h period did not compensate for the 3MJ HP, HF or HC preloads (208). Conversely the energy from a HP 'preload entrée plus main course' was significantly less than the energy consumed at a 'main course' only (202).

An alternative approach to reducing overall energy intake is to extend the duration of satiety arising from a meal and abstain from 'between meal' snacking. In the third study, the 50g protein liquid preload (1MJ) did not have an advantage over carbohydrate-based preloads at meals consumed 4h later (298). Increasing the amount

of protein above 50g may prolong satiety and in turn reduce energy intake at the buffet lunch. However there is a practical limit to the level of protein in a meal or food/beverage. The preload composition (i.e. protein and energy content) in study three was chosen to allow comparison with previous studies (297, 299).

Whilst dietary protein is more satiating than other macronutrients, the findings described above indicate that there are additional dietary factors that impart considerable influence on appetite. A reduction in energy density and portion size and an increase in volume are associated with satiety. Therefore, future studies should explore combining protein with other food characteristics that contribute to satiety and reduce *ad libitum* intake to extend the duration of satiety provided by a protein rich food beyond 3h.

Decreasing the energy density (7.32kJ/g to 5.23 kJ/g) and portion size (900g to 500g) of an 'amorphous' pasta meal reduced *ad libitum* consumption at the meal by up to 3.1MJ and ~1.5MJ respectively. These effects were additive (-3.9MJ for the lowest portion size and energy density) (194). The impact of increasing portion size on food consumption is maintained when the serving is more obviously increased (i.e. packet of potato crisps) (300). Similarly, a 25% reduction in the energy density of various foods consumed over two days decreased *ad libitum* energy intake by -2.4MJ/d (301), which indicates that these effects may not be compensated for over subsequent meals. A 25% reduction in the portion size of the same foods over two days produced a 10% reduction in intake (-0.97MJ/d), and the effect of combining the two treatments was additive (-3.4MJ/d) (301). Importantly, these decreases in consumption were not associated with changes in perceptions of fullness or hunger.

Increasing the volume of a beverage (2MJ) from 300mL to 600mL by the addition of water reduced consumption 30 min later by ~0.5MkJ (153). Similarly, increasing the volume of a milkshake from 300ml to 600 ml by the addition of air reduced energy intake by -~0.4MJ 20 min later (151). The association between increased antral area and fullness ratings may explain the impact of food volume on appetite (r^2 = -0.66, P < 0.0001). Antral distension is also an inverse predictor of energy intake (r^2 = -0.90, P < 0.0001) (302).

Based on these results, a meal rich in protein (~200g meat or 50g protein), low in energy density and a large volume (either by increased air or water) would theoretically maximise the duration of the satiety sensation. Since the duration may be affected by the energy density and amount of protein, a mid-meal snack of similar composition that is proportionally lower in protein and total energy may have a similar effect over a shorter period. Understanding the postprandial responses (appetite ratings, *ad libitum* intake, appetite related hormones and gastric emptying rate) to various combinations of these attributes warrants further investigation. This also has practical relevance because macronutrients are consumed as part of mixed meals in the free-living setting.

It would be valuable to compare the timing of spontaneous requests for food with the plasma concentration of appetite hormones to further explore their relative contribution to actual intake. Replicating the acute effects of macronutrients in the free-living setting is also required to observe the relative contribution of these hormones and environmental influences (Chapter 2.1) to intake. However such studies

present the logistical challenge of measuring food intake, which is consistently underreported by overweight subjects (188), and collecting blood samples.

Gastric Emptying

Liquid preloads were selected in all studies in this thesis to minimise the variation in gastric emptying rate and highlight the impact of the macronutrients on appetite hormones. Solids foods, the predominant form in which energy is consumed, empty from the stomach more slowly (303) and may also require more extensive digestion compared to liquids.

In Study 3 the diminished cholecystokinin response after the combined whey/fructose beverage (32g total protein) compared to the whey beverage (57g total protein) indicates a possible dose response, which would presumably influence gastric emptying rate (298). Postprandial ghrelin suppression is greater when gastric emptying rate is reduced (76). Accordingly, the weaker cholecystokinin response observed after the combined preload (and assumed impact on gastric emptying) in Study 3 may explain the likeness in ghrelin responses after the whey/fructose and the fructose preloads – where 32g of protein seemingly had no impact on prolonging ghrelin secretion. Likewise, the similarity in GLP-1 following the whey/fructose and fructose treatments may also reflect an effect of 'faster' gastric empting or a possible threshold effect such that a given amount of protein is required to prolong postprandial changes in GLP-1.

In support of the proposed relationship between dietary protein, gastric emptying and gut hormones, a recent study reported GLP-1, cholecystokinin and gastric emptying

profiles similar to those reported in this thesis, after lean subjects consumed mixed HP (14.4g fat) and HC (12.2g fat) preloads (yoghurts) (94). An small increase in ghrelin 3h after the HC treatment was observed, but was not associated with a reduction in plasma glucose (94).

Interestingly, another recent study showed that GLP-1 stimulation was higher and ghrelin suppression was prolonged when glucose was infused into more distal regions of the small intestine, compared to restricting infusion to the proximal 0-60 cm section (77). This suggests that foods which are rapidly digested and absorbed in the proximal small intestine, and have little or no residue reaching the distal small intestine, may produce a lower or shorter postprandial change in these hormones. Conversely, foods that require more extensive digestion and move distally along the intestine may continue to stimulate postprandial changes in GLP-1 and ghrelin. This may partly contribute to prolonged elevation of GLP-1 and suppression of ghrelin after proteins, relative to the carbohydrates that are rapidly absorbed. This raises the possibility that the digestive requirements of dietary protein may contribute to its effect on satiety, in addition to the nutrient type. This hypothesis may also apply to the longer satiety that is often attributed to slowly digested carbohydrates (low GI) compared to rapidly digested and absorbed carbohydrates (high GI) (Chapter 5.6). Future studies should investigate relationships between physical form, gastric emptying rate, digestibility, appetite and associated regulatory hormones.

Subject selection

Dietary characteristics that influence acute appetite regulation have relevance for weight management in overweight and obese subjects. Accordingly, it has been important to confirm the greater sensation of satiety (and associated appetite hormone responses) after protein consumption in this group. Study 2 showed that energy intake and appetite ratings were independent of bodyweight, despite differences in glucose, insulin, ghrelin and GLP-1 between the lean and overweight subjects (299). In that study design, overweight and lean subjects consumed the buffet lunches together to replicate the free-living setting. However it is possible that eating behaviour may have differed between the lean and overweight subjects if eating alone or separated by bodyweight group (22, 170, 304). The subjects in all studies were limited to males; therefore these findings require confirmation in females, which may differ due to an influence of menstrual cycle on eating behaviour (305). Reduced glycemic control and insulin sensitivity in subjects with type-2 diabetes (82) and women with polycystic ovarian syndrome (306) may be associated with further dysregulation of appetite regulatory hormones and warrants investigation.

Relationship of acute appetite to energy balance

These studies only assessed food intake at the next meal; compensation for reduced intake at subsequent meals cannot be excluded. Preliminary studies indicate that protein-rich dietary patterns consumed *ad libitum* do affect energy intake sufficiently to produce a negative energy balance and weight loss (11, 12). The impact of such dietary patterns on appetite regulatory hormones over longer periods requires investigation. A study in overweight men and women reported a small change in the 3h postprandial ghrelin profile after 12wk weight loss and 4 wk weight maintenance,

independent of the high protein/low fat and standard protein/high fat dietary treatments and test meals (307).

Methodological limitations

A number of methodological issues arose in these studies. Assessing appetite ratings is highly variable and subjective in nature (Chapter 4.2). Attempts were made to reduce the variability by use of categorical scales (299) and a computer based methodology (298) with no apparent reduction in variability. Combining the responses to various questions that measure the same construct reduced inter-individual variation. Reducing the number of questions, the use of pictorial/visual cues to indicate prospective intake and recording meal requests are alternate strategies that could be considered for assessment of appetite in future studies.

While knowledge of appetite hormones has improved considerably in recent years, the most appropriate form of some hormones to assess is not certain. During the course of this these studies, commercial kits to assess active ghrelin concentration became available. One study has shown an effect of macronutrient composition on postprandial active ghrelin (308), but this is not consistently observed (94).

The studies in this thesis are among the first assess the impact of dietary composition on multiple appetite hormones. Studies published at the same time reported similar gut hormone profiles after semi solid (94) and solid preloads of mixed macronutrient composition (309). However there is an ever-increasing number of peripheral hormones that appear to contribute to acute appetite regulation (310), and more still that have an impact on long-term energy balance (310). Peptide YY (PYY 3-36) is of

particular interest. This hormone is produced by gut L cells (309). Administration at a physiological dose reduces appetite ratings and inhibits food intake by 33% over 24h (309). When these studies were designed there was insufficient evidence to establish an a priori hypothesis for PYY, and indeed other potential appetite regulatory hormones, in the effect of macronutrients on postprandial responses. A recent study has shown that consumption of protein-rich mixed meals increased PYY concentration over 3h and this was associated with reduced appetite ratings and *ad libitum* intake in lean and obese subjects (308).

Ideally future studies will simultaneously analyse a broader range of appetite hormones in a complex mathematical model, and in conjunction with other parameters such as gastric emptying, to fully understand the impact of dietary intake on the mechanisms involved in acute appetite regulation. Technologies such as multiplex assays will make this more achievable in the near future.

Studies in this thesis did not address the contribution of dietary fat to appetite responses. The contribution of fat to cholecystokinin and gastric emptying may be important. Indeed, plasma concentration of ghrelin is suppressed after infusion of fat into the duodenum, and this is attenuated when fat digestion is inhibited. Similarly the postprandial increase in PYY is abolished when fat digestion is inhibited (311).

In conclusion, the studies in this thesis have shown that dietary proteins consumed as liquids provide greater satiety and reduce *ad libitum* food intake relative to carbohydrate in overweight and obese adults after 3h. This relationship may be partly explained by the prolonged elevation of GLP-1 and cholecystokinin and suppression

of ghrelin after protein ingestion, independent of the source of protein and body weight status. Future studies should explore additional dietary strategies to extend the duration of satiety, such as reducing energy density or combining with a slowly digested starch. The contribution of gastric emptying rate to these responses and involvement of other gut derived appetite hormones warrant further investigation. Additionally, the contribution of these acute observations to longer-term energy balance requires demonstration.

Appendix 1 Appetite questionnaire; Visual analogue scale

Please indicate how you are feeling **at this moment** by placing **a vertical mark** at the appropriate point on each scale below. Please do not make a cross or slopping mark. Mark all scales.

Not Nauseated————————————————————— Nauseated

Hungry   ————————————————————— Not hungry

Empty   ————————————————————— Full

Thirsty   ————————————————————— Not thirsty

Satiated   —————————————————————Not satiated

How strong is your desire to eat?
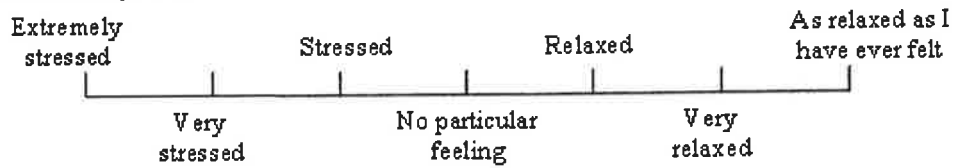
Weak   —————————————————————Strong

How much food do you think you could eat?

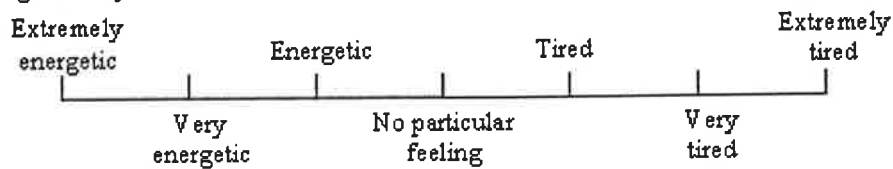None   —————————————————————A large amount

Appendix 2 Appetite questionnaire; Categorical scale

Please indicate how you are feeling at this moment by placing a circle at the appropriate point on each scale below. Mark all scales.

How relaxed do you feel?

| Extremely stressed | | Stressed | | Relaxed | | As relaxed as I have ever felt |
| Very stressed | | No particular feeling | | Very relaxed | |

How energetic do you feel?

| Extremely energetic | | Energetic | | Tired | | Extremely tired |
| Very energetic | | No particular feeling | | Very tired | |

How hungry do you feel?

| Extremely hungry | | Hungry | | Satisfied | | Extremely full |
| Very hungry | | No particular feeling | | Full | |

How much food would you like to eat now?

| A very large amount | | A moderate amount | | A small snack | | None |
| A large amount | | No particular amount | | One mouthful | |
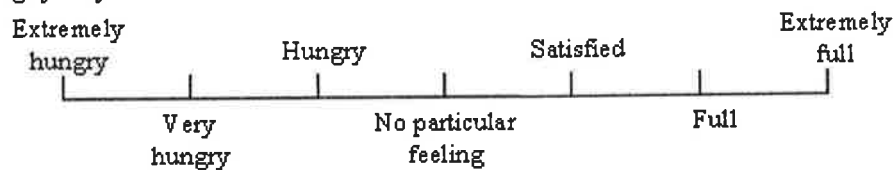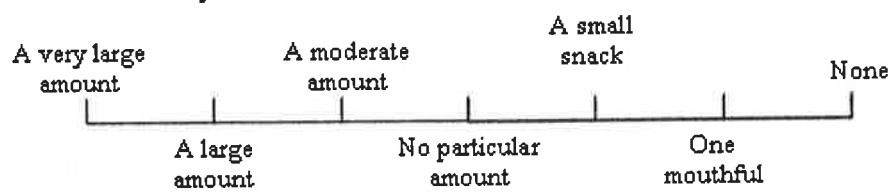
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