Gastrointestinal Mechanisms in the ‘Anorexia of Ageing’ – Effects of Dietary Protein

A thesis by

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De visschers weten dat de zee gevaarlijk is, en de storm geducht, maar hebben nooit kunnen inzien dat de gevaren redenen waren om op 't strand te blijven kuieren.

The fishermen know that the sea is dangerous and the storm terrible, but they have never found these dangers sufficient reason for remaining ashore.

Vincent van Gogh, The Hague, 1882

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THESIS ABSTRACT

Involuntary weight loss in older people reflecting of a decline in appetite and energy, mainly protein, intake, is associated with the development of undernutrition and increased morbidity and mortality, and is termed the 'anorexia of ageing'. A common strategy for management of undernutrition in older people is the use of nutritional supplements which are usually high-energy drinks, rich in whey protein.

In younger adults, whey protein, when compared to other proteins, is perceived as a 'fast-acting' protein, with a rapid satiating effect. Given that protein is the most satiating macronutrient in younger people, and its substitution for other macronutrients is often advocated to promote weight loss, it is possible that the satiating effects of increased protein ingestion could counteract some, or all, of the positive effects of increased protein ingestion in older people on muscle mass and function. Despite the increasing use of protein-rich drinks by older people, information about their effects on energy intake, appetite and underlying gastrointestinal mechanisms in this age group is limited. The primary aim of this thesis was to determine the effects of dietary protein on energy intake, appetite and underlying gastrointestinal mechanisms, including antropyloroduodenal motility, gastric emptying and plasma gut hormone concentrations in healthy older when compared to younger adults.

The studies produced clear-cut results - ingestion of whey protein was less suppressive of feeding behaviour in older than younger adults, so that there was an increase in total energy intake in the elderly. Younger adults showed suppression of perception of appetite after protein ingestion when compared to control, while older adults increased their appetite. Energy intake at a buffet meal was not affected by the timing of protein ingestion before the meal. Young women, in contrast to men, did not show suppression of *ad libitum* energy

intake after oral protein preloads. Older compared to younger adults, and women compared to men, had slower gastric emptying of whey protein drinks. Ageing appears especially to affect the initial phase of gastric emptying of protein. In older adults, plasma CCK and GIP concentrations after protein ingestion were higher compared to young adults.

In conclusion, the regulation of appetite and energy intake is impaired in the elderly. In particular, the acute suppression of energy intake by whey protein is less in healthy older, than younger, adults, resulting in increased overall energy intake in the older adults.

DECLARATION

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

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ABSTRACTS AND PRESENTATIONS

Giezenaar C, Luscombe-Marsh ND, Hutchison AT, Horowitz M, Chapman I, Soenen S. *Effects of whey protein on suppression of energy intake, gastric emptying and gut hormone concentrations in men and women.* 10th Asia Pacific Conference on Clinical Nutrition (APCCN), Adelaide, Australia, November 2017 (Oral presentation; **awarded with best student poster prize**).

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CHAPTER 1: THESIS OVERVIEW

OUR POPULATION IS AGEING

The world population, including that of Australia, is ageing. The percentages of Australia's population older than 65 increased from 9% in 1976 to 15% in 2016. By 2056, it is anticipated that these percentages will further increase to 22% of Australia's population (1). Currently, Australian life expectancy is 82.1 years for men and women combined, which is the sixth highest among countries within the Organisation for Economic Co-operation and Development (OECD) (2).

Much of the health expenditure and burdens of poor health in Australia and other developed countries relates to people over the age of 65 years (3), and health expenditure is expected to increase seven-fold for those aged over 65 years, and twelve-fold for those aged over 85 years between 2010 and 2050 (4). Undernutrition in older people is associated with substantial reductions in functional independence and quality of life with increases in health care utilization. Optimising the health of older people is, therefore, important to limit increasing health care costs.

ENERGY INTAKE IN OLDER PEOPLE – THE 'ANOREXIA OF AGEING'

With healthy ageing, mean food and energy intake decrease (5), which has been termed the 'anorexia of ageing' (6). For example, in a cross sectional study, older people aged 72 years (range: 65 - 94 years) old had a ~30% lower energy intake than younger adults aged 26 years (19-35 years) old (7). A longitudinal study of seven years follow up of 156 healthy older individuals reported a decrease in energy intake of 19 kcal/day/year in women and 25 kcal/day/year in men (8).

CHANGES IN BODY WEIGHT AND COMPOSITION WITH AGEING – UNDERNUTRITION IN OLDER PEOPLE

Weight loss is more common than weight gain in older people (9) and may lead to undernutrition (10, 11); between 20-85% (depending on the diagnostic method used) of the ageing population in nursing homes and acute hospital care and 5-20% of the community-dwelling population, are undernourished (10, 12). Low body weight and weight loss, especially if involuntary, are strong predictors of poor outcomes in older people. Weight loss is associated with a 70% increase in mortality, whereas weight stability and weight gain are not associated with increased mortality (12). The adverse effects of being overweight and of obesity are also less in older than young people; a BMI of ~27-30 kg/m^2 in older people is associated with maximum life expectancy compared to a BMI of 20-25 kg/m^2 for young adults (13). A major factor contributing to the development of undernutrition and associated adverse effects is that weight loss in older people predominantly reflects loss of skeletal muscle (14), accounting for functional impairments including reduced grip strength and gait speed, greater rates and durations of hospitalisation, a higher number of individuals moving from home into supported accommodation and increased mortality (10).

EFFECTS OF AGEING ON MUSCLE PROTEIN SYNTHESIS

In older adults, insufficient protein intake facilitates muscle loss by limiting muscle anabolism (15). Furthermore, older people may experience 'anabolic resistance', which increases the threshold of protein ingestion that is needed to stimulate postprandial protein anabolism (16). Importantly, providing sufficient amounts of protein and essential amino acids are ingested [including free essential amino acids (17, 18), whey (19), casein (20) or meat protein (21)], ageing does appear to not impair the capacity for muscle protein synthesis, Muscle protein synthesis dose-dependently increases after ingestion of 2.5, 5 or

10 g of essential amino acids (22) and 10, 20 or 35 g of whey protein (23). The highest rate of muscle protein synthesis was reported after the highest loads; 10 g essential amino acid and 35 g whey protein. Whole-body muscle protein synthesis (synthesis minus breakdown) was reported to be increased in older adults after protein ingestion of 1.5 g/kg/day (~120 g/day) compared to 0.8 g/kg/day (~70 g/day) (24). A consensus statement by the PROT-AGE study group set up by European Union Geriatric Medicine Society, in cooperation with other scientific organisations, to review dietary protein needs with ageing, recommended that dietary protein intake in older people should be increased from ~0.8-1.0 g/kg body weight (the recommendation for younger adults) to ~1.2-1.5 g/kg for older adults (25).

EFFECTS OF DIETARY PROTEIN ON MUSCLE MASS AND FUNCTION IN OLDER PEOPLE

A Cochrane review of protein and energy supplementation studies in older people (n = 3,058) concluded that protein-energy supplementation resulted in a small weight gain and reduced mortality, but only when participants were undernourished, or when supplementation was ≥ 400 kcal/day (26). No firm conclusions could be made regarding the potential benefits of protein-energy supplementation in a community population as the studies included differed substantially in design and the outcomes assessed. A systemic review of body composition measurements in older people (n = 1,287) concluded that nutritional supplementation has some protective, and beneficial, effects on muscle mass and strength, which are increased further when combined with physical exercise (27). In undernourished elderly, protein-rich supplementation after hospital discharge produced modest increases in handgrip strength (~1 kg at 8 weeks) (28). In frail older people (n = 65), dietary protein supplementation (120 kcal/day for 24 weeks) improved physical performance by ~10-20% (29). In sarcopenic patients (n = 130), intake of a whey-protein supplement (20 g twice daily for 13 weeks)

compared to control resulted in a larger improvement in muscle mass (protein vs. control group: + 0.17 kg) and function (repeated chair stands; protein vs. control group: -1.01 s) (30).

PROTEIN INTAKE IN OLDER PEOPLE

Due to the increasing awareness of the major muscle loss that accompanies weight loss in older people, and its particular adverse effects, there has been a recent, and substantial, increase in the preferential administration of protein in these supplements in an attempt to preserve, or even increase, muscle mass and function (26, 27). A wide range of high-protein supplements, usually whey-protein, are being used increasingly frequently with the aim of increasing energy-protein intake in (undernourished) older people in both institutionalised and community-dwelling populations (26, 31).

Whey is a milk protein and major protein source (in dairy) within the diet. It is a liquid by-product of the cheese making process. Whey protein has a high essential amino acid content, particularly leucine. Protein metabolism appears to be dependent on the quantity of essential amino acids ingested – there was no difference in muscle synthesis after older people ingested either 18 g essential amino acids or 40 g balanced amino acids (18 g essential amino acids plus 22 g non-essential amino acids) (32). Leucine, in particular, may play an important role – muscle protein synthesis in older people was not increased after a 6.7 g essential amino acid mixture containing 26% of leucine, but increased when the leucine content was increased to 42% (33). Whey protein, compared to casein and casein hydrolysate (all 20 g), resulted in higher muscle protein synthesis in healthy older adults (19). In younger adults whey empties from the stomach relatively quickly when compared to casein (34), so that the absorption of amino acids into the circulation and the onset of satiety is rapid, but relatively short-lived (28).

EFFECTS OF DIETARY PROTEIN ON APPETITE, ENERGY INTAKE AND BODY WEIGHT IN YOUNG ADULTS

There has been substantial interest in the mechanisms underlying the effects of protein ingestion on body weight and body composition in young adults, driven primarily by attempts of overweight young adults to lose weight, and evidence that high protein diets promote weight loss by increasing satiety (35). Short-term studies of the effect of oral preloads (36-39) in young adults have shown that protein is more satiating than carbohydrate and lipid, resulting in greater reduction of hunger and increase of fullness and in decreased energy intake after a protein preload compared to carbohydrate and fat preloads (36). The landmark study of Weigle *et al*. indicated that a diet containing 30% of protein was more satiating, associated with decreased energy intake and greater weight loss over time, than an iso-caloric diet containing 15% of protein (40).

THE GAP IN KNOWLEDGE AND AIMS OF THE THESIS

Given that protein is the most satiating macronutrient in young people, and its substitution for other macronutrients is often advocated to promote weight loss in overweight young adults, it is possible that the satiating effects of increased protein ingestion could counteract some, or all, of positive effects on muscle in older people. Yet, despite the increasing use of protein-rich drinks by older people, information about their effects on energy intake in this age group is lacking.

The primary aim of this thesis was to determine the effects of dietary protein on energy intake, appetite and underlying gastrointestinal mechanisms, including antropyloroduodenal motility, gastric emptying and plasma gut hormone concentrations in healthy older when compared to younger adults.

OUTLINE OF THE THESIS

The acute effects of dietary protein intake on energy intake are largely unknown. This thesis will explore the age-related physiological changes in energy intake and appetite and related gastrointestinal mechanisms after whey protein ingestion, with the aim of developing an optimal strategy for protein supplementation to increase energy intake and muscle mass and function in older people, particular those at risk of undernutrition. **Chapter 2** summarises the current literature relating to the effects of ageing on antropyloroduodenal motility, gastric emptying and gut hormones associated with appetite and energy intake regulation. **Chapter 3** presents a meta-analysis aiming to determine the effect of ageing on appetite and energy intake. In **chapter 4**, the techniques used of the studies in this thesis are presented. **Chapters 5 and 6** describe the load effects of intraduodenally administered (thereby bypassing oro-gastric effects) whey protein, on energy intake, antropyloroduodenal motility, perceptions of appetite and gastrointestinal symptoms, glucose, gut hormone and amino acid concentrations. **Chapter 7** presents a study relating to the effects of oral whey protein loads on energy intake, gastric emptying, perceptions of appetite and gastrointestinal symptoms. **Chapter 8** describes the effect of timing of whey protein intake before the meal on subsequent energy intake and perceptions of appetite and gastrointestinal symptoms. **Chapters 9 and 10** present the gender effects of whey protein intake on energy intake, perceptions of appetite and gastrointestinal symptoms, gastric emptying, glucose and gut hormone concentrations after oral loads in younger and older adults. **Chapter 11** examines the effects of ageing on blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP and GLP-1 in response to oral protein loads. **Chapter 12** describes the effects of substitution, or addition, of carbohydrate and fat to whey protein on energy intake, perceptions of appetite and gastrointestinal symptoms, gastric emptying, glucose and gut hormone concentrations

in older men. Finally, this thesis concludes with a general discussion (**Chapter 13**) and summary of the results of the above-described studies.

CHAPTER 2: EFFECT OF AGEING ON ENERGY INTAKE REGULATION – A LITERATURE REVIEW

INTRODUCTION

The regulation of appetite and energy intake is complex, and influenced by the interaction of both central and peripheral mechanisms, all of which are potentially affected by ageing. The co-ordination between interrelated intragastric and small intestinal sensory and motor mechanisms is precise, and triggered by the interaction with ingested nutrients. Intragastric mechanisms include slowing of gastric emptying, increased antral area (distension of the distal stomach), and inhibition of plasma ghrelin concentrations. Small intestinal mechanisms include suppressed antral and duodenal motility and increased pyloric motility, which result in slowing of gastric emptying, and stimulated gut hormone secretion, i.e. cholecystokinin (CCK), glucagon-like-peptide-1 (GLP-1), peptide-tyrosine-tyrosine (PYY) and gastric-inhibitory-peptide (GIP). Blood glucose concentrations are mainly regulated through the secretion of insulin and glucagon in the post-prandial and post-absorptive phase. Intragastric, small intestinal, and blood glucose mechanisms are important factors in the regulation of appetite and subsequent energy intake. The age-related changes in the gastrointestinal mechanisms and glucose regulation influencing feeding are reviewed in this chapter.

ENERGY INTAKE AND APPETITE IN YOUNG AND OLDER INDIVIDUALS

It has been established clearly that there are differences in appetite and energy intake between young and older people. Fasting hunger ratings, measured by a visual analogue scale (VAS), are lower in healthy older when compared to young individuals (41-46), whereas fasting fullness ratings appear comparable between the age groups (42-44, 47-52). On average, daily energy intake in older individuals is less when compared to young individuals (42, 44, 45, 48, 53-56). Interestingly, there is evidence that, after a nutrient

preload, energy intake at a subsequent meal is less suppressed in older compared to young adults (42, 46, 48, 57), and there is a linear decrease in energy intake compensation with age of approximately 2.4% per year of increased age (58). When older individuals are given a nutritional supplement prior to a meal, energy intake at a subsequent meal is not significantly different from energy intake without a nutritional supplement (59-62). Also in longer-term studies, after 21 days of overfeeding or underfeeding, older individuals do not compensate as well as young individuals for the increase or decrease in energy intake during subsequent *ad libitum* diet periods, compared to their energy intake during the baseline weight maintenance period (56, 63). Young people compensate for their body weight gain, or loss, by reducing, or increasing their energy intake during the *ad libitum* period, whereas older people sustain a higher energy intake after an overfeeding period, and a lower energy intake after an energy restriction period compared to energy intake during a weight maintenance period (63). These observations indicate that there is a reduction in the effectiveness of homeostatic mechanisms controlling appetite and food intake in the ageing population.

INTRAGASTRIC MECHANISMS IN YOUNG AND OLDER ADULTS

Intragastric mechanisms of relevance to appetite control include gastric emptying, antral area and concentration and activity of the orexigenic hormone ghrelin.

Gastric emptying and antral area

Gastric emptying is a coordinated activity of the proximal and distal stomach, the pylorus and the upper small intestine (**Figure 2.1**). During meal intake, the proximal stomach relaxes in order to increase space for the meal (**Figure 2.2**). Solid foods are redistributed from the proximal to the distal stomach, and ground by the antrum to small particles, called ‘lag phase’, whereas emptying of liquids does not usually involve a lag phase.

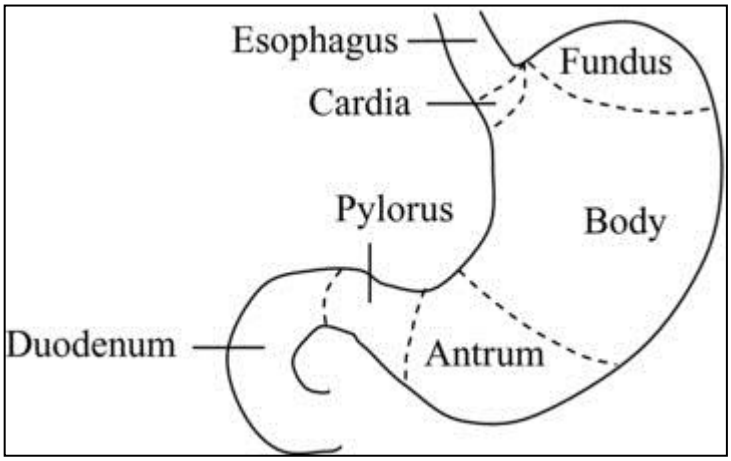

Figure 2.1: Diagram of the stomach areas (64).

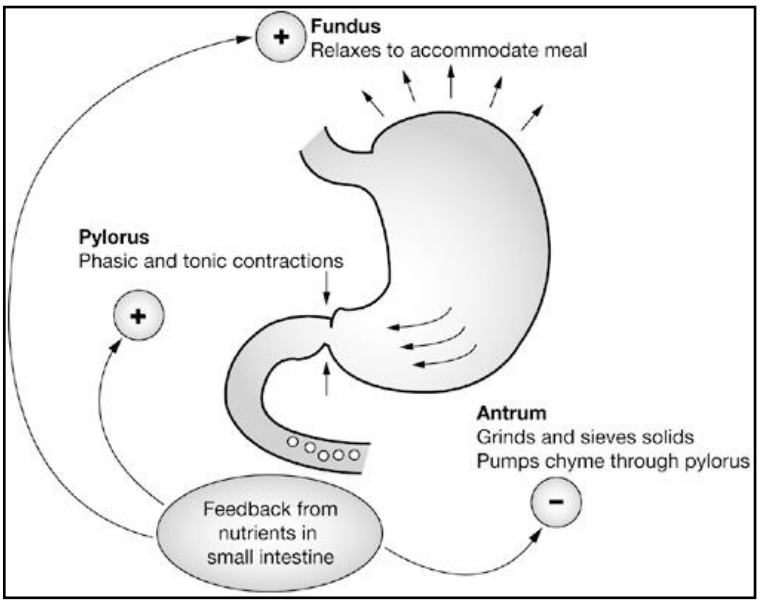

Figure 2.2: Motor patterns associated with gastric emptying (65).

The distal stomach, i.e. antrum, grinds the solid food into smaller particles (< 2 mm) to form a semifluid partly digested food mass, called 'chyme'. The antrum and the pylorus regulate the amount and rate of chyme that is transported from the stomach into the duodenum. The contractions of the distal stomach and the pylorus are regulated by pacemaker cells called 'interstitial cells of Cajal' (66). The antral area reflects an interplay of passive and active forces favouring distension by volume and gravity and resistance to distension occurring as a result of muscular contractions. Because of the active regulation, the antral area/pylorus is

the major determinant for the rate of gastric emptying (67), which typically ranges between 1-4 kcal/min (68). Solid foods empty in an overall linear pattern after a lag phase and low energy liquids empty in an overall exponential pattern; the pattern becomes more linear as the energy content of the ingested material increases (69). Gastric emptying is related to feelings of fullness; a number of studies have shown a positive linear relationship between postprandial fullness and antral gastric distension, but not for proximal gastric distension and postprandial hunger (42, 70, 71). Antral content is relatively stable compared to the content of the proximal stomach; the proximal stomach progressively reduces its content after meal ingestion and releases the gastric distension faster than the antral stomach (42). The perception of fullness is triggered by gastric stretch receptors, located mainly in the distal stomach and activated by gastric distension (72). Because of the absence of a correlation between antral area, or distal stomach content and feelings of hunger, it has been suggested that hunger and fullness are regulated by different mechanisms (42, 70).

Current evidence indicates that ageing is associated with a slowing, albeit modest, of gastric emptying, which may potentially contribute to reduced food intake in older people as a result of increased postprandial fullness (50). Older compared to young individuals, have been reported to have slower gastric emptying times after mixed nutrient (50, 51, 73-77), glucose (78) and lipid (79) preloads (solid or liquid) in some, but not all studies (42, 80, 81). One study reported no effect of ageing on gastric emptying of a solid mixed nutrient meal, but slower emptying in older, compared to young, adults for the gastric emptying of a glass of orange juice (82). Only one study reported an accelerating effect of ageing on gastric emptying (83). Cholecystokinin (CCK) plays an important role in the slowing of gastric emptying (see below), and fasting and postprandial concentrations are increased in older, compared to younger, adults (43, 48, 51, 53, 79). Gastric distribution of a meal may also be affected by ageing – increased retention in the distal stomach may result in increased antral distension, possibly as a result of autonomic dysfunction (50). Ageing is also associated with

diminished perception of gastric distension, which may contribute to the less precise control of food intake in older when compared to young people (84). The effect of ageing on gastric emptying of pure protein, and the relation between subsequent energy intake and gastric emptying in older people are unknown.

Ghrelin

Ghrelin is produced and released from the fundus of the stomach when the stomach is empty (**Figure 2.3**). It exerts its effect via the 'growth hormone secretagogue' receptor (GHS-R) and releases growth-hormone (85). GHS receptors are present in the hypothalamus, pituitary, adrenal, thyroid, pancreas, myocardium, spleen, ovary, enteric neurons and stomach tissue (86). Ghrelin stimulates food intake by increasing gastric acid production and gastric motility, and by inhibition of insulin via neuropeptide Y (NPY) and its production of agouti-related peptide (AgRP), which stimulate appetite via central effects. Plasma ghrelin levels are decreased in people with obesity and increased in those with cachexia or anorexia, when compared to healthy individuals (87).

The majority of studies have found that plasma active (acylated) ghrelin (52, 88) and total ghrelin (41, 48, 52, 88-90) concentrations during fasting are not affected by increasing age. However, Di Fransesco *et al.* and Rigamonti *et al.* reported that older, compared with young adults, had lower levels of plasma active (91) and total (92) ghrelin concentrations during fasting, providing another possible mechanism for the ageing-associated decline in appetite and food intake.

In young adults, plasma total ghrelin concentrations have been reported to decrease after an oral nutrient preload (41, 52, 91) and to start to increase again approximately two hours after preload administration (41, 91), whereas at this time they remain similar to baseline in older participants (52). This suggests altered ghrelin production or sensitivity in older individuals.

Sturm *et al.*, however, reported that the absolute decline in plasma total ghrelin concentrations after an oral mixed nutrient preload was comparable in both age groups (48). Higher plasma ghrelin concentrations have been observed in older compared to young individuals during an energy restricted period and a subsequent *ad libitum* period (55). Plasma total ghrelin concentrations were lower in young, than in older individuals after a two-week period of caloric restriction, but in both age groups there was an increase in plasma ghrelin concentrations during the *ad libitum* period compared to an energy balance period (55). These observations suggest that ghrelin sensitivity is decreased in this group i.e. higher levels may be required to achieve the same orexigenic effect as in young people. The effect of ageing on stimulation of ghrelin in response to protein is unknown.

SMALL INTESTINAL MECHANISMS IN YOUNG AND OLDER ADULTS

Small intestinal mechanisms include gut motility and concentrations and activity of the anorexigenic hormones.

Gut motility

Stomach and small intestinal motility consists of three phases which follow a pattern in the fasting state, called 'the migrating motor complex (MMC)'; phase I, in which no motility is present (~40 min), phase II in which irregular contractions are present (~50 min) and phase III, which shows strong and regular contractions (~5-10 min) (93). The MMC is stimulated by different gut hormones and activation of the parasympathetic and enteric nervous system (94). Agents, including hormones, which induce phase III contractions with gastric origin include motilin (95), erythromycin (96), atropine (97) and ghrelin (98). Hormones that

induce phase III contractions with duodenal origin include somatostatin (99) and xenin (100).

Serotonin has no effects on the MMC when administered intraduodenally, however, it increases the frequency and migration velocity of phase II contraction when administered intravenously (101, 102).

Studies performed employing recording with a balloon kymograph showed that gastric motor activity is inhibited after truncal vagotomy (103) but phase III contractions were still present in the small intestine (104) suggesting that the vagus nerve is involved in gastric motility, but not the motility of the small intestine. The latter is now thought to be regulated primarily by the enteric nervous system. Contraction of the smooth muscle cells of the gastrointestinal tract is initiated by the release of excitatory neurotransmitters from motor neurons of the enteric nervous system at the same time as slow waves generated by the interstitial cells of Cajal (94). During phase I, slow waves are present, but there is no secretion of an excitatory stimulus from the enteric nerve system so that no contractions occur during phase I. During phase II and III, both slow waves and excitatory stimuli are present, leading to the generation of contractions (94).

After meal consumption, fasting motility changes to postprandial motility in the segments where the chyme is present; characterised by irregular antral contractions to enable mixing and digestion of the food consumed (105). These contractions last ~1 hour for each 200 kcal ingested. In the segments where no chyme is present, fasting motility continues (106). The transportation of (unabsorbed) nutrients from the stomach to the small intestine via the pylorus is associated with gastric acid production (107), pancreatic secretion (108) and inhibition of gastric and jejunal motility (109, 110). The latter effect is known as the 'ileal brake' effect and is exerted by gastrointestinal hormones (111) (**Figure 2.1**). Intraduodenal infusion of lipids (112), carbohydrates (113) and hydrolysed-whey protein (114) in young adults, suppresses subsequent energy intake (114) by increasing neural and hormonal

feedback and stimulating increases in phasic (isolated pyloric pressure waves; IPPW's) and tonic pressures of the pylorus, to regulate nutrient delivery to the intestine, resulting in slowing of the rate of gastric emptying.

Healthy ageing is associated with increased small intestinal responses to nutrients, including greater stimulation of phasic pyloric pressure waves by intraduodenal lipid infusion (45), a greater satiating effect of intraduodenal glucose (44), and altered gut hormone responses (42, 43, 48); all of these changes may contribute to the observed modest slowing in gastric emptying (53). The effect of ageing on small intestinal responses to intraduodenal protein has not been investigated.

Anorexigenic hormones

Another aspect of the small intestinal and gastrointestinal mechanisms influencing energy intake are the anorexigenic hormones [cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), peptide tyrosine tyrosine 3-36 (PYY), gastric inhibitory polypeptide (GIP), insulin and glucagon] which are present in low concentrations in the peripheral blood during fasting conditions, with a substantial increase during and after meal intake associated with meal termination and suppression of subsequent energy intake (115).

Cholecystokinin (CCK)

CCK is released from L-cells in the mucosal epithelium of the duodenum in response to meals, particularly those containing fat or protein (**Figure 2.3**) (116-118). CCK is transported via the blood and has rapid, short-lived, effects (119) which effects are mediated by CCK_A receptors, present in the pancreas and on the afferent vagus nerve and enteric neurons, and also CCK_B receptors, present in the stomach and on the afferent vagus nerve

(120, 121). Receptor binding influences gastric emptying, gut motility, secretion of gastric acid, enzyme secretion of the pancreas and contraction of the gallbladder (116-118).

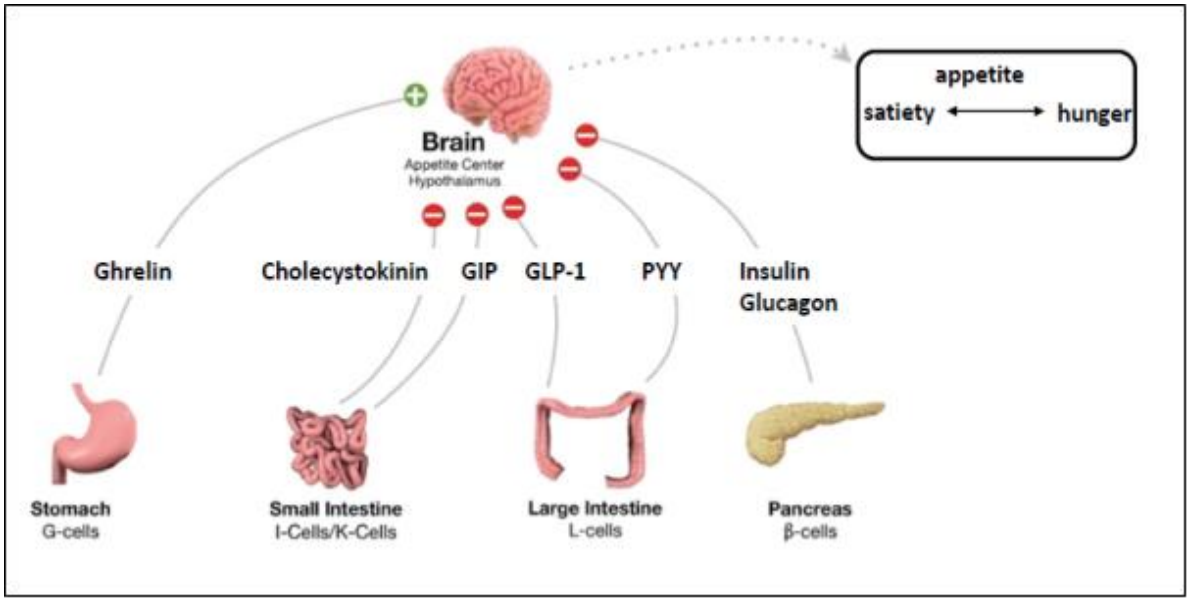

Figure 2.3: Hormone production sites. Adapted from (122).

CCK acts to reduce hunger and suppress food intake: infusions of CCK reduce food intake in young and older adults (43) while administration of CCK antagonists results in increased hunger and food intake, indicative of a physiological role for this inhibitory effect (123). Older people appear to have higher plasma CCK concentrations, on average, than young adults under all conditions studied: during fasting (43, 48, 51, 53), after ingestion of an oral nutrient preload (51), during ID infusion of lipids and glucose (53), and during intravenous infusion of saline and exogenous CCK (43). Only one study failed to find any differences in CCK concentrations between young and older people (41). Older people seem to retain their sensitivity to the satiating effects of CCK as the suppression of energy intake induced by intravenous infusion of CCK, compared to the control day, was higher in the older adults compared with the young adults (32% vs. 16%) (43). The effect of ageing on the CCK response to protein in unknown.

Gastric inhibitory protein (GIP)

GIP is secreted by K-cells located in the duodenum and proximal jejunum mainly as a response to intake of carbohydrates and lipids (**Figure 2.3**) (124). GIP has a very short half-life time of 5-7 min, before it is rapidly degraded by dipeptidyl peptidase-4 (DPP-IV) (125). The amino acid sequence of GIP is almost identical to GLP-1 (see below) (124). GIP exerts its effects via GIP receptors, which are located on the pancreatic islands, gut, adipose tissue, heart, pituitary, adrenal cortex and in several regions of the brain.

The effect of GIP on energy intake is not clear (126). Potentially GIP exerts an anorexigenic effect by stimulating insulin secretion (127), promoting ß-cell proliferation (128) and regulating fat metabolism in adipocytes (129) (incorporation of fatty acid into triglycerides and stimulation of lipoprotein lipase activity and stimulation of fatty acid synthesis). GIP stimulates the secretion of glucagon in a dose-dependent matter (130) and one study reported a modest acceleration of gastric emptying by peripheral infusion of GIP (131). The physiology of GIP is poorly understood because of the absence of a specific GIP receptor antagonist suitable for human use.

Older compared with young people, have similar plasma GIP concentrations during fasting (132-134). After oral ingestion of glucose (132, 134, 135) and during a 1 kcal/min intraduodenal glucose infusion (baseline to 60 min) (133), GIP levels have been reported to be higher in older compared to young subjects and to also return to baseline slower (132). Higher post-nutrient GIP levels in older than young adults may reflect reduced DPP-IV levels in older compared to young people and/or alterations in glucose-induced insulin secretion and the β-cell response, potentially resulting in increased feelings of satiety (135). The sensitivity to GIP in the ageing population has not been evaluated, and the effect of ageing on the GIP response to protein in unknown.

Glucagon-like peptide1 (GLP-1)

GLP-1 is released from proglucagon L-cells located predominantly in the mucosal epithelium of the ileum and colon (**Figure 2.3**) (136). GLP-1 has a short half-life of 1-2 min as it is, like GIP, rapidly broken down by DPP-IV (137). GLP-1 exerts its effects primarily by the GLP-1 receptor, present on peripheral and neurons of the central nervous system (CNS), cells in the pancreatic islands, gastrointestinal tract, lungs, kidneys, and heart (138-140).

GLP-1 suppresses appetite and food intake probably in part by inhibiting gastrointestinal motility and thereby the transit of nutrients through the gastrointestinal tract, contributing to the ileal brake effect (141, 142), by glucose-dependent stimulatory and inhibitory effects on insulin and glucagon secretion, respectively (143). The physiological role of GLP-1 on gastric emptying was determined by a study using exendin 9-39 amide, a GLP-1 receptor antagonist. The study reported accelerated gastric emptying and increased postprandial glucose concentrations after a mixed nutrient preload during intravenous infusion of exendin 9-39 amide compared to control. Exogenous infusion of GLP-1 slowed of gastric emptying and also reported decreased energy intake compared to a control saline infusion in young healthy men (144).

In most studies, older and young adults have been reported to have comparable circulating plasma GLP-1 concentrations during fasting (48, 53, 133). However, Ranganath *et al.* reported higher concentrations in older compared with young females (132), while, in contrast, Trahair *et al.* found a trend towards lower fasting GLP-1 concentrations in older, compared to young, men and women (133). After a mixed nutrient oral preload (48) or during intraduodenal glucose or lipid infusion (53, 133) comparable GLP-1 plasma concentrations were observed in young and older individuals. In contrast, Ranganath *et al.* reported higher GLP-1 plasma concentrations in older compared with young people in response to oral carbohydrate (132). MacIntosh *et al.* reported an inverse relationship

between hunger ratings and GLP-1 levels in young, but not in older, subjects (53), consistent with the concept that older people are less sensitive to the satiating effects of GLP-1. The sensitivity to GLP-1 in the older population has not been evaluated, and the effect of ageing on the GLP-1 response to protein is unknown.

Peptide tyrosine-tyrosine (PYY)

PYY is released from endocrine L-cells in the mucosal epithelium of the ileum and colon (**Figure 2.3**) (145) as PYY(1-36) and metabolised in to PYY(3-36) by DPP-IV (146, 147). PYY exerts its effects by binding to the NPY receptor family, and neuropeptide Y2 receptors (148, 149). PYY probably has a major role in the ileal brake effect, which may contribute to its anorexigenic effect (150, 151). Plasma PYY concentrations during fasting appear to be largely unaffected by ageing (51, 53), although, after an oral mixed-nutrient preload, meal-induced rises may be more prolonged in older people (51), which may contribute to increased feelings of satiety. However, during a two hour intraduodenal glucose or lipid infusion, PYY concentrations in both age groups were comparable (53). This may be because in this study the nutrients were infused into the duodenum, and would, accordingly, be expected to be absorbed higher in the gastrointestinal tract, i.e. since PYY is produced in the colon, it is not surprising that only a low PYY response (and no difference between age groups) was observed (53). No studies have evaluated the sensitivity to PYY in an ageing population have been conducted. The effect of ageing on the PYY response to protein is unknown.

Glucose regulation in young and older adults

Glucose is the main form of fuel for the body. In response to food intake, particularly carbohydrates, blood glucose concentrations increase, and insulin released from the pancreas to promote incorporation of glucose into fat, liver and skeletal muscle cells. Glucagon has the opposite effect – when glucose concentrations are low glucagon stimulates the liver to

convert glycogen intro glucose. Due to insulin resistance associated with ageing, older people have higher blood glucose concentrations compared to young adults (152).

Insulin

Insulin is released from ß-cells in the pancreas and is required for the transportation of glucose into the body cells (**Figure 2.3**) (153). Intranasal administration of insulin in young men (154) and women (155) suppressed subsequent energy intake, and increased insulin concentrations have been correlated with decreased food intake in various studies in young adults (156-159). Whey protein results in greater increases in insulin concentrations and more suppression of energy intake compared to other high-protein meals (tuna, egg and turkey) (160). Insulin may suppress energy intake via the stimulation of alpha-melanocyte-stimulating hormone (αMSH) and cocaine- and amphetamine-regulated transcript (CART) neurons, by inhibiting NPY and AgRP production in the arcuate nucleus (161), and by decreasing ghrelin levels (162) (163) (164).

Circulating insulin concentrations probably increase with increasing age. Plasma insulin concentrations in older compared to young individuals during fasting were higher in five studies (20, 48, 89, 133, 165), comparable in four studies (42, 44, 52, 132) and lower in one study (43). After oral mixed-nutrient preloads higher peak plasma insulin concentrations have been reported in older than young subjects (41, 89); a 9-fold increase in insulin concentrations was seen in the older group versus a 3-fold increase in the young group in one study (41). During intraduodenal glucose infusion, the rise in serum levels of insulin was reported to be greater in older than in young subjects (44). Two hours after an oral preload, higher insulin concentrations in older compared with young subjects were observed (52). Another study found increased insulin concentrations throughout a day in older than in young adults when subjects were given two standardised oral meals (166). However, four other studies found that after oral carbohydrate preloads, mixed nutrient preloads and during

intraduodenal glucose infusion, there was no difference in insulin levels between older and young participants (42, 48, 132, 133), although levels returned slower to baseline in older compared with young subjects (132). Insulin resistance in the ageing population may be explained by an increase in visceral fat mass, rather than by age per se – intra-abdominal fat mass correlates significantly with insulin resistance regardless of gender (167). Increased insulin concentrations in older people could possibly result in a decreased appetite as insulin stimulates leptin production, and inhibits ghrelin expression. The effect of ageing on the insulin response to protein is unknown.

Glucagon

Glucagon is secreted from α-cells in the pancreas and released in the portal vein in fasting conditions and in response to exercise (**Figure 2.3**). Glucagon acts on the liver to promote glycogenolysis and gluconeogenesis to increase blood glucose levels (69). Glucagon mediates its effects via the glucagon receptor, which is expressed mainly in the liver and kidneys, but is also found in the gut, adrenal glands, brain, heart, pancreas, spleen and adipocytes (168).

Exogenous administration of glucagon was found to suppress hunger and energy intake in multiple old studies (169-171). A recent study confirmed these results and reported decreased energy intake in response to intravenous infusion of glucagon, either by itself or in combination with GLP-1, in healthy young men (144). Glucagon had no effect on gastric emptying or appetite scores, indicating that glucagon suppresses energy intake through in an alternative way. A possible mechanism is though increasing glucose concentrations and reducing hunger contractions (172) although another studies has found the suppressive capacities of glucagon independent of glucose concentrations (169).

Fasting glucagon concentrations have been reported to be similar between young and older people (173, 174) (175). Glucagon concentrations after intravenous glucose and arginine

infusions (173), after 250 kcal, 500 kcal and 1000 kcal mixed nutrient meals (175), and during 2 hyperglycaemic clamps (basal plasma glucose + 5.4 mmol/L or basal plasma glucose + 12.8 mmol/L) (176), were also not different between younger and older age groups. One study found reduced glucagon responses after an oral glucose tolerance test in older compared to young men and women (134). The effect of ageing on the glucagon response to protein is unknown.

CONCLUSIONS

In conclusion, the literature suggests that ageing is associated with slower gastric emptying, greater stimulation of phasic pyloric pressure waves in response to intraduodenal infusion of lipid and glucose, and altered hormone responses, potentially contributing to increased satiety in the older population. The effect of ageing on the gastric emptying, antrapyloroduodenal motility, ghrelin, CCK, GLP-1, GIP, insulin, glucagon and glucose responses to protein are unknown.

CHAPTER 3: AGEING IS ASSOCIATED WITH DECREASES IN APPETITE AND ENERGY INTAKE - A META-ANALYSIS IN HEALTHY ADULTS

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Candidate	Caroline Giezenaar		
Contribution	Searching, identification and critical review of the literature data interpretation, statistical analysis and drafting the manuscript.		
Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	October 2017

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

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

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

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

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ABSTRACT

Background and aims: It is not well recognised that in the elderly weight loss is more common than weight gain. The aim of this analysis was to determine the effect of ageing on appetite (hunger/fullness) and energy intake, after overnight fasting and in a postprandial state, by meta-analyses of trials that included at least two age groups (> 18 years). We hypothesised that appetite and energy intake would be less in healthy older compared with younger adults.

Methods: Following a PubMed-database systematic search up to 30 June 2015, 59 studies were included in the random-effects-model meta-analyses.

Results: Energy intake was 16%–20% lower in older (*n* = 3574/~70 years/~71 kg/~25 kg/m^2) than younger (*n* = 4111/~26 years/~69 kg/~23 kg/m^2) adults [standardised mean difference: −0.77 (95% confidence interval −0.90 to −0.64)]. Hunger was 25% [after overnight fasting; weighted mean difference (WMD): −17 (−22 to −13) mm) to 39% (in a postprandial state; WMD: −14 (−19 to −9) mm] lower, and fullness 37% [after overnight fasting; WMD: 6 mm (95% CI: 1 to 11 mm)] greater in older than younger adults.

Conclusions: In conclusion, appetite and energy intake are less in healthy older than younger adults, suggesting that ageing *per se* affects food intake.

INTRODUCTION

The world population is ageing rapidly. For example, the proportion of the world's population over 60 years will double from 11% to 22% between 2000 and 2050. As healthcare costs are incurred largely by older people, this will have dramatic societal impacts, so that, largely as a result of population ageing, it is projected that government spending in Australia on health will increase tenfold per capita by 2055 (177). Reducing morbidity in the older population is, accordingly, a major public health goal. A very large proportion of the increases in healthcare costs are accounted for by increasing rates and duration of hospital admissions in older people. During hospitalisation nutritional status often declines in older patients, due to a lack of adequate energy intake (178).

It is often not recognised that after age ~65 years weight loss, particularly lean tissue, is more common than weight gain - this has been well documented in cross-sectional and longitudinal studies (5, 8, 179-182). In the elderly, both low body weight and weight loss are strong predictors of poor outcomes (180, 183), including the development of pathological undernutrition and sarcopenia and reduced functional capacity and frailty (184). Data form animal studies suggest that caloric restriction, and probably more importantly diet composition, play a role in longevity by reducing the risk of developing type 2 diabetes, hypertension, cardiovascular disease and cancer which may be related to the body composition during life, i.e. less fat and more lean tissue (185, 186). The loss of body weight in older people is usually associated with disproportionate loss of lean body tissue, with average decreases of up to 3 kg of lean body mass, mainly skeletal muscle, per decade after the age of ~50 years (14). Furthermore, the adverse effects of overweight and obesity are much less in older than young adults, so that the body mass index (BMI) associated with maximum life expectancy increases with age; ~27-30 kg/m^2 in people over 65 years compared to 20-25 kg/m^2 in younger adults (13). There is no sound evidence that in people

over 70 years a BMI > 30 kg/m^2 is associated with any reduction in life expectancy. Consistent with this, the lower end of the 'optimum' BMI range is higher in older than young adults at about 22 kg/m^2 (187).

Weight loss in older people occurs because there is a decrease in daily energy intake (188) which is greater than the decrease in energy expenditure (189). The decrease in energy intake, and the reduction in appetite which underlies it, has been called the 'physiological anorexia of ageing' (6, 190). The reduction in energy expenditure in the elderly is due to reduced physical exercise, loss of energy-demanding lean tissue, and decreased metabolic cost of metabolizing the smaller amount of consumed food (191-193). The American National Health and Nutrition Examination Survey (NHANES) III cross sectional studies reported a decline in energy intake, between the ages of 20-29 and 70-79 years, of 38% (1138 kcal/day) in men and 27% (522 kcal/day) in women and energy intake measured with 24-h recall interviews (179). We recently showed that energy intake was 16% lower in older than younger men, when energy intake was measured with a more accurate technique: of a single *ad libitum* buffet-style meal at the research facility (194).

An important strategy for maintaining good health in older people is the prevention and management of weight loss in the elderly. It is important, therefore, to accurately characterise this problem. Many of the studies in the area have used different methods to measure energy intake and included relatively few subjects, so there is benefit in combining these data. The aim of this analysis was to determine (i) the magnitude of decrease in energy intake and appetite by ageing; (ii) whether the age-effect on energy intake is present both after overnight fasting and in the postprandial state; (iii) whether the age-effect on energy intake is affected by the method of energy-intake measurement, by meta-analyses of studies which included two age groups of healthy (younger and older) adults. We hypothesised that appetite and energy intake would be ~20-25% less in healthy older when compared with younger adults.

MATERIALS AND METHODS

Search strategy, study selection, data extraction and quality assessment

We performed a search of English-language publications in the PubMed database for studies that reported original data of appetite and/or energy (food) intake in 'healthy' adults up to 30 June 2015. We used 'ageing/ageing' in combination with 'appetite', 'hunger', 'fullness', and 'food/energy intake' as keywords (search terms: ("ageing"[MeSH Terms] OR "ageing"[All Fields] OR "ageing"[All Fields]) AND ("appetite"[MeSH Terms] OR "appetite"[All Fields]); ("ageing"[MeSH Terms] OR "ageing"[All Fields] OR "ageing"[All Fields]) AND ("hunger"[MeSH Terms] OR "hunger"[All Fields]);("ageing"[MeSH Terms] OR "ageing"[All Fields] OR "ageing"[All Fields]) AND fullness[All Fields]); ("ageing"[MeSH Terms] OR "ageing"[All Fields] OR "ageing"[All Fields]) AND "energy intake"[All Fields]); ("ageing"[MeSH Terms] OR "ageing"[All Fields] OR "ageing"[All Fields]) AND "food intake"[All Fields]) with filters for animal and non-English publications. We searched for a broad and heterogeneous range of studies, and not only intervention studies, reporting data on appetite and energy intake in both younger and older adults. These data are often reported as 'subject characteristics' at baseline, particularly in the case of energy intake, and not as primary study outcomes. Two researchers (CG and SS) performed screening of studies by titles and abstracts and, subsequently, full texts. References from the retrieved publications and bibliographies of relevant reviews were checked to identify potential additional articles. Studies were included if they reported mean ± SD/SEM energy intake (kcal) and/or appetite (i.e., hunger and/or fullness) of at least two age groups - 'younger' and 'older' adults. Study subjects were required to be 'healthy' and at least 18 years old, without using age restrictions in defining the 'younger' and 'older' age groups. Usually the older groups were made up of people over 60-65 years. Animal studies and non-English publications were excluded. Characteristics were extracted from the original reports

using a standardised data extraction form. When SD's or SEM's of appetite or energy intake were missing in the publication or it was stated that these data were measured but not given, the investigators were contacted by e-mail with a request to provide these data - requested and received twice regarding data of appetite and once requested but not received regarding data of energy intake. We recorded the study's author(s), year of publication, study design, number and gender distribution of the participants, and mean ± SD: age (years), body weight (kg), body mass index (BMI, kg/m^2) for both age groups (**Table 3.1, Appendix 1**). The usual quality filters for randomised trials or observational epidemiologic studies did not apply since the primary aim of this meta-analysis was to determine the magnitude of decrease in energy intake and appetite by ageing rather than to determine the effect of an intervention. We determined whether studies reported inclusion and exclusion criteria and data on attrition, and whether potential confounders were considered, for example whether the younger and older groups were matched for body weight and/or BMI. When data of interventions were used, we reported whether randomization of study conditions was used and whether the study subjects and research personnel were blinded. This meta-analysis is reported in accordance with the recommendations and criteria outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (195).

Data analysis

Meta-analyses were performed with REVMAN software (version 5.2; the Cochrane Collaboration Oxford, United Kingdom) using the DerSimonian and Laird random-effects model with a 95% confidence interval, to account for measurement variability among the included studies. For this analysis, the number of participants' means and SD's of energy intake and hunger and fullness were extracted for both age groups, i.e., younger and older adults. For all data, the SD's were calculated, when necessary, from SE's, and when data were not provided in numerical form they were estimated from the figures. Cochran's test

for heterogeneity was used to determine whether the studies included in the meta-analysis were evaluating the same underlying sizes of effect. A threshold of $P < 0.1$ was used to decide whether heterogeneity (genuine variation in effect sizes) was present. I^2, an estimate of the proportion of total observed variability that is due to genuine variation rather than random error within studies, was used to quantify the degree of inconsistency among studies; it was considered substantial when it was > 50% (196). Sensitivity analyses were performed on studies that may cause bias in the results. Differences in energy intake between younger and older adults were analysed using

Standardised mean differences (SMD's). The SMD is used when it is necessary to standardise the results of several studies to a uniform scale - when studies assess the same outcome (e.g., energy intake) but measure it in a variety of ways (e.g., kcal/meal or kcal/day for energy intake). The SMD expresses the size of the effect in each study relative to the variability observed in that study. The SMD is calculated by dividing the difference in mean outcome between groups (younger and older adults) by the SD of outcome among participants (196). Data relating to hunger and fullness were defined as mean difference between the younger and older adults. Percentage differences between the younger and older adults for the outcomes were calculated for each study and averaged.

RESULTS

The PubMed search identified 5044 potential articles. The review flow diagram is, following the recommendations of the PRISMA statement (195), depicted in **Figure 3.1**. We screened 2703 titles or abstracts following exclusion of 2341 animal studies or non-English articles. We screened 88 publications in full text of which 59 studies fulfilled the inclusion criteria. There were 7 studies which included more than 2 age groups (197-203) - we extracted the data of energy intake and/or appetite of the youngest (≥ 18 years) and oldest age group for

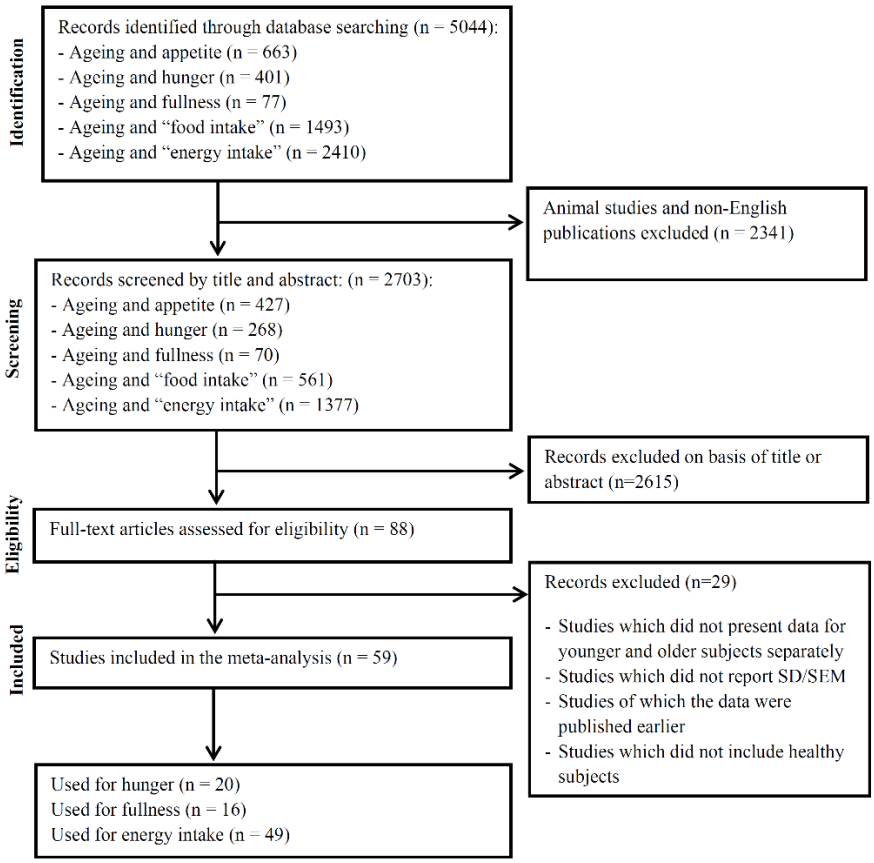

Figure 3.1: Flow diagram for the selection of studies.

each of these studies. There were 14 studies which presented data of multiple groups within the younger and older study groups (7, 200, 204-215), i.e., gender, country, and/or level of physical activity - we combined the male and female or country or level of physical activity groups by calculating their mean energy intake/ appetite score and pooling their SD's to create a single pair-wise comparison.

No studies were excluded based on quality of study, although many studies did not report sufficient information for a clear bias assessment. All studies, except 4 (205, 216-218), reported inclusion and/or exclusion criteria, and stated that the participants met these criteria. Of the studies measuring energy intake (49 studies), 18 studies matched the younger and older participants for body weight (48, 54, 55, 194, 197, 209, 216, 218-228) and 13 studies for BMI (42-45, 49, 54, 207, 216, 218, 219, 226, 228, 229) and 11 studies considered gender

as a confounder (7, 49, 200, 203-205, 208-210, 212, 214). No studies considered confounders for hunger or fullness.

In crossover studies [17 studies (42-46, 48, 49, 57, 84, 194, 198, 201, 214, 215, 230-232)], selection bias and performance bias were possible sources of bias. Thirteen studies (42-46, 48, 49, 84, 194, 201, 214, 215, 232) were randomised of which one (194) detailed a method through random numbers, in the other 6 randomization was not discussed. No studies reported the use of allocation concealment. Performance bias scored worse, 3 studies were double-blind (46, 49, 194) and 2 single-blind (44, 57), in the other 12 blinding was not discussed. In the studies measuring energy intake over a prolonged period of time, all studies, except 2 (63, 219), had a method to check compliance.

Effect of age on energy intake

Three different methods of measuring energy intake were distinguished: (i) energy intake of a single *ad libitum* buffet-style meal at the research facility after overnight fasting [during a 'control' condition; e.g., no preload, water preload or saline intraduodenal infusion (43, 49, 194)] and in a postprandial state after a nutrient preload [> 0 kcal; range 180 (194) - 729 (201) kcal], administered orally or infused directly into the small intestine [i.e., intraduodenally (44, 194)] – all intervention crossover studies, (ii) energy intake of provided food items during a prolonged period (~4 days - 2 weeks) and, (iii) energy intake of weighed food records (~3-14 days), 24-hour food intake recalls, or food frequency questionnaires – we used the observational data for energy intake for the latter two categories. There were 3 studies measuring energy intake of a buffet-style meal in a postprandial state which consisted of multiple nutrient-preload conditions (44, 46, 194) - we extracted the energy intake data of the condition which had the largest effect to suppress energy intake by the nutrient ingestion in the younger-subject group (i.e., the nutrient preload with the highest energy content).

Forty-nine unique studies presented data on energy intake; (i) 10 studies (311 subjects) reported energy intake of 'a buffet-style meal' after overnight fasting (42, 43, 46, 48, 49, 84, 194, 201, 214, 215) and 9 studies (266 subjects) in a postprandial state (42, 44-46, 48, 57, 194, 201, 214) – of which 6 studies (203 subjects) measured energy intake both after overnight fasting and in a postprandial state (42, 46, 48, 194, 201, 214); (ii) 7 studies (339 subjects) reported energy intake 'during a prolonged period' (7, 54-56, 63, 192, 219) and; (iii) 37 studies (7035 subjects) reported energy intake of weighed-food records [30 studies (42-44, 48, 49, 53, 191, 200, 204-212, 214, 215, 217, 218, 220-225, 227, 228, 233)], 24-hour food intake recalls [6 studies (197, 202, 203, 216, 226, 229)], or food frequency questionnaires [1 study (199)] - of which 8 studies also reported energy intake of a buffet-style meal (42-45, 48, 49, 214, 215).

Twenty-six studies were conducted in the United States (7, 46, 55, 56, 63, 191, 192, 200, 204-206, 208, 211, 214, 215, 217, 219, 221-224, 226-230, 233), 10 in Europe (54, 57, 197, 199, 201-203, 207, 212, 218), 8 in Australia (42-44, 48, 49, 53, 194), 2 in Asia (210, 216), 2 in Canada (209, 220), and 1 in Chile (225). The oldest study was published in 1966 (217) and the most recent in 2014 (194). The largest study included 2685 subjects (229) and the smallest 10 subjects (84). The mean age of the youngest group within a study was 19 years (the older group in that study had a mean age of 55 years) (233) and of the oldest group 77 years (the younger group in that study had a mean age of 22 years) (48).

Energy intake in the total group

In the total group of 7685 subjects, energy intake after overnight fasting was less in the older (n = 3574, ~70 years, body weight ~71 kg, BMI ~25 kg/m^2) than the younger adults (n = 4111, ~26 years, ~69 kg, ~23 kg/m^2), with a SMD of -0.77 [95% CI: -0.90 to -0.64] (**Figure 3.2**) and significant heterogeneity (I^2 = 76%, P < 0.001). As a group, the older adults had on average 18 ± 9% (mean ± SD) lower energy intake than the younger adults.

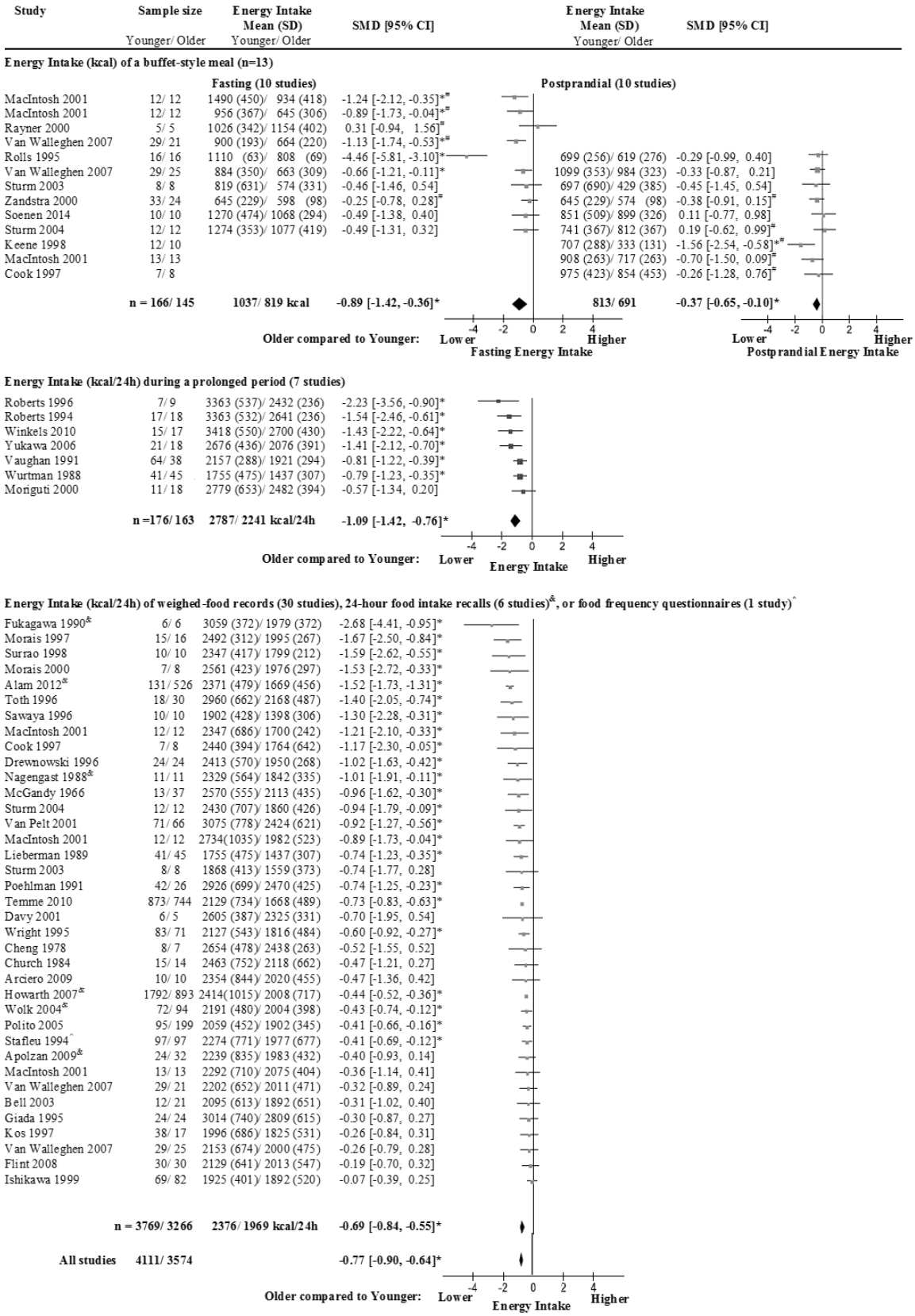

Figure 3.2: Energy intake. Mean ± SD of energy intake (kcal) and a plot of the standardised mean difference (SMD; mm) of energy intake in older compared with younger subjects with the DerSimonian and Laird random-effect model. The horizontal lines denote the 95%

confidence interval; ■ point estimates (the size of the square corresponds to its weight); ♦ the pooled estimate of age effect. Three different methods of measuring energy intake were distinguished: (i) energy intake of a single *ad libitum* buffet-style meal at the research facility after overnight fasting and in a postprandial state after a nutrient preload, administered orally or infused directly into the small intestine; (ii) energy intake of provided food items during a prolonged period; and (iii) energy intake of weighed food records, 24-h food intake recalls, or food frequency questionnaires. In the total group of 7685 subjects, energy intake was less (SMD: −0.77 (95% CI −0.90 to −0.64), I^2 = 76%, p < 0.001) in the older than the younger adults. * P < 0.05 energy intake significantly less in older than younger adults within the study; # data were derived from a figure of the original publication.

Heterogeneity was not affected by introducing a maximum age of the younger and a minimum age of the older age groups (I^2 = 78%, n = 6620 subjects, P < 0.001); i.e., after excluding studies in which the mean age or the maximum age, when age was reported as a range, of the younger adult group was > 40 years old [studies excluded: mean age of 61 (212); age range of 30-49 (210), 42-54 (202), 20-64 (200)] and after excluding studies in which the mean age or the minimum age, when age was reported as a range, of the older adult group was < 65 years old [studies excluded: mean age of 55 (233), 57 (207), 59 (218), 62 (211), 63 (224) years; age range of 36-53 (204), 50-69 (210)]. In the studies included in this sensitivity analysis energy intake was less in the older than the younger adults with a SMD of -0.87 [95% CI: -1.03 to -0.72]. As a group, the older adults (n = 2992) had on average 19 ± 9% lower energy intake than the younger (n = 3628) adults.

Heterogeneity was not affected by excluding the 'small-intestinal' studies, i.e., subjects were intubated with a catheter to deliver the nutrients directly into the small intestine [I^2 = 77%, n = 7617, P < 0.001; 3 studies excluded (43, 49, 194)]. In the studies included in this sensitivity analysis energy intake was less in the older than the younger adults with a SMD of -0.77 [95% CI: -0.90 to -0.63]. As a group, the older adults (n = 3540) had on average 17 ± 9% lower energy intake than the younger (n = 4077) adults.

Heterogeneity was not affected by excluding the 'larger' studies, i.e. with > 100 subjects per age group [I^2 = 59%, n = 2432, P < 0.001; 4 studies excluded (57, 201, 210, 231)]. In the

studies included in this sensitivity analysis energy intake was less in the older than the younger adults with a SMD of -0.77 [95% CI: -0.92 to -0.63]. As a group, the older adults (n = 1212) had on average 18 ± 9% lower energy intake than the younger (n = 1220) adults.

In 1555 females [17 studies (7, 48, 49, 199, 200, 203-205, 208-210, 212, 215, 218, 221, 223, 233)], energy intake after overnight fasting was less in older (n = 763) than younger participants (n = 792), with a SMD of -0.70 [95% CI: -0.95 to -0.45] and significant heterogeneity (I^2 = 73%, P < 0.001). As a group, the older females (1559 kcal) had on average 16 ± 9% lower energy intake than the younger females (1844 kcal).

In 2030 males [28 studies (7, 44-46, 49, 54, 63, 84, 191, 194, 200, 202-212, 215, 217, 219, 222, 224, 225)] energy intake after overnight fasting was less in older (n = 1045) than younger participants (n = 985), with a SMD of -0.95 [95% CI: -1.20 to -0.75] and significant heterogeneity (I^2 = 73%, P < 0.001). As a group, the older males (2033 kcal) had on average 18 ± 10% lower energy intake than the younger males (2486 kcal).

Within an individual study, energy intake was significantly less in older than younger adults in 5 of 10 studies which determined energy intake of a single *ad libitum* buffet-style meal after overnight fasting at the research facility (43, 46, 49, 214, 215), 6 of 7 studies which determined energy intake by provided food items during a prolonged period (7, 54, 55, 63, 192, 219), and 24 of 37 studies which determined energy intake by weighed-food records, 24-hour food intake recalls, or food frequency questionnaires (42, 43, 45, 48, 49, 191, 197, 199, 200, 202, 203, 205, 206, 208, 209, 211, 212, 216, 217, 220-223, 229) (**Figure 3.2**). There were no studies in which energy intake after overnight fasting was significantly higher in older than younger adults.

Energy intake of a buffet-style meal

In the subgroup of 311 subjects, in which energy intake was measured of a single *ad libitum* buffet-style meal at the research facility after overnight fasting (42, 43, 46, 48, 49, 84, 194,

201, 214, 215), energy intake was less in the older than the younger adults, with a SMD of -0.89 [95% CI: -1.42 to -0.36] (**Figure 3.2**) and significant heterogeneity (I^2 = 77%, P < 0.001). As a group the older adults (n = 145, energy intake of 819 kcal/meal), had on average 20 ± 15% (~218 kcal/meal) lower energy intake of a buffet-style meal after overnight fasting than the younger adults (n = 166, energy intake of 1037 kcal/meal).

In the subgroup of 266 subjects, in which energy intake was measured of a buffet-style meal during postprandial conditions (42, 44-46, 48, 57, 194, 201, 214), energy intake was less in the older than the younger adults, with a SMD of -0.37 [95% CI: -0.65 to -0.10] (**Figure 3.2**) and no significant heterogeneity (I^2 = 15%, P = 0.31). As a group the older adults (n = 126, energy intake of 691 kcal/meal) had on average 16 ± 20% (~122 kcal/meal) lower energy intake of a buffet-style meal in a postprandial state than the younger adults (n = 140, energy intake of 814 kcal/meal).

In the subgroup of 203 subjects, in which energy intake was measured of a buffet-style meal both after overnight fasting and in a postprandial state (42, 46, 48, 194, 201, 214), energy intake decreased less in the older adults (decrease in energy intake of on average 10% or ~79 kcal from 798 kcal after overnight fasting to 719 kcal in the postprandial state) than in the younger adults (decrease in energy intake of on average 21% or ~212 kcal from 1000 kcal after overnight fasting to 788 kcal in the postprandial state).

Energy intake during a prolonged period

In the subgroup of 339 subjects, in which energy intake from provided food items was measured during a prolonged period (~4 days - 2 weeks) (7, 54-56, 63, 192, 219), energy intake was less in the older than the younger adults, with a SMD of -1.09 [95% CI: -1.42 to -0.76] **(Figure 3.2**) and non-significant heterogeneity (I^2 = 42%, P = 0.11). As a group the older adults (n = 163, energy intake of 2241 kcal/24 h) had on average 19 ± 6% (~546 kcal/24

h) lower energy intake during a prolonged period than the younger adults (n = 176, energy intake of 2787 kcal/24 h).

Energy intake of weighed-food records, 24-hour food intake recalls, or food frequency questionnaires

In the subgroup of 7035 subjects, in which energy intake was measured using weighed food records (42-44, 48, 49, 53, 191, 200, 204-212, 214, 215, 217, 218, 220-225, 227, 228, 233), 24-hour food intake recall (197, 202, 203, 216, 226, 229), or food frequency questionnaires (199), energy intake was less in the older than the younger adults with a SMD of -0.69 [95% CI: -0.84 to -0.55] (**Figure 3.2**) and significant heterogeneity (I^2 = 77%, P < 0.001). As a group the older adults (n = 3266, energy intake of 1969 kcal/24 h,) had on average 17 ± 8% (~407 kcal/24 h) lower energy intake than the younger adults (n = 3769, energy intake of 2376 kcal/24 h).

In the subgroup of 4311 subjects, in which energy intake was measured using weighed food records energy intake was less in the older than the younger adults with a SMD of -0.63 [95% CI: -0.77 to -0.49] and significant heterogeneity (I^2 = 49%, P = 0.001). In the subgroup of 2530 subjects, in which energy intake was measured using 24-hour food intake recalls energy intake was less in the older than the younger adults with a SMD of -0.63 [95% CI: -0.77 to -0.49] and significant heterogeneity (I^2 = 92%, P = 0.001).

Effect of age on appetite

Hunger

Twenty studies (561 subjects) reported hunger after overnight fasting (41-46, 48-51, 84, 88-90, 194, 198, 214, 215, 230, 231). Twelve of these studies (344 subjects) evaluated hunger also after nutrient ingestion (60 min after oral nutrient consumption/ start of the small

intestinal nutrient infusion; i.e., a time point which was reported in the majority of the studies (41, 42, 44, 48, 50, 51, 89, 90, 194, 231) - 2 studies did not measure appetite up to 60 min and, therefore, the data of 30 min (214) and 15 min (46) were included); 10 studies (98 subjects) after oral mixed macronutrient (protein, carbohydrate and fat) consumption (41, 42, 46, 48, 50, 51, 89, 90, 214, 231) and 2 studies (46 subjects) during intraduodenal infusion of protein (194) or fat (44). All studies reporting hunger were intervention studies, 14 crossover (42-46, 48, 49, 84, 194, 198, 214, 215, 230, 231) and 6 non-controlled studies (41, 50, 51, 88-90),

Nine studies were conducted in Australia (42-45, 48-50, 84, 194), 6 in Europe (41, 51, 88-90, 231), and 5 in the United States (46, 198, 214, 215, 230). The largest study included 54 subjects (214) and the smallest 10 subjects (84). The mean age of the youngest group within a study was 22 years (the older group in that study had a mean age of 77 years) and the oldest group 81 years (the young group in that study had a mean age of 38 years) (90).

Hunger, measured after overnight fasting (41-46, 48-51, 84, 88-90, 194, 198, 214, 215, 230, 231), was less in the older (n = 285, 74 years, 72 kg, 25 kg/m^2) than the younger adults (n = 276, 27 years, 69 kg, 24 kg/m^2), with a weighted mean difference (WMD) of -17 mm [95% CI: -22 to -13 mm] (**Figure 3.3**) and significant heterogeneity (I^2 = 52%, P = 0.004). Heterogeneity was not significant when the 'small-intestinal' studies were excluded (43-45, 49, 194) (I^2 = 51 %, n = 444 subjects, P = 0.010). As a group the older adults (43 mm) had

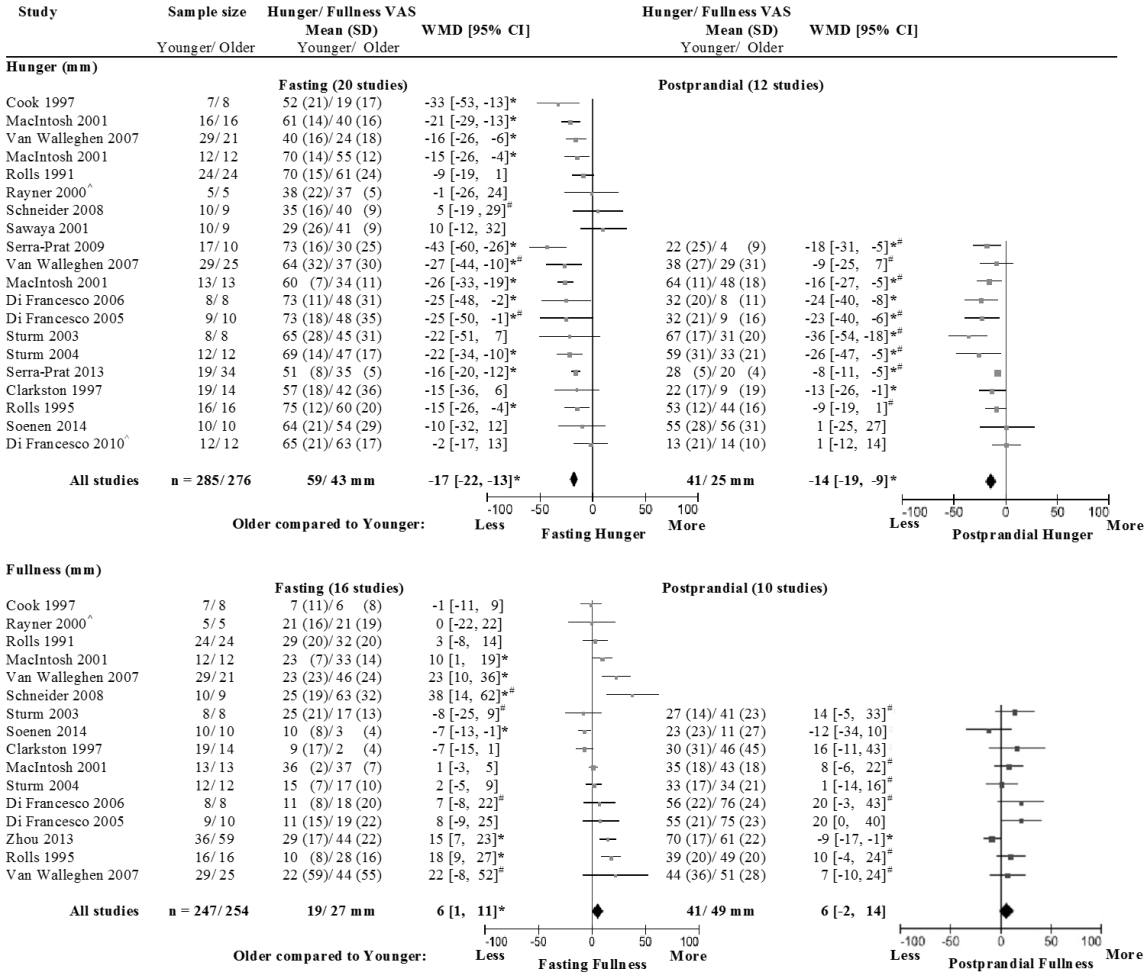

Figure 3.3: Appetite. Mean ± SD of appetite [hunger and fullness; visual analogue scale (VAS; mm)] after overnight fasting and in a postprandial state and a plot of the weighted mean difference (WMD; mm) of appetite in older compared with younger subjects with the DerSimonian and Laird random-effect model. The horizontal lines denote the 95% confidence interval; ■ point estimates (the size of the square corresponds to its weight); ♦ the pooled estimate of the age effect. Older compared to younger adults were less hungry (WMD: −17 mm (95% CI −22 to −13 mm), I^2 = 52%, P = 0.004) and more full (WMD: 6 mm 95% CI 1 to 11 mm, I^2 = 76%, P < 0.001) after overnight fasting and less hungry (WMD: −14 mm (95% CI −19 to −9 mm), I^2 = 53%, P = 0.01) in a postprandial state, whereas fullness was comparable (WMD: 6 mm (95% CI −2 to 14 mm), I^2 = 54%, P = 0.02). ***** P < 0.05 appetite (hunger/fullness) significantly different in older than younger adults within the study; # data were derived from a figure of the original publication; ^ data were provided by the investigators by e-mail upon request.

on average 25 ± 24% (~16 ± 13 mm) lower hunger after overnight fasting than the younger adults (59 mm).

Hunger, measured in a postprandial state (41, 42, 44, 46, 48, 50, 51, 89, 90, 194, 214, 231), was less in the older than the younger adults, with a WMD of -14 mm [95% CI: -19 to -9 mm] (**Figure 3.3**) and significant heterogeneity (I^2 = 53%, P = 0.010). As a group the older adults had on average 39 ± 30% (~15 ± 11 mm) lower hunger in a postprandial state than the younger adults. In the group of 344 subjects, hunger was decreased by 26 mm from 66 mm after overnight fasting to 40 mm in a postprandial state in the younger adults and by 20 mm from 45 mm after overnight fasting to 25 mm in a postprandial state in the older adults. Within an individual study, hunger was significantly less in older than younger adults in 12 (41, 43-46, 49, 51, 84, 89, 90, 214, 215) of 20 studies after overnight fasting and 8 (41, 42, 44, 48, 50, 51, 89, 90) of 12 studies in a postprandial state. There were no studies in which hunger was significantly higher in older than younger adults.

Fullness

Sixteen studies (501 subjects) reported fullness after overnight fasting (42, 44-46, 48-51, 84, 88, 89, 194, 198, 214, 215, 232). Ten of these studies (335 subjects) evaluated fullness also after nutrient ingestion; 8 studies after oral mixed macronutrient consumption (42, 46, 48, 50, 51, 89, 214, 232) and 2 studies during intraduodenal infusion of protein (194) or fat (44). All studies reporting fullness were intervention studies, 12 crossover (42, 44-46, 48, 49, 84, 194, 198, 214, 215, 232) and 4 non-controlled studies (50, 51, 88, 89).

Eight studies were conducted in Australia (42, 44, 45, 48-50, 84, 194), 4 in the United States (46, 198, 214, 215), 3 in Europe (51, 88, 89), and 1 in Asia (232). The largest study included 95 subjects (232) and the smallest 10 subjects (84). The mean age of the youngest group was 22 years and 77 years of the oldest group (48).

Fullness, measured after overnight fasting, was greater in the older (n = 254, 73 years, 71 kg, 25 kg/m^2) than the younger adults (n = 247, 26 years, 67 kg, 23 kg/m^2), with a WMD of 6 mm [95% CI: 1 to 11 mm] and significant heterogeneity (I^2 = 76%, P < 0.001; **Figure 3.3**).

Heterogeneity was not affected by excluding the 'small-intestinal' studies (I^2 =73%, n = 416 subjects, $P < 0.001$; 4 studies excluded (44, 45, 49, 194). As a group the older adults (27 mm) had on average 37 ± 73% (~8 ± 13 mm) higher fullness after overnight fasting than the younger adults (19 mm) adults.

Fullness, measured in a postprandial state, was not significantly different between the older and the younger subjects with a WMD of 6 mm [95% CI: -2 to 14 mm] and significant heterogeneity (I^2 = 54%, P = 0.020). In the group of 335 subjects, fullness was increased by 23 mm from 18 mm after overnight fasting to 41 mm in a postprandial state in the younger adults and by 26 mm from 23 mm after overnight fasting to 49 mm in a postprandial state in the older adults.

Heterogeneity decreased by introducing a maximum age of the younger and a minimum age of the older age groups (I^2 = 0%, n = 240 subjects, P = 0.51); i.e., after excluding studies in which the mean age or the minimum age, when age was reported as a range, of the older adult group was < 65 years old [one study excluded: range of 50-59 (232)] - there were no studies in which the mean age or the maximum age, when age was reported as a range, of the younger adult group was > 40 years old. In the studies included in this sensitivity analysis fullness was significantly different between the older and the younger adults with a WMD of 9 mm [95% CI: 2 to 14]. As a group the older adults had on average 21 ± 32% (~9 ± 10 mm) higher fullness in a postprandial state than the younger adults.

Within an individual study, fullness was greater in older than younger adults in 6 (46, 49, 88, 194, 215, 232) of 16 studies after overnight fasting. In contrast, fullness was less in older than younger adults in 1 (194) of 16 studies after overnight fasting and 1 (232) of 10 studies in a postprandial state.

DISCUSSION

This meta-analysis examined the effect of ageing on appetite and energy intake in adults, including data from > 7500 subjects on energy intake and > 500 subjects on appetite derived from 59 studies. Energy intake was less in healthy older (~70 years) than younger (~26 years) adults. The calculated reduction fell into quite a narrow range at 16-20%, despite studies being done in the fasting and fed state, and energy intake being calculated by a variety of methods, including intake at an acute study meal, during prolonged periods or using weighed food records, 24-hour food intake recalls, and food frequency questionnaires, i.e., a robust finding regardless of the method of intake evaluation. The results of this analysis show that older people (~73 years) feel less hungry than younger adults (~26 years), both fasting (25%) and after they have consumed some food (39%), and also feel more full in the fasting state (37%). These age-related differences are substantial, and likely to be a major cause of the reduced energy intake by older people.

Our results indicate a reduction in energy intake of approximately 20% between the ages of 26 and 70 years, i.e. about 0.5% per year. This is consistent with previous reports of reduced energy intake of approximately 30% between the ages of 20 and 80 years (7, 179), and with the results of individual prospective studies. For example, a 7-year New Mexico longitudinal study of 156 persons aged 64-91 years, reported a decrease of 19 kcal/day/year in women and 25 kcal/day/year in men (8), while a Swedish longitudinal study of 98 people found an even greater decline of energy intake of 610 kcal/day in men and 440 kcal/day in women, between the ages of 70 and 76 years (5). A population-based study indicated that older people aged 60-74 years consume ~500-700 kcal/day less than their younger counterparts aged 20-39 years (179). Our gender analyses indicated that energy intake was less in both older than younger males (18%) and females (16%), to a similar extent in both sexes.

The regulation of energy intake may be diminished in the elderly. Older subjects have a reduced suppression of energy intake after oral (46), or small intestinal nutrient (194), ingestion. In this meta-analysis in the subgroup of 203 subjects [6 studies (42, 46, 48, 194, 201, 214)], in which energy intake was measured during a single *ad libitum* buffet-style meal at the research facility both after overnight fasting and in the postprandial state, energy intake decreased on average 11% less in the older than young adults. Older people do not show the ability to regulate food intake after prolonged over- or under-feeding as young individuals (56, 63). This indicates that after an anorectic insult (for example, major surgery), older people are likely to take longer than young adults to regain the weight lost, remain undernourished longer, and be more susceptible to subsequent superimposed illnesses, such as infections.

Our results indicate a reduction in hunger of approximately 25% and increase in fullness of approximately 35% between the ages of 27 and 74 years, i.e. changes of about 0.5% per year for hunger and about 0.7% per year for fullness respectively. Scores for appetite are predictive of energy intake in both healthy young and older subjects (47). Appetite and energy intake are dependent on the precise co-ordination of interrelated intragastric (i.e., gastric emptying (68), antral area and motility (the distal stomach) (42, 68), and plasma ghrelin concentrations (48, 113, 234, 235) and 'small intestinal' mechanisms [pyloric motility (236) and gut hormone secretion including cholecystokinin (CCK) (235), glucagon-like polypeptide-1 (GLP-1) (237), peptide tyrosine tyrosine (PYY) and gastric inhibitory polypeptide (GIP)]. These gastrointestinal mechanisms affecting appetite and energy intake are modulated by ageing (238). Healthy older people, as a group, have slightly slower gastric emptying (50) mediated by increased pyloric motility (42, 45, 48, 53), greater gastric antral area (42), decreased perception of gastric distension (84), lower plasma ghrelin (92) and higher CCK concentrations than young adults, differences that all favour reductions in appetite and energy intake. Ageing is associated with insulin resistance and impaired glucose

tolerance (239) which may be influenced by changes in small intestinal hormone (GLP-1, GIP) secretion (133). Also, thyroid hormone concentrations are known to change with ageing which may be regarded as a physiologic process that can affect appetite (240, 241). There may be a decrease in appetite-stimulating free thyroid hormones with increasing age in men (242). Serum thyroid stimulating hormone (TSH) concentrations may be higher and free thyroid hormones lower in older, when compared to younger, men (243). Older people have an increased prevalence of both hypo- (up to 5%) and hyperthyroidism (0.5-3%) than younger patients. In the elderly the symptoms of both conditions can overlap with other age-related diseases (e.g., unexplained, weight loss, anorexia, weakness, fatigue, depression, constipation) (243). The senses of smell and taste deteriorate with age (244), leading to a reduced capacity to enjoy food and develop sensory-specific satiety, (198) the normal decline in pleasantness of the taste of a particular food after it has been consumed, leading to a decrease in its consumption and a tendency to shift consumption to other food choices during a meal. Age-related reduction in sensory-specific satiety favours a less varied, more monotonous diet, and the development of micronutrient deficiencies.

Physiological anorexia and seemingly minor weight loss predisposes to the development of pathological under-nutrition, cachexia and adverse effects (245) and is, accordingly, associated with increased morbidity and mortality. For example, in a large study of community-dwelling Americans aged 65 years or older, weight loss in excess of 5% body weight over 3 years occurred in 17% and was associated with a 70% increase in mortality, irrespective of the initial weight, whereas weight stability and weight gain were not associated with increased mortality (246). Not uncommonly, pathological anorexia and weight loss are superimposed on the 'physiological anorexia of ageing' (190). This can be the result of a variety of conditions that become more frequent with age, including acute and chronic medical conditions (gastrointestinal disease, malabsorption syndromes, infection, hypermetabolism, micronutrient deficiencies, increased energy requirements), medications

(which may cause malabsorption of nutrients, gastrointestinal symptoms, and loss of appetite), psychological factors (depression, dementia and Alzheimer's disease, and bereavement), social factors (poverty, difficulties with shopping, meal preparation and self-feeding, living alone, social isolation and loneliness) and physical factors (poor dentition leading to problems with chewing, immobility (stroke), Parkinson disease, and impaired vision). Because the majority of these factors are at least partly responsive to treatment, their recognition is important. For example, increased cytokine levels, due to the stress of ageing per se, or the amplified stressful effects of other pathologies, may provide an explanation for some of the decline in appetite and energy intake in older people (247). Increased cortisol and catecholamines stimulate the release of interleukin 6 and tumour necrosis factor alpha (248).

Although only a limited number of studies have examined the effects of undernutrition on appetite and energy intake, there is evidence of substantial differences between undernourished and well-nourished older people, which may potentially result from being undernourished and/or contribute to the undernourished state (41, 48, 90). Undernourished older adults had significantly reduced hunger in the fasted state and in the postprandial state' and significant greater fullness in the fasted state when compared to healthy older (90) and young adults (48, 90). In undernourished older women energy intake was not suppressed by a mixed-nutrient preload, unlike in well-nourished older and young women (48). In another study of undernourished older subjects, concentrations of CCK were higher than in well-nourished older subjects (249), suggesting that increased CCK activity may be a cause of undernutrition in older people, and/or act to perpetuate it.

Limitations of the meta-analysis are that we used a single database and that there is variability in study design and characteristics indicated by high heterogeneity - we performed a-priori determined meta-analyses depending on the method used to determine

energy intake and sensitivity analyses when possible, i.e. effect of sex and age, and observed that the effects of ageing on appetite and energy intake were comparable in these analyses.

In summary, this meta-analysis of 59 studies supports previous reports that appetite and energy intake are reduced in healthy older compared with younger adults, with a 16-20% lower energy intake, 25-39% lower hunger and 37% more fullness in those aged on average 70-74 years compared to 26-27 years, a robust finding regardless of the method of intake evaluation. These age-related differences in healthy adults are consistent with a reduction of food intake with ageing i.e., a physiological anorexia of ageing. The reduction in energy intake in this analysis equates to approximately 0.5% per year of increasing age, and is likely to contribute to loss of weight in older people and the development of pathological under-nutrition in predisposed older people.

CHAPTER 4: METHODOLOGIES

INTRODUCTION

This chapter provides an overview of the methodologies used in the data collection for the studies presented in **chapters 5-12** of this thesis. All of the methodologies are well established, including measurements of *ad libitum* energy intake using a standardised buffet meal (250, 251), perception of appetite and gastrointestinal symptoms by visual analogue scales (47), antropyloroduodenal motility by manometry (252), gastric emptying by 3-dimensional (3D) ultrasonography (68) and stable isotope breath testing (253, 254), blood glucose concentrations and plasma concentrations of gut hormones including insulin, glucagon, ghrelin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) by radioimmunoassay (251, 255) and amino acids by precolumn derivatization with 6-aminoquinolyl-N hydroxysuccmimidyl carbamate (256).

ETHICAL APPROVAL

All protocols for the studies described in this thesis were approved by the Royal Adelaide Hospital Human Research Ethics Committee. Each subject provided written, informed consent prior to their enrolment in the studies. All studies were conducted in accordance with the Declaration of Helsinki. Each study was registered on the Australia and New Zealand Clinical Trial Registry, and their registration numbers are provided in their respective chapters.

SUBJECTS

Subjects were recruited by advertisement on notice boards at the Royal Adelaide Hospital and the universities in Adelaide, through advertisement websites, and from an existing database of volunteers who had participated previously in research studies within our

department. Subject demographics and inclusion and exclusion criteria are provided in each chapter. All subjects were paid an honorarium of $18 per hour for their time spent at our clinical facilities.

ENERGY INTAKE

A buffet or cafeteria-style meal is commonly used to quantify *ad libitum* energy intake after preload ingestion (**Chapters 5, 7, 8 -10, 12**) (250, 251). A variety of food and drink items (**Table 4.1, Figure 4.1**) were presented to the subjects, in excess of what they were expected to consume. Subjects were instructed to consume food until they felt comfortably full in a room by themselves to limit external distractions (257).The amount of food eaten (g) was quantified by weighing each food item before and after consumption. Energy intake (kcal) and proportions of intake of protein, carbohydrate, and fat were calculated using commercially available software (Foodworks; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake (kcal) was calculated both as the intake at the meal and as the cumulative energy intake, defined as the sum of meal energy intake and preload/infusion energy content. Suppression of energy intake at the buffet meal by the preload/infusion was calculated as percentage (%) of energy intake of the control day.

Figure 4.1: The *ad libitum* meal as presented to the study subjects.

Table 4.1: Composition of the buffet meal

Food items	**Amount served (g)**	**Energy content (kcal)**	**Protein (g)**	**Carbohydrate (g)**	**Fat (g)**
Wholemeal bread, 4 slices[a]	125	308	13.4	53.1	4.7
White bread, 4 slices[a]	125	304	10.8	59.5	2.6
Cheese, sliced[b]	85	346	21.9	0.9	28.3
Ham, sliced[c]	100	95	16.6	3.4	1.7
Chicken, sliced[d]	100	104	18.8	3.6	1.6
Margarine[e]	20	108	0.0	0.0	12.0
Mayonnaise[f]	20	137	0.4	0.7	14.7
Tomato, sliced	100	13	1.0	1.9	0.1
Cucumber, sliced	100	11	0.5	1.9	0.1
Lettuce	100	5	0.9	0.4	0.0
Apple	170	89	0.5	21.3	0.2
Banana	190	166	3.2	37.8	0.2
Fruit salad[g]	140	81	0.4	17.1	1.3
Strawberry yogurt[h]	175	162	8.8	24.2	3.3
Chocolate custard[i]	100	105	3.2	16.4	3.0
Milkyway[j]	12	52	0.3	8.7	1.8
Orange juice, unsweetened[k]	300	117	1.8	21.9	2.6
Iced coffee[l]	375	254	12.0	37.1	6.4
Water	600	0	0.0	0.0	0.0
Total	**2937**	**2457**	**114.5**	**309.9**	**84.6**

[a]Sunblest, Tiptop, George Weston Foods Ltd, Enfield, NSW, Australia; [b]Coon Tasty Cheese slices, Australian Cooperative Foods Ltd, Sydney Olympic Park, NSW, Australia; [c]KRC boneless leg ham, George Weston Foods Ltd, Enfield, NSW, Australia; [d]Inghams chicken

breast, Inhams Enterprises Pty Ltd, Burton, SA, Australia; [e]Vita-Lite canola, Peerless Holdings Pty Ltd, Braybook, VIC, Australia; [f]MasterFoods, Mars Food Australia, Berkeley Vale, NSW, Australia; [g]Goulburn Valley, SPC, Ardmona Operations Ltd, Shepparton, VIC, Australia; [h]Yoplait, LD&D Foods Pty Ltd, Docklands, VIC, Australia; [i]Yogo, LD&D Foods Pty Ltd, Docklands, VIC, Australia; [j]Mars Chocolate Australia, Wendouree, VIC, Australia; [k]Nippy's Fruit juices Pty Ltd, Regency Park, SA, Australia ; [l]Farmers Union, LD&D Foods Pty Ltd, Docklands, VIC, Australia.

PERCEPTION OF APPETITE AND GASTROINTESTINAL SYMPTOMS

Validated visual analogue scales (**Chapters 5, 7, 8 -10, 12**) are the most commonly used tool to measure perceptions of appetite and gastrointestinal symptoms in research studies (47, 258). Hunger, fullness, desire to eat, prospective food consumption, nausea and bloating were measured by visual analogue scales at baseline and at 15 min intervals throughout the studies. The questionnaires consisted of 100 mm horizontal lines, where 0 represented that the sensation was 'not felt at all' and 100 represented that the sensation was 'felt the greatest'. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation felt at the specified time points and the distance from 0 mm was measured and recorded.

ANTROPYLORODUODENAL MOTILITY

Manometry (**Chapters 5 and 6**) is a well-established technique to measure motility in the antral area of the stomach, the pylorus and the proximal area of the duodenum (252, 259). Contractions are measured using a manometric catheter which is perfused with degassed water and saline through side holes. The water column of the catheter transmits the detected changes in pressures to external transducers, where they are digitalised and displayed as pressure waves on a computer screen.

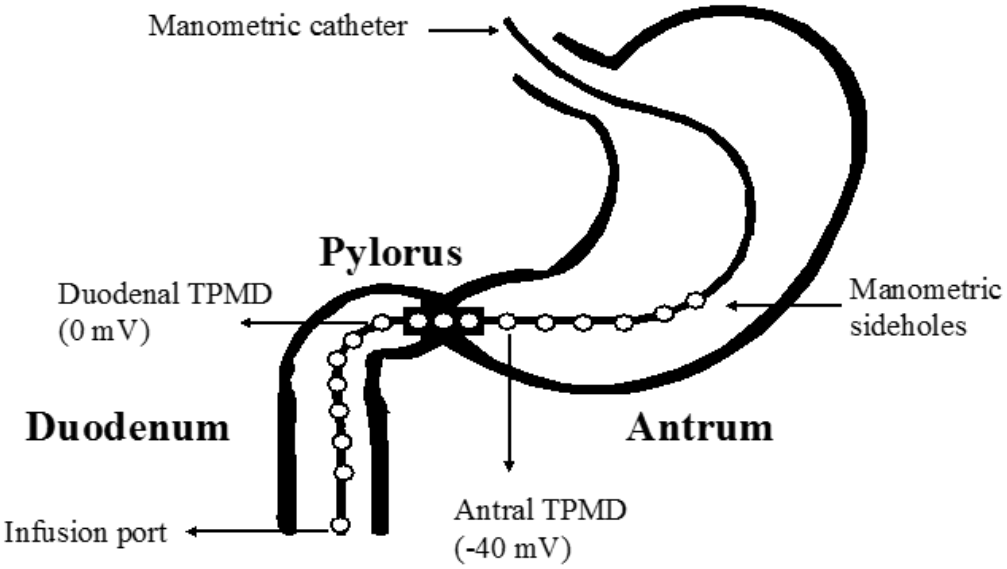

Figure 4.2: Schematic representation of the manometric catheter used for intraduodenal protein infusion, incorporating six antral and seven duodenal side holes, spaced 1.5 cm apart, a pyloric sleeve sensor and an infusion port.

The silicon rubber manometric catheter (Dentsleeve International, Mui Scientific, Mississauga, ON, Canada) has a total length of 1 m, a small-diameter of 3.5 mm and 16 side holes, each from a different channel, separated by 1.5 cm intervals. After correct positioning (**Figure 4.2**), the side holes of channels 1-6 are located in the antrum (stomach), a 4.5 cm sleeve sensor (channel 7) with channels 8 and 9 on the back of the sleeve are located across the pylorus, and channels 10-16 are located in the duodenum. The infusion port at the end of the catheter, an additional channel, is located in the proximal small intestine 14.5 cm from the pylorus and used for the administration of intraduodenal protein (114).

Intubation was done via an anaesthetised nostril (Lignocaine 5%, Orion Laboratories Pty Ltd, Calcatta, WA, Australia) into the stomach, after which the catheter passed into the duodenum through peristalsis (260). The length of intubation was dependent on the height of the subject, but was not longer than 75 cm. Correct positioning of the catheter, with the sleeve sensor straddling the pylorus, was maintained by continuous measurement of the transmucosal potential difference (TMPD) between the most distal antral channel (channel 6, ~-40 mV) and the most proximal duodenal channel (channel 10, ~0 mV) and a reference

electrode attached to an intravenous cannula filled with sterile saline positioned subcutaneously in the left forearm (261). All manometric channels were perfused with degassed, distilled water at a rate of 0.15 mL/min, except for the two transmucosal-potential-difference channels, which were perfused with degassed 0.9% saline (260). Baseline motility was recorded for 10 min after the occurrence of a phase III of the migrating motor complex (MMC), upon which the intraduodenal protein infusion was commenced. The set-up of the study day is pictured in **Figure 4.3**.

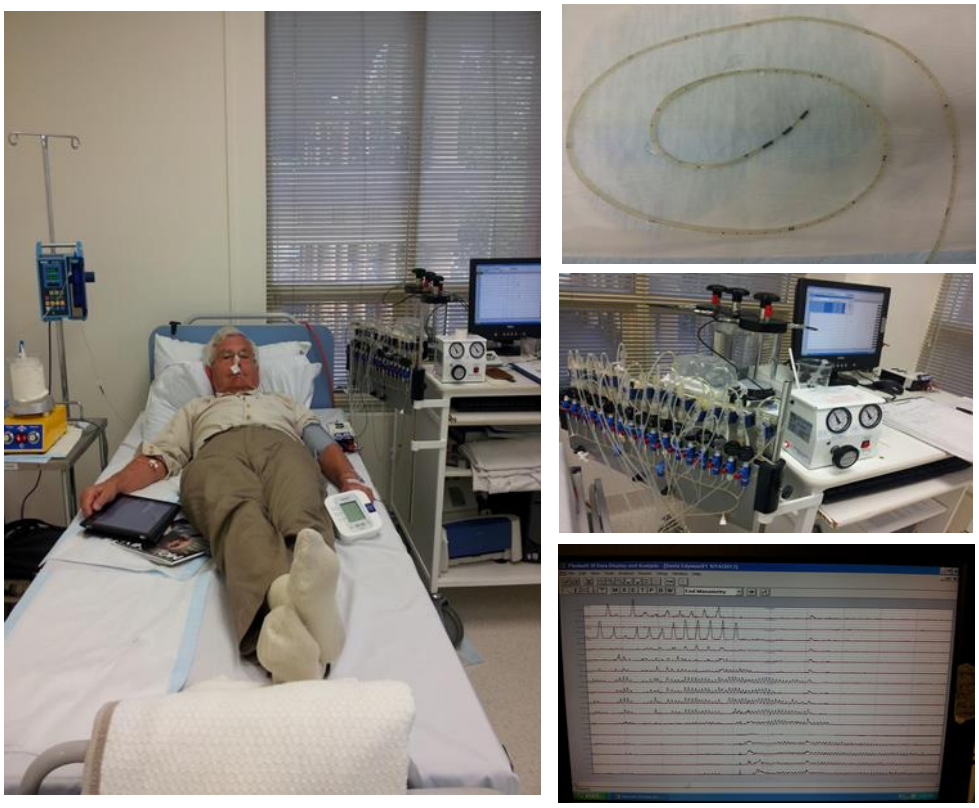

Figure 4.3: left: study set up with the infusion pump on the left and the manometry system on the right of the patient. Right top: the 3.5 mm silicone catheter; middle: a close up of the manometry system; bottom: a typical phase III of the migrating motor complex as displayed on the Flexisoft software.

Antropyloroduodenal pressure waves were recorded continuously and digitised using a computer-based system that ran commercially available software (Flexisoft version 3; Oakfield Instruments Ltd, Eynsham, England) and were stored for subsequent analysis. Data were analysed for basal pyloric pressures, the number and amplitude of isolated pyloric pressure waves (IPPWs) and the number and amplitude of antral and duodenal pressure

waves. Basal pyloric pressure was calculated by subtracting the mean basal pressure (with phasic pressures excluded) recorded at the most distal antral channel from the mean basal pressure recorded at the sleeve with custom-written software (A. Smout, University Medical Centre, Amsterdam, the Netherlands) modified to our requirements (252). Pressure waves were defined by an amplitude of 10 mm Hg or more with a minimum time interval of 15 s between peaks for IPPWs and antral pressure waves and 3 s for duodenal pressure waves (262). Baseline fasting values were calculated from 10 min before to the start of intraduodenal infusion as the mean of the study days.

GASTRIC EMPTYING

Gastric emptying (**Chapters 7, 9, 10 and 12**) was measured with a Logiq™ 9 ultrasonography system (GE Healthcare Technologies, Sydney, NSW, Australia) with TruScan Architecture (i.e. built-in magnetically sensed 3D positioning and orientation measurement) including a 3D sensor, attached to a 3.5C broad-spectrum 2.5-4-MHz convex transducer, and a transmitter placed at the level of the stomach immediately behind the subject (**Figure 4.4**).

3D ultrasonography has a good correlation with the 'gold standard' technique of scintigraphy ($r = 0.92$, $P < 0.001$) (68).

The stomach was scanned by a continuous translational movement along its long axis (~10 s). As the transmitter produces a spatially varying magnetic field that is distorted by conductive metals, all metal objects were removed from the subjects to minimise interference during image acquisition. During each scan, subjects were instructed to sit still and hold their breath at the end of inspiration. If gastric contractions were observed, the acquisition was paused until the contraction wave had passed. The raw data (original scan

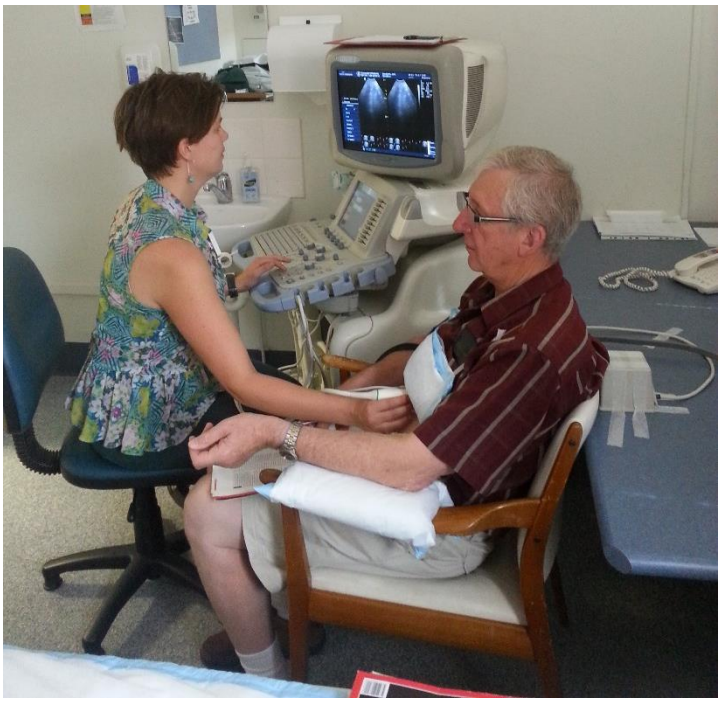

Figure 4.4: Set up of study subject and 3-D ultrasound machine.

planes) were transferred for 3D reconstructions and volume estimation using EchoPAC-3D software (GE Vingmed Sound, Horten, Norway; **Figure 4.5**). Gastric retentions were calculated as total gastric volumes minus baseline 'empty' gastric volume at each time point expressed as a percentage of the maximal gastric volume (100%), i.e., volume of the ingested drink. When ultrasound images lacked sufficient clarity to determine the volume of the stomach, data were imputed by linear interpolation. The time at which 50% of the preload drink had emptied from the stomach (50% gastric emptying time; T50; min) and 'complete' emptying time (T100; min) of the drink, defined as the time when the residual volume of the drink in the stomach was ≤ 5%, were calculated (263).

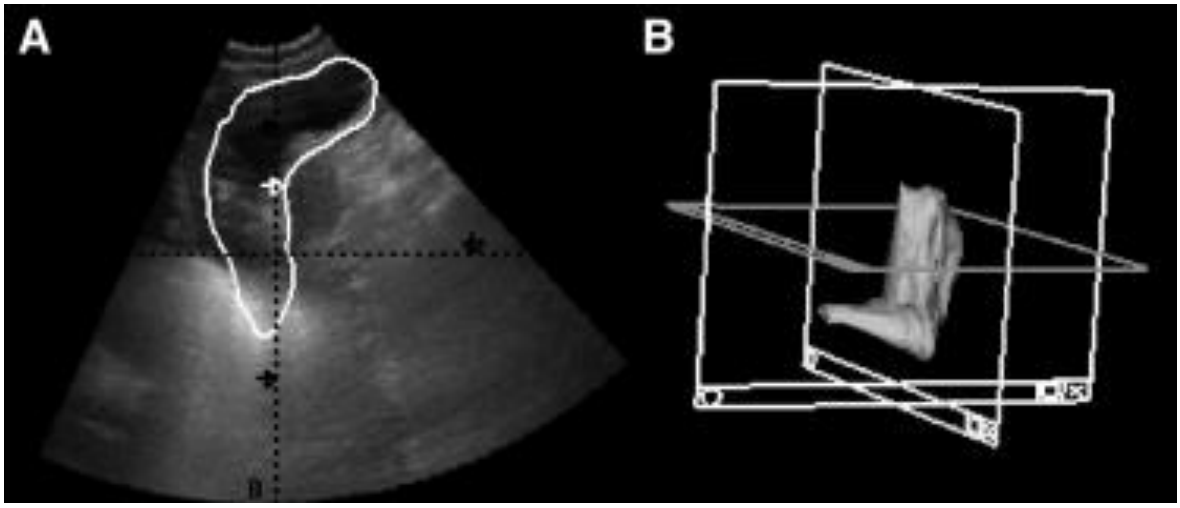

Figure 4.5: A) Ultrasonic image of the stomach with highlighted region of interested, and B) 3D reconstructed volumetric image of the stomach (68).

CONCENTRATIONS OF BLOOD GLUCOSE AND PLASMA INSULIN, GLUCAGON, GHRELIN, CCK, GIP, GLP-1, PYY AND AMINO ACIDS

For frequent blood sampling (**Chapters 6, 9-12**), an intravenous cannula was inserted into an antecubital vein. Venous blood samples were collected in EDTA-coated tubes. No inhibitors were added (264). Blood glucose (millimoles per liter) was determined immediately after collection by the glucose oxidase method using a portable glucometer (Optium Xceed, Abbott Laboratories, Australia).

Plasma was obtained by centrifugation for 15 min at 3200 rpm at 4°C and samples were stored at -80°C for further analysis of hormone concentrations.

Total plasma insulin (milliunits per liter) was measured by enzyme-linked immunosorbent assay (ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden).

Radioimmunoassays were used to measure plasma concentrations of total glucagon (detection limit of 20 pg/mL), total ghrelin (detection limit of 40 pg/mL), CCK-8 (detection limit of 1 pmol/L), total GIP (detection limit of 2 pmol/L), total GLP-1 (detection limit of 3 pmol/L) and total PYY (detection limit of 1.5 pmol/L). Samples from individual subjects

were measured in the same run. Intra- and inter-assay coefficients of variation are provided in **chapters 6 and 9-12**.

Plasma concentrations (mmol/L) of free amino acids asparagine, aspartic acid, alanine, arginine, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, were measured (**Chapter 6**) using precolumn derivatization with 6-aminoquinolyl-N hydroxysuccmimidyl carbamate (AQC) performed at the Australian Proteome Analysis's Facility established under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS).

CHAPTER 5: EFFECTS OF INTRADUODENAL PROTEIN ON APPETITE, ENERGY INTAKE, AND ANTROPYLORODUODENAL MOTILITY IN HEALTHY OLDER COMPARED WITH YOUNG MEN IN A RANDOMISED TRIAL

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Candidate contribution

Candidate	Caroline Giezenaar		
Contribution	Data interpretation, statistical analysis and drafting of the manuscript.		
Overall percentage	50%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am second author on the paper.		
Signature		Date	October 2017

Co-Author Contributions

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

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

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

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

Name of Co-Author	Stijn Soenen
Contribution	Conception and design of the study, data collection and

	interpretation, statistical analysis, drafting of the manuscript and overall responsibility for the study.		
Signature		Date	October 2017
Name of Co-Author	Amy T Hutchison		
Contribution	Data collection and interpretation, and drafting of the manuscript.		
Signature		Date	October 2017
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Contribution	Conception and design of the study, data interpretation and drafting of the manuscript.		
Signature		Date	October 2017
Name of Co-Author	Ian Chapman		
Contribution	Conception and design of the study, data interpretation and drafting of the manuscript.		
Signature		Date	October 2017
Name of Co-Author	Natalie Luscombe-Marsh		
Contribution	Conception and design of the study, data interpretation and drafting of the manuscript.		
Signature		Date	October 2017

ABSTRACT

Background: Protein-rich supplements are used widely for the prevention and management of undernutrition in older people. The use of protein supplements in older people could, however, be counter-productive, by reducing appetite and overall energy intake.

Objective: To determine whether ageing influences the effects of protein loads, administered directly into the small intestine, on energy intake, antropyloroduodenal motility and appetite.

Design: Intraduodenal infusions (240 ml, 60 min) of saline (0 kcal/control) and (hydrolysed whey) protein loads of 30 kcal, 90 kcal and 180 kcal followed by *ad libitum* buffet meal in 10 young (19-29 years) and 10 healthy older (68-81 years) men. Suppression of energy intake (kcal) at the meal by protein infusion compared to control was calculated.

Results: In young subjects, there was dose-responsive suppression of energy intake at the buffet meal by protein (suppression at 30 kcal 7 ± 8% $P = 0.19$; 90 kcal 17 ± 8% $P = 0.05$; 180 kcal 33 ± 7% $P = 0.002$), whereas in older subjects there was suppression only after the 180 kcal load (30 kcal 7 ± 4% increase $P = 0.13$; 90 kcal 6 ± 7% increase $P = 0.29$; 180 kcal 17±6% suppression $P = 0.016$). Suppression of energy intake by protein was less in older than young subjects ($P < 0.005$). In young subjects total energy intake (meal + infusion) on the 180 kcal protein-infusion day was decreased compared to the control day ($P = 0.041$), whereas in older it was increased during the 30 kcal ($P = 0.033$) and 90 kcal ($P = 0.016$) days.

Energy intake was inversely related to isolated pyloric pressure waves ($r = -0.32$ $P = 0.013$) and positively related to antral ($r = 0.30$ $P = 0.021$) and duodenal ($r = 0.35$ $P = 0.006$) pressure waves. Suppression of energy intake by protein was inversely related to the change in isolated pyloric pressure waves ($r = -0.35$ $P = 0.027$) and positively related to duodenal pressure waves ($r = 0.32$ $P = 0.044$).

Conclusions: Intraduodenal protein suppresses appetite and energy intake less in healthy older than young adults. In older subjects intraduodenal protein at low doses increased overall energy intake, supporting the use of protein supplements in undernourished older people.

INTRODUCTION

As for young people, the number of older people who are overweight or obese increased substantially over recent decades (265). However, healthy ageing is associated with a reduction of appetite and energy, including protein, intake; the 'anorexia of ageing' (6, 7). These changes predispose older people to weight loss, particularly loss of skeletal muscle, and reduced functional capacity, and the development of pathological undernutrition, sarcopenia cachexia and frailty (6, 14, 180, 190, 266). The causes of the 'anorexia of ageing' are poorly defined, but likely to be multiple. Potential mechanisms include central and/or peripheral reductions in feeding drives and increased activity of central and/or peripheral satiety signals (267). Peripheral mechanisms, notably those related to the gastrointestinal tract, are important in regulating appetite and energy intake, particularly in the short-term after nutrient ingestion. They include interrelated 'intragastric' mechanisms, such as variations in the rate of gastric emptying and gastric distension (42, 50, 70, 74), and 'small intestinal' mechanisms, such as changes in antropyloroduodenal motility and the release of appetite-regulating hormones (70, 112-114, 234, 268). Changes in antropyloric motility in response to nutrient ingestion are independently related to subsequent energy intake in young subjects (269), and may therefore have a causative role. Compared to young adults, older people have a reduced perception of proximal gastric distension, and greater distension of the distal stomach, i.e. antral area, and slightly slower gastric emptying (42, 50, 74, 84), differences that would favour reductions in energy intake.

A common strategy to increase energy intake and body weight in undernourished older people is the use of protein-enriched supplements, usually high-energy drinks rich in carbohydrates and whey protein (a major protein source in dairy). Despite the widespread use of such supplements by older people, evidence for their efficacy is limited, and their 'optimal' composition unknown (26, 27, 31). The high satiating effects of dietary protein in

younger adults have been extensively studied, driven primarily by attempts by overweight younger adults to lose weight (270). The effects of dietary protein on energy intake and underlying gastrointestinal mechanisms in older people are, however, largely unknown, which is surprising given the potential for protein-enriched supplements given to older people - to increase body weight and muscle mass - could reduce subsequent energy intake, which could counteract any associated protein-induced beneficial effects on muscle mass (20, 271, 272).

In this study we aimed to characterise the effect of ageing on powerful 'subgastric' small intestinal mechanisms, by infusing hydrolysed (resembling partially digested protein) whey protein directly into the duodenum, and so 'bypassing' orosensory and gastric factors. We hypothesised that small intestinal administration of protein at loads lower than (0.5 kcal/min), similar to (1.5 kcal/min) and at the upper end (3 kcal/min) of normal gastric emptying rates [1-4 kcal/min (273)] would reduce energy intake, antropyloroduodenal motility, and perceptions of appetite in a load-related fashion and, that the acute suppression of energy intake at a buffet meal would be less in healthy older persons than in young adults.

SUBJECTS AND METHODS

Subjects

The study included 10 healthy young men [age (mean ± SEM): 23 ± 1 years (range 19-29 years), body weight: 73 ± 2 kg (62-87 kg), height: 1.82 ± 0.02 m, BMI: 22 ± 1 kg/m^2] and 10 healthy older men [age: 74 ± 1 years (68-81 years), body weight: 79 ± 2 kg (66-92 kg), height: 1.74 ± 0.02 m, BMI: 26 ± 1 kg/m^2]. Body weight of the two groups did not differ significantly. The older subjects had a lower height and, accordingly, higher BMI than young subjects ($P < 0.05$). Subjects were recruited by advertisement. Based on our previous work (114) we calculated that 10 subjects per group would allow us to detect a minimum

suppression in energy intake after the higher protein preload (180 kcal over 60 min), compared to the control, infusion of 397 kcal, with $\alpha = 0.05$ and power of 80%. Exclusion criteria were smoking, alcohol abuse, diabetes, gastrointestinal surgery (apart from uncomplicated appendectomy), significant gastrointestinal symptoms (pain, reflux, diarrhoea, or constipation) or use of medications known to potentially affect energy intake, appetite or gastrointestinal motor function, and for older people: impaired cognitive function [score < 25 on Mini Mental State (274)], depression [score > 11 on the Geriatric Depression Questionnaire (275)] and undernutrition [score < 24 on the Mini Nutritional Assessment (276)]. The Royal Adelaide Hospital Research Ethics Committee approved the study protocol, and the study was registered as a clinical trial with the Australia and New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number 12612000906853). All subjects provided written informed consent prior to their inclusion in the study.

Protocol

Subjects were studied on 4 occasions, separated by at least 3 days, to determine the effects of three intraduodenal protein loads and a saline control, each infused for 60 min, on energy intake, antropyloroduodenal motility, perceptions of appetite and gastrointestinal symptoms in a randomised (by using the method of randomly permuted blocks; www.randomization.com), double-blind, crossover design.

Protein solutions were prepared by dissolving whey protein hydrolysate powder (18.1% Hydrolysed Whey Protein 821, Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in varying amounts of saline and water to achieve the desired loads; i.e. 0.5, 1.5 and 3 kcal/min, which equated to 30, 90 and 180 kcal or 8, 24 and 48 g of protein or 0.11 ± 0.01 (range 0.09 – 0.13), 0.32 ± 0.03 (0.26 – 0.39) and 0.63 ± 0.06 (0.52 – 0.78) g of protein per kg body weight, and to ensure they were iso-osmotic (680 mOsmol/L). Infusions were

prepared on the morning of each study by a research officer who was not involved in the data analysis. The infusion apparatus was covered at all times, so both the investigator and the subject were blinded to the treatment. The infusions were administered at a rate of 4 mL/min (240 mL over 60 min).

Subjects were provided with a standardised evening meal [beef lasagne (McCain Foods Pty Ltd, Wendouree, VIC, Australia), ~591 kcal] to consume on the night before each study, and instructed to fast overnight from solids and liquids and to refrain from strenuous physical activity until they attended the laboratory at the University of Adelaide Discipline of Medicine, Royal Adelaide Hospital, at ~08.30 h. On arrival, a small-diameter (3.5 mm) 16-channel (side holes spaced at 1.5 cm intervals with channels 1-6: in the antrum, channel 7: a 4.5 cm sleeve sensor - including channels 8 and 9 on the back of the sleeve - across the pylorus, channels 10-16: in the duodenum) manometric catheter (total length: 100 cm, Dentsleeve International, Mui Scientific, Mississauga, ON, Canada) was inserted into the stomach through an anesthetised nostril and allowed to pass into the duodenum by peristalsis. The correct positioning of the catheter, with the sleeve sensor straddling the pylorus, was maintained by continuous measurement of the transmucosal potential difference between the most distal antral channel (channel 6, ~-40 mV) and the most proximal duodenal channel (channel 10, ~0 mV), and a reference electrode attached to an intravenous cannula filled with sterile saline positioned subcutaneously in the left forearm. The infusion port of the catheter was located in the proximal small intestine 14.5 cm from the pylorus. All manometric channels were perfused with degassed, distilled water, except for the two transmucosal-potential-difference channels, which were perfused with degassed 0.9% saline, at a rate of 0.15 mL/min.

Once the catheter was positioned, fasting motility was observed until phase III of the interdigestive migrating motor complex occurred. Immediately after cessation of phase III activity, during motor quiescence (phase I of the migrating motor complex), a visual

analogue scale (VAS) questionnaire to assess perceptions of appetite and gastrointestinal symptoms was completed and baseline antropyloroduodenal motility was measured for 15 min upon which the intraduodenal infusion commenced. During the infusion, antropyloroduodenal motility was measured continuously, and VAS ratings were obtained at 15 min intervals. After 60 min the infusion was terminated and both the intraduodenal catheter and subcutaneous cannula were removed. Subjects were then presented with a standard, cold, buffet-style meal in excess of what they were expected to consume and instructed to eat freely for up to 30 min until comfortably full. The composition of the buffet meal is presented in **Table 4.1**. Immediately after completion of the meal (t = 90 min), final VAS was completed and the subject was allowed to leave the laboratory.

Measurements

Energy intake

The amount eaten (g) was quantified by weighing the buffet meal before and after consumption. Energy intake (kcal) at the buffet meal and proportions of intake of protein, carbohydrate and fat were calculated using commercially available software (Foodworks; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake was calculated both as the intake at the buffet meal, and as total energy intake, defined as the sum of energy intake at the buffet meal and energy content of the intraduodenal infusion. Absolute and percentage suppression of energy intake (kcal) at the buffet meal by a given protein infusion compared to control was calculated.

Perceptions of appetite and gastrointestinal function

Perceptions of hunger, desire to eat, prospective consumption, and fullness, as well as nausea and bloating, were rated by using validated VAS questionnaires. These questionnaires consisted of 100 mm horizontal lines, where 0 represented that the sensation was ‘not felt at

all' and 100 represented that the sensation was 'felt the greatest'. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation felt at the specified time points. Baseline fasting ratings were calculated as mean of the four study days. Protein overall ratings were calculated as mean of the three protein-infusion study days.

Antropyloroduodenal motility

Antropyloroduodenal pressure waves were recorded continuously and digitised by using a computer-based system that ran commercially available software (Flexisoft v3; Oakfield Instruments, GS Hebbard) and stored for subsequent analysis. Data were analysed for basal pyloric pressures (BPPs) and number and amplitude of isolated pyloric pressure waves (IPPWs) and antral and duodenal pressure waves. BPP was calculated by subtracting the mean basal pressure (with phasic pressures excluded) recorded at the most distal antral channel from the mean basal pressure recorded at the sleeve with custom-written software modified to our requirements (272). Pressure waves were defined by an amplitude > 10 mmHg with a minimum time interval of 15 sec between peaks for IPPWS and antral pressure waves and 3 sec for duodenal pressure waves. Baseline fasting values were calculated from 10 min before to start of intraduodenal infusion as mean of the four study days.

Data analysis

Statistical analyses were performed using SPSS software (version 21, IBM). Between-subject effects were determined by using ANOVA. Within-subject and interaction effects were determined by using repeated-measures ANOVA. Post-hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when ANOVAs revealed significant effects. Relations of energy intake with AUCs (which were calculated by using the trapezoidal rule) for antropyloroduodenal pressures and appetite were evaluated

by between- and within-subject correlations (277, 278). Statistical significance was accepted at $P < 0.05$. All data are presented as mean ± SEM.

RESULTS

The study protocol was well tolerated by all subjects.

Energy and macronutrient intakes

Energy intake at the buffet meal

Energy intake at the buffet meal after the intraduodenal infusions of saline (0 kcal, control) and protein loads of 30, 90 and 180 kcal was: 1270 ± 150 kcal, 1123 ± 151 kcal, 1028 ± 163 kcal and 851 ± 161 kcal in young subjects and; 1068 ± 93 kcal, 1129 ± 91 kcal, 1123 ± 98 kcal and 899 ± 103 kcal in older subjects. The lower (~16%) energy intake at the buffet meal during the control day in older compared with young subjects was not statistically significant ($P = 0.27$). The interaction effect of age x protein-load for energy intake at the buffet meal was significant ($P = 0.039$). Energy intake was dose-responsively suppressed by protein in the young subjects (suppression at 30 kcal 7 ± 8% $P = 0.19$; 90 kcal 17 ± 8% $P = 0.05$; 180 kcal 33 ± 8% $P = 0.002$, **Figure 5.1**); whereas there was suppression in the older subjects only with the 180 kcal infusion (30 kcal 7 ± 4% increase in intake $P = 0.13$; 90 kcal 6 ± 7% increase in intake $P = 0.29$; 180 kcal 17 ± 6% suppression $P = 0.016$). Suppression of energy intake at the buffet meal by the protein loads was less in older than young subjects ($P < 0.05$, **Figure 5.2**).

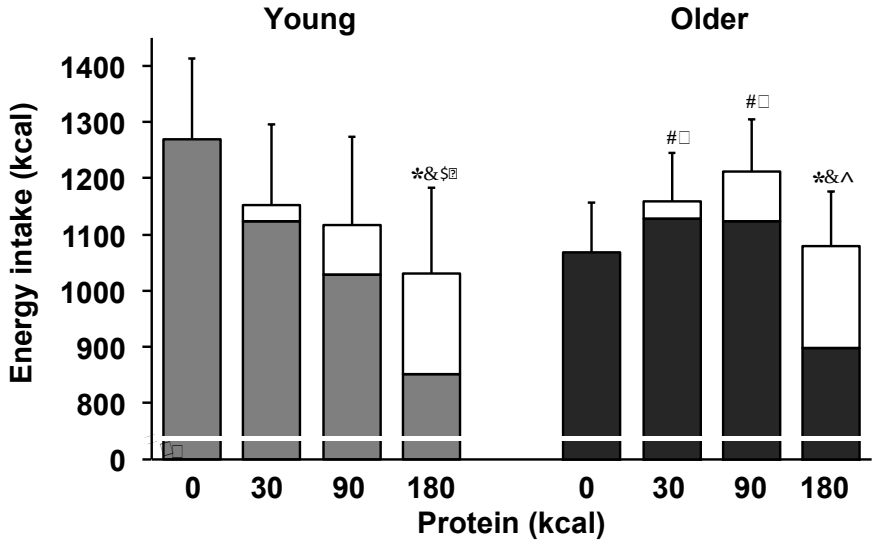

Figure 5.1: Data are means ± SEMs. Energy intake (kcal) in young ■ (n = 10) and older ■ (n = 10) subjects after intraduodenal infusions □ of saline (0 kcal, control) and whey protein loads of 30, 90 and 180 kcal for 60 min at 4 mL/min. In young subjects there was a dose-responsive suppression of energy intake at the buffet meal by protein compared to control, whereas in older subjects there was suppression only with the 180 kcal infusion. Between-subject effects were determined by using ANOVA. Within-subject and interaction effects were determined by using repeated-measures ANOVA.
There was a significant age x protein-load interaction for energy intake at a buffet meal ($P = 0.039$) and total energy intake (buffet meal + infusion) ($P = 0.039$).
* $P < 0.05$ Within age-group, lower energy intake at a buffet meal after protein-load infusion of 180 kcal compared to control in young and in older subjects.
$^{\&}$ $P < 0.05$ Within age-group, lower energy intake at a buffet meal after protein infusion of 180 kcal compared to 30 kcal load in young and in older subjects.
$^{\wedge}$ $P < 0.05$ Within age-group, lower energy intake at a buffet meal after protein infusion of 180 kcal compared to 90 kcal load in older subjects.
$^{\$}$ $P < 0.05$ Within age-group, lower total energy intake (energy intake at the buffet meal plus energy content of the infusion) during 180 kcal protein-load infusion day compared to control day in young subjects.
# $P < 0.05$ Within age-group, higher total energy intake during 30 and 90 kcal protein-load infusion day compared to control day in older subjects.

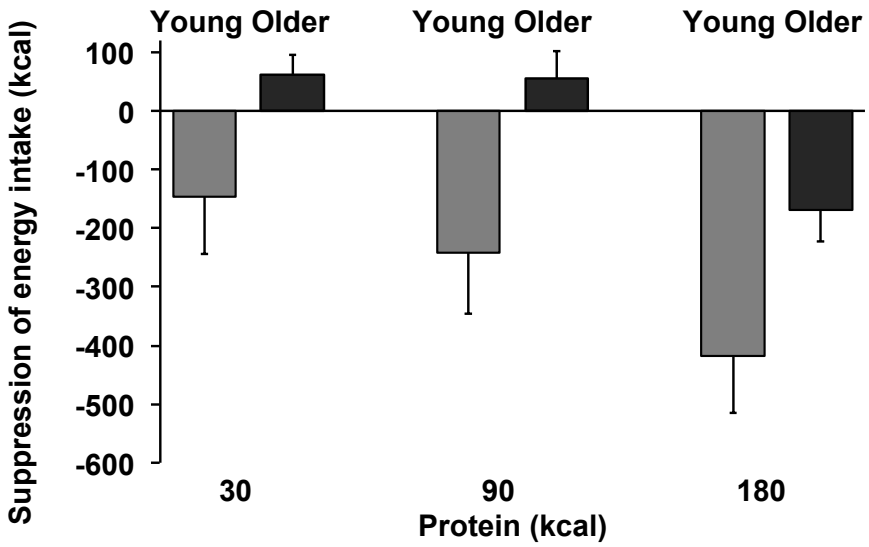

Figure 5.2: Data are means ± SEMs. Suppression of energy intake (kcal) at the buffet meal in young ▭ (n = 10) and older ■ (n = 10) subjects after intraduodenal infusions of whey protein loads of 30, 90 and 180 kcal compared to after intraduodenal infusion of saline (0 kcal, control) for 60 min at 4 mL/min. Suppression of energy intake at the buffet meal by protein was less in older than young subjects. Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA. The interaction age x protein-load was not significant ($P = 0.57$). Main effects of age ($P < 0.05$) and protein-load ($P < 0.001$) were significant.

Macronutrient intake

Proportions of intake of protein, carbohydrate and fat at the buffet meal during the control day were not significantly different (P > 0.05) between young and older subjects (**Table 5.1**). The young subjects increased their proportion of carbohydrates and decreased their proportion of fat intake after the intraduodenal protein infusions reaching level of significance after the 180 kcal load when compared to control ($P = 0.006$ and $P = 0.036$). The interaction effect of age x protein-load for proportions of intake of protein ($P = 0.23$), carbohydrate ($P = 0.07$) and fat ($P = 0.07$) was not significant.

Total energy intake

In young subjects total energy intake (i.e. energy intake at the buffet meal plus energy content of the infusion) on the 30 kcal (1153 ± 151 kcal, ↓~9% $P = 0.29$) and 90 kcal (1118

Table 5.1: Proportions of intake of protein, carbohydrate and fat at a buffet meal after intraduodenal protein infusions in young and older men

	Young men (n = 10)				Older men (n = 10)			
	0 kcal	30 kcal	90 kcal	180 kcal	0 kcal	30 kcal	90 kcal	180 kcal
Protein (%)	20±1	21±1	20±1	18±1	21±1	20±1	20±1	20±1
Carbohydrate (%)	44±3	47±2	49±3	53±3[1]	49±3	49±3	49±3	48±3
Fat (%)	36±4	32±2	31±2	29±3[1]	30±2	31±2	31±2	32±2

[1] $P < 0.05$ Significantly different intake of carbohydrate and fat compared to control in young subjects.
Intake of protein was not significantly different between protein loads ($P > 0.05$).
Proportions of intake of protein, carbohydrate and fat at the buffet meal during the control day were not significantly different between age groups ($P > 0.05$).
The interaction effect of age x protein-load for proportions of intake of protein ($P = 0.23$), carbohydrate ($P = 0.07$) and fat ($P = 0.07$) was not significant.

± 163 kcal, ↓~12% $P = 0.20$) protein-infusion days was non-significantly less than, total energy intake on the control day (1270 ± 150 kcal), and significantly less on the 180 kcal day (1031 ± 153 kcal, ↓~19% $P = 0.041$, **Figure 5.1**). In contrast, in older subjects total energy intake was significantly increased on the 30 kcal, (1159 ± 91 kcal, ↑~9% $P = 0.033$) and 90 kcal (1213 ± 98 kcal, ↑~14%, $P = 0.016$) protein-infusion days when compared to the control day (1068 ± 88 kcal) and not significantly different on the 180 kcal day compared to the control day (1079 ± 103 kcal, ↑~1%, $P = 0.86$). The interaction effect of age x protein-load for total energy intake was significant ($P = 0.039$).

Antropyloroduodenal motility

Baseline isolated pyloric pressure wave (IPPW) number (young vs. old: 2 ± 1vs. 6 ± 1 per 60 min, $P = 0.024$) and amplitude (young vs. old: 7 ± 2 vs. 21 ± 3 mmHg, $P = 0.001$) were higher in older than young subjects, while fasting basal pyloric pressures and, number and

amplitude of IPPWs and antral and duodenal pressure waves were not significantly different between study days in both age groups ($P > 0.05$).

In young and older subjects IPPW number was increased and antral and duodenal pressure wave number was decreased by the 180 kcal protein load compared to control ($P < 0.05$). Basal pyloric pressures of the protein loads were not different from control in both age groups ($P > 0.05$). Antral pressure wave amplitude was higher during the 30 kcal infusion in older than young subjects ($P < 0.05$, **Table 5.2**). Duodenal pressure wave amplitude was lower during the 180 kcal infusion in older than young subjects ($P < 0.05$).

Perceptions of appetite and gastrointestinal symptoms

Appetite

Baseline ratings of hunger (young vs. old: 67 ± 5mm vs. 45 ± 9mm $P = 0.042$), desire to eat (young vs. old: 69 ± 4mm vs. 37 ± 8mm $P < 0.002$) and prospective food consumption (young vs. old: 70 ± 4mm vs. 47 ± 6mm $P = 0.005$) were lower in older than young subjects; fullness was not significantly different between the age groups (young vs. old: 11 ± 3mm vs. 6 ± 2mm, $P = 0.20$). Ratings of hunger, desire to eat, prospective food consumption and fullness were not different from baseline during the infusions (t = 0-60 min) in both age groups ($P > 0.05$), with the exception of ratings of prospective food consumption ($P = 0.020$) being decreased by the 30 kcal infusion in young subjects (**Figure 5.3**).

The age x protein-load interaction for hunger ($P = 0.38$), desire to eat ($P = 0.51$), prospective food consumption ($P = 0.07$) and fullness ($P = 0.91$) was not significant. Ratings of hunger, desire to eat and fullness during the intraduodenal infusions were not significantly different between age groups or protein loads ($P > 0.05$).

Ratings of prospective food consumption (change AUC from baseline to 60 min) of the 30 kcal protein load (372 ± 192mm vs. 19 ± 189mm $P = 0.017$) were significantly higher than

Table 5.2: Number and amplitude of IPPWs and antral and duodenal pressure waves and basal pyloric pressures during 60 min intraduodenal protein infusions in young and older men

	Young men (n = 10)				Older men (n = 10)				
Protein	0 kcal	30 kcal	90 kcal	180 kcal	0 kcal	30 kcal	90 kcal	180 kcal	*P*[1]
IPPWs									
Number/60 min	41±10	42±9	65±12	76±12[2]	44±11	50±18	75±8	85±14[2]	0.980
Amplitude (mmHg)	30±5	32±4	38±5	39±3	34±7	38±6	38±4	47±8	0.884
BPP (mmHg)	1±2	1±2	-1±2	3±2	1±5	1±2	-1±1	-1±1	0.640
Antral pressure waves									
Number/60 min	93±17	102±41	67±21	35±15[2]	73±26	112±40	18±6	17±6[2]	0.604
Amplitude	11±8	12±7[5]	8±3	0±6	28±6	76±16[2,5]	28±12[3]	11±2[2,3]	0.003
Duodenal pressure waves									
Number/60 min	641±81	565±125	461±62	380±75[2]	467±85	546±72	376±72	223±46[2]	0.708
Amplitude (mmHg)	25±2[5]	28±1	26±1	25±2[5]	38±6[5]	31±3	27±2	16±2[2,3,4,5]	0.005

Data are mean ± SEM. Intraduodenal infusions consisted of saline (0 kcal, control) and whey protein loads of 30, 90 and 180 kcal for 60 min at 4 mL/min. IPPWs, isolated pyloric pressure waves. BPP, basal pyloric pressure. Between-subject effects were determined by using ANOVA. Within-subject and interaction effects were determined by using repeated-measures ANOVA.
[1] *P* age x protein-load interaction.
[2] $P < 0.05$ Significantly different from control.
[3] $P < 0.05$ Significantly different from 30 kcal protein load.
[4] $P < 0.05$ Significantly different from 90 kcal protein load.
[5] $P < 0.05$ Significantly different between young men and older men.

control in older subjects; whereas ratings of prospective food consumption of the 30 kcal protein load were significantly lower than control in young subjects (-503 ± 238 mm vs. -59

± 201 mm $P = 0.007$). Furthermore, in the older subjects mean prospective food consumption (change AUC from baseline to 60 min of the three protein loads vs. control: 230 ± 215 mm vs. 19 ± 189 mm $P = 0.019$) was increased by the protein loads compared to control; whereas in the young mean prospective food consumption decreased by the protein loads compared to control (protein vs. control: -326 ± 213 mm vs. -59 ± 201mm $P = 0.020$).

Ratings of prospective food consumption (change AUC from baseline to 60 min) of the 30 kcal protein load (young vs. older -503 ± 238 mm vs. 372 ± 192 mm $P = 0.010$) were significantly higher in older when compared with young subjects. Change in mean ratings of prospective food consumption (AUC young vs. old: -267 ± 94 mm vs. 211 ± 74 mm $P = 0.001$) by the protein loads when compared with control was significantly less in older than young subjects.

Nausea and bloating

Ratings of nausea and bloating during the intraduodenal infusions were not significantly different between age groups or protein loads ($P > 0.05$, **Table 5.3**). The interaction effect of age x protein-load for ratings of nausea ($P = 0.65$) and bloating ($P = 0.33$) was not significant. Ratings of nausea and bloating were not different from baseline in both age groups ($P > 0.05$).

Table 5.3: Ratings (AUC) of nausea and bloating during 60 min intraduodenal protein infusions in young and older men

	Young men (n = 10)				Older men (n = 10)			
	0 kcal	30 kcal	90 kcal	180 kcal	0 kcal	30 kcal	90 kcal	180 kcal
Nausea (mm)	657±227	773±300	559±178	922±329	196±61	184±61	151±45	244±71
Bloating (mm)	626±187	803±203	726±203	546±214	457±168	470±166	786±292	641±317

Ratings of nausea ($P = 0.06$) and bloating ($P = 0.79$) were not significantly different between age groups.
Ratings of nausea ($P = 0.33$) and bloating ($P = 0.56$) were not significantly different between protein-loads.
The interaction effect of age x protein-load for ratings of nausea ($P = 0.65$) and bloating ($P = 0.33$) was not significant.

Relations between antropyloroduodenal motility and perceptions of appetite with energy intake

Energy intake at the buffet meal was, within-subjects, inversely related to IPPW number ($r = -0.32$ $P = 0.013$) and positively related to antral pressure wave number ($r = 0.30$ $P = 0.021$) and duodenal pressure wave number ($r = 0.35$ $P = 0.006$) and, between-subjects, positively related to ratings of desire to eat ($r = 0.47$ $P = 0.037$) and prospective food consumption ($r = 0.57$ $P = 0.008$).

Suppression of energy intake at the buffet meal by protein compared to control was, within-subjects, inversely related to the change in IPPW number ($r = -0.35$ $P = 0.027$) and positively related to change in duodenal pressure wave number ($r = 0.32$ $P = 0.044$) and amplitude ($r = 0.48$ $P = 0.002$) by protein from control.

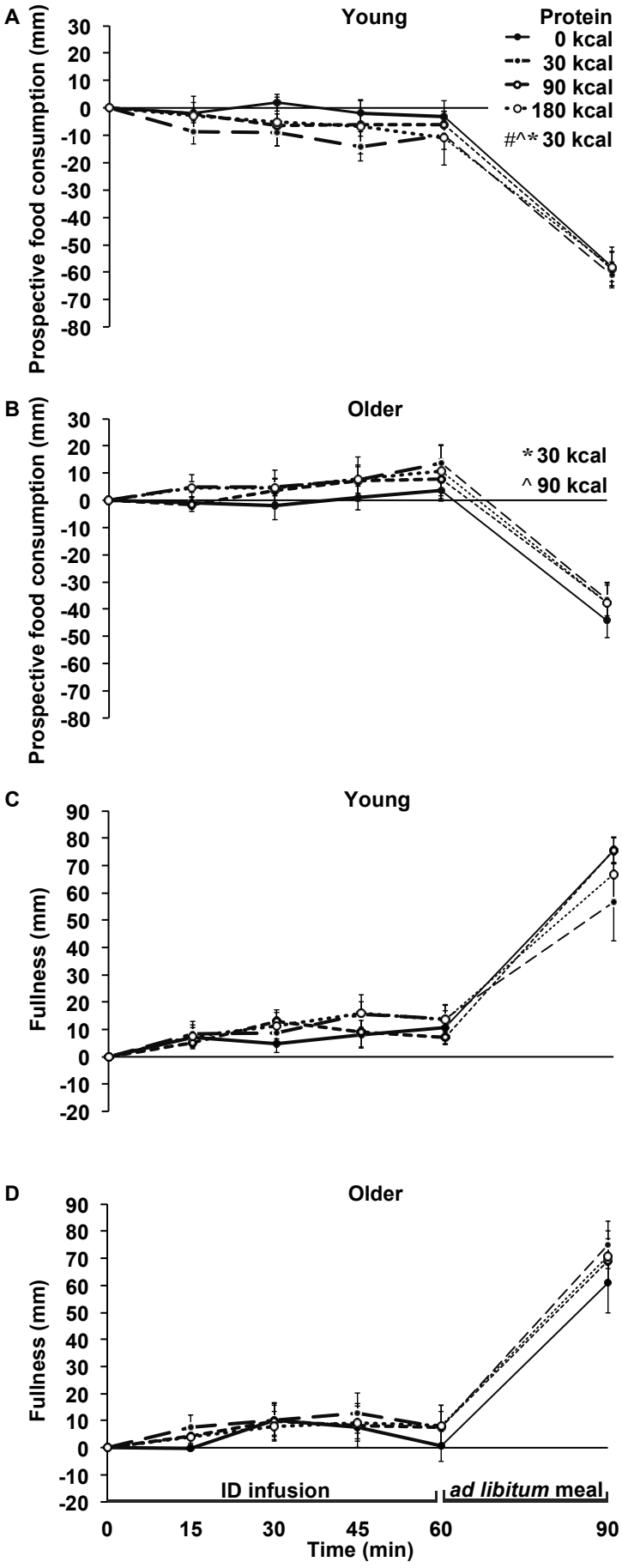
A
Young
Protein
0 kcal
30 kcal
90 kcal
180 kcal
#^* 30 kcal
Prospective food consumption (mm)
B
Older
* 30 kcal
^ 90 kcal
Prospective food consumption (mm)
C
Young
Fullness (mm)
D
Older
Fullness (mm)
ID infusion
ad libitum meal
Time (min)

Figure 5.3: Data are means ± SEMs. Change from fasting, baseline (t = 0 min) visual analogue scores (mm) of prospective food consumption (**A and B**) and fullness (**C and D**) in young (n = 10) and older (n = 10) subjects during intraduodenal infusions of saline (0 kcal, control) and whey protein loads of 30, 90 and 180 kcal for 60 min and after the *ad libitum* buffet meal (t = 90 min). Between-subject effects were determined by using ANOVA. Within-subject, main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.
The age x protein-load interaction for prospective food consumption ($P = 0.07$) and fullness ($P = 0.91$) was not significant.
Fullness was not significantly different between age groups ($P = 0.63$) or protein loads ($P = 0.42$).
# $P < 0.05$ Time effect (t = 0-60 min) for prospective food consumption.
^ $P < 0.05$ Protein-load effect compared to control for prospective food consumption.
* $P < 0.05$ Age effect (AUC t = 0-60 min) for prospective food consumption.

DISCUSSION

This study examined the influence of ageing on the effects of intraduodenal protein administration on appetite and subsequent *ad libitum* energy intake. The protein infusion rates (0.5, 1.5 and 3 kcal/min) were lower than, similar to, and at the upper end of normal gastric emptying rates, 1-4 kcal/min (273). Consistent with previous studies, older subjects were less hungry at baseline and ate less, 201 kcal or ~16%, on the control day than younger subjects (6). The major finding of our study was that protein-induced suppression of energy intake was significantly less in older than young subjects. In particular, while total energy intake (at buffet meal plus infusion) was suppressed by the intraduodenal protein infusions in young subjects, there was no suppression at any dose in older subjects, who actually had *increased* total energy intake.

These results are consistent with previous results indicating a reduced responsiveness in older people to the suppressive effects of nutrients on appetite and energy intake (46, 63). In the fasting state healthy older people exhibit lower hunger and higher fullness ratings than young adults (41-46, 49, 88, 214, 215, 279). As in younger people, those hunger ratings are related positively and fullness ratings negatively to subsequent *ad libitum* energy intake (279). In response to oral or gastric nutrient administration, the reductions in hunger ratings

and subsequent energy intake are less in older than young adults (46, 48). We have shown that in older people the suppression of hunger ratings by intraduodenal infusions of fat and carbohydrate are less than in young adults (45) and the current results which show less suppression of hunger ratings and subsequent energy intake by protein infusions are, accordingly, not surprising. As in previous studies appetite ratings were positively related to subsequent energy intake and, consistent with the effect of protein on energy intake, decreased less during the protein infusions in older than young subjects. Thus, while older people are less hungry and eat less than younger adults, they appear to be less susceptible to further suppression of appetite and eating behaviour by ingestion of energy and nutrients, including protein. This is consistent with the attenuated homeostatic mechanisms in older people as evidenced by an impaired ability to compensate for modifications in diet (63).

The finding of an age-related reduction in the satiating effects of protein is important. In young adults protein is the most satiating macronutrient in young adults when orally ingested (280), and at least as satiating as fat when infused intraduodenally (112), and there is good evidence that high protein diets promote satiety and aid deliberate weight loss in overweight younger adults (281). While beneficial in *those* circumstances, protein-enriched nutritional supplements given to older people for management of undernutrition, could have unintended adverse effects if satiating effects are undiminished (or increased) by age, by increasing satiety and reducing *ad libitum* energy intake. The use of high protein supplements by older people for this purpose is widespread, and increasing, in response to greater awareness of the prevalence of undernutrition and sarcopenia in older people, and evidence that protein supplementation may increase muscle mass and function (20, 271).

The reduction in suppression of appetite and feeding responses to protein in older people seen in the present study may, therefore, point to a beneficial effect of ageing. If timing and preparation are optimised, it may be possible to give enough protein to older people to preserve, or increase muscle mass and function, without suppressing energy intake. Indeed

our observations suggest that optimal protein administration may increase overall energy intake in older people. All protein doses had a suppressive effect on total energy intake during the study (total of energy in the infusion plus that in the buffet meal) in young subjects, with a substantial 19% suppression at the highest dose. In contrast, total energy intake was not suppressed by any protein dose in the older subjects, and significantly increased with the 30 kcal (9%) and 90 kcal (14%) protein doses; 90 kcal of protein (22.5 g) increased total energy intake by ~145 kcal, and similar amounts of protein could reasonably be given as protein supplements several times during the day. This raises the intriguing possibility that appropriately designed protein supplements administered in divided doses, might act to increase energy intake in undernourished people by meaningful amounts (> 200-300 kcal/day), without the need to encourage and supervise additional energy intake.

We assessed antropyloroduodenal motility to examine potential mechanisms responsible for protein-induced suppression of feeding behaviour and potential age-related differences in that suppression. Gastrointestinal mechanisms involved in satiation are numerous and include variations in gastric distension (70), gastric emptying (261) - neither a factor in this intraduodenal infusion study - gut hormone secretion (e.g. cholecystokinin, glucagon-like peptide-1, peptide tyrosine tyrosine and gastric inhibitory peptide, ghrelin) (114), pancreatic signals (e.g. insulin) (39), plasma amino acid concentrations (e.g. branched-chain amino acids) (282), diet induced thermogenesis (283), and gluconeogenesis (284). Our group has shown that pyloric motility, particularly as reflected in the number of IPPWs, is an independent negative predictor of subsequent energy intake in young subjects (269). In the present study, pyloric motility (IPPWs) was modestly greater, in the short time period of the fasting state in the older than young subjects. In both age groups IPPW number increased and antral and duodenal pressure number decreased by the highest (180 kcal) protein load when compared with control. Furthermore in the combined subject group there was an inverse relationship between IPPW and energy intake, and a positive relationship between

antral and duodenal pressures and energy intake, providing further support for a relation between antropyloroduodenal motor activity and feeding behaviour.

This study has several limitations, which reduce our ability to draw stronger conclusions. The subject numbers were relatively small. Nevertheless the findings were clear-cut. In the current study protein was infused directly into the duodenum, to allow exploration of small intestinal effects by bypassing higher neural, oral and gastric mechanisms that may affect energy intake, including variations in nutrient taste, gastric distension and gastric emptying rate. It seems that the older men had a somewhat better tolerance to the intraduodenal protein infusion that the young men, i.e. lower ratings of nausea and bloating however not statistically significant. We studied only men, as they appear to have the greatest ability to regulate energy intake in response to energy manipulation (46), and in women particularly the menstrual cycle may have a confounding effect on appetite and energy intake. The results do not, therefore, necessarily apply to the effects of ageing in women. Further studies are needed to determine if this age-related reduction in protein's satiating effect is also present in women, and when the protein is administered orally and as part of a mixed macronutrient supplement, with the ultimate aim of developing the most effective form of protein/nutritional supplement for older people, which combines the greatest anabolic effect on muscle with the least suppression of appetite and energy intake. Nevertheless, our findings provide support for the use of protein supplements in undernourished and/or sarcopenic older people, as they provide no evidence that they suppress feeding behaviour and counteract attempts to increase body weight.

In summary, older men had less suppression of appetite and subsequent *ad libitum* energy intake by intraduodenal protein infusions than young men, associated with antropyloroduodenal motility. At lower doses protein administration to older people even increased overall energy intake. Future studies are needed to characterise the effects of different oral protein loads in direct comparison to saline and carbohydrate controls in older

healthy and malnourished men and women compared with young subjects and, thereby, provide comprehensive insights into the underlying mechanisms. This should lead to improved, evidence-based, strategies for the use, i.e. type, dose and timing, of pure oral protein supplements, to increase energy intake in older undernourished individuals or to decrease energy intake as part of a weight loss diet strategy in older obese people.

CHAPTER 6: DOSE-DEPENDENT EFFECTS OF RANDOMISED INTRADUODENAL WHEY-PROTEIN LOADS ON GLUCOSE, GUT HORMONE, AND AMINO ACID CONCENTRATIONS IN HEALTHY OLDER AND YOUNGER MEN

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Contribution	Data interpretation, statistical analysis and drafting of the manuscript.		
Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am first author on the paper.		
Signature		Date	October 2017

Co-Author Contributions

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

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

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

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ABSTRACT

Background: Protein-rich supplements are used widely for the prevention and management of malnutrition in older people. We have reported that healthy older, compared to younger, adults have less suppression of energy intake by whey-protein - effects on appetite-related hormones are unknown.

Objective: To determine the effects of intraduodenally administered whey-protein on glucose, gut hormone and amino acid concentrations, and their relation to subsequent *ad libitum* energy intake at a buffet meal, in healthy older and younger men.

Design: Hydrolysed whey-protein (30kcal, 90kcal and 180kcal) and a saline control (~0kcal) were infused intraduodenally for 60min in 10 younger (19-29y, 73±2kg, 22±1kg/m^2) and 10 older (68-81y, 79±2kg, 26±1kg/m^2) healthy men in a randomised, double-blind fashion.

Results: Plasma insulin, glucagon, gastric inhibitory peptide (GIP), glucagon-like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY) and amino acid concentrations, but not blood glucose, increased, while ghrelin decreased during the whey-protein infusions. Only plasma GIP concentrations were greater in older than younger men. Energy intake correlated positively with ghrelin and negatively with insulin, glucagon GIP, GLP-1, PYY and amino acids concentrations (all P <0.05).

Conclusions: In conclusion, intraduodenal whey-protein infusions showed comparable load-dependent responses in plasma gut hormone and amino acid concentrations in healthy older and younger men, which correlated to subsequent energy intake.

INTRODUCTION

A growing awareness of the extent and adverse effects of aging-related muscle loss, including reduced functional capacity and decreased quality of life (6, 190, 285), has stimulated the development of nutritional strategies designed specifically to preserve and/or restore skeletal muscle mass and function. A 'common' nutritional strategy for management of malnutrition in older people is the use of nutritional supplements, which are usually high-energy drinks rich in whey protein (e.g., 10-30 g protein) (26, 27, 286). In younger as well as older adults higher postprandial plasma amino acid concentrations induce greater muscle protein synthesis (17, 19, 23), which provides a rationale to increase protein intake in older people. Whey, when compared to casein or soy, protein results in greater muscle protein synthesis in young and older men (19). On the other hand, weight loss and under-nutrition are common in older adults, often associated with and/or caused by reduced appetite and food intake, termed the 'anorexia of aging' (6, 190), and associated with serious adverse effects. Given that protein is the most satiating macronutrient in young people, and its substitution for other macronutrients is often advocated to promote weight loss in overweight young adults (270, 287), the satiating and weight loss promoting effects of increased protein ingestion could potentially counteract some or all of the muscle benefits of increased protein ingestion in older people. Yet, despite the increasing use of protein-rich drinks by older people, information about their effects on appetite related gastrointestinal mechanisms in this age group is lacking.

Potential mechanisms involved in the regulation of energy intake include variations in gut hormone release and action [e.g., ghrelin, glucose-dependent insulinotropic polypeptide/ gastric inhibiting polypeptide (GIP), glucagon-like polypeptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY)], as well as plasma amino acid concentrations (112, 114, 288). We have recently shown that in young men there is a dose-dependent effect of intraduodenal

whey protein infusion on plasma gut hormones (114) and amino acid concentrations (289). Older, compared to younger, adults had greater responses in plasma concentrations of insulin [in response to intraduodenal glucose infusions (44)], GIP [after oral glucose intake (132, 135)] and GLP-1 [after oral glucose (132)] and mixed macronutrient intakes (231)], but not PYY [in response to intraduodenal infusions of glucose or lipid (53)]. Effects of aging on ghrelin after oral mixed macronutrient intakes were inconsistent (48, 52, 88, 89, 91).

Healthy aging is associated with a reduced responsiveness to the suppressive effects of nutrients, including protein, on appetite and energy intake (46, 63). Consistent with this, we have recently demonstrated that suppression of energy intake by protein ingested either orally [30 g (120 kcal), 70 g (280 kcal) whey-protein loads (263)] or infused directly into the small intestine at rates encompassing the normal rate of gastric emptying of nutrients [0.5 kcal/min (7.5 g, 30 kcal), 1.5 kcal/min (22.5 g, 90 kcal), 3.0 kcal/min (45 g, 180 kcal) (194)], and thereby bypassing 'oral' and 'gastric' effects, is less in older than younger men.

The aim of the study was to further characterise the effects of intraduodenal whey protein loads on blood glucose and plasma insulin, glucagon, ghrelin, GIP, GLP-1, PYY and amino acid concentrations and, their relationships including those with subsequent *ad libitum* energy intake, in older and younger men. We hypothesised that intraduodenally administered whey protein would result in load-related responses, related to subsequent energy intake, of glucose, gut hormones and amino acids, and that these responses would be greater in older than younger men.

SUBJECTS AND METHODS

Subjects

Our original study characterised the effect of aging on energy intake, perceptions of appetite and gastrointestinal symptoms, and antropyloroduodenal motility in response to infusion of

hydrolysed whey protein directly into the duodenum (thereby bypassing orosensory and gastric factors) at loads lower than (0.5 kcal/min), similar to (1.5 kcal/min) and at the upper end (3 kcal/min) of normal gastric emptying rates (1 – 4 kcal/min), and a saline control, at a rate of 4 mL/min for 60 min [previously published (194)]. We have now further characterised effects on blood glucose and plasma insulin, glucagon, ghrelin, GIP, GLP-1, PYY and amino acid concentrations and, their relationships, including those with subsequent energy intake. The Royal Adelaide Hospital Research Ethics Committee approved the study protocol (approval ID: 120504, approval date: 1 May 2012), and the study was registered as a clinical trial with the Australia and New Zealand Clinical Trial Registry (www.anzctr.org.au, ACTRN12612000906853). All subjects provided written informed consent prior to their inclusion in the study.

Protocol

Each subject was studied on 4 occasions, separated by 7 – 14 days, in randomised order. The protocol was described in detail previously (194).

Subjects were provided with a standardised evening meal [beef lasagna (McCain Foods, Wendouree, VIC, Ausrtalia), ~591 kcal] to consume on the night before each study, and were instructed to fast overnight from solids and liquids and to refrain from strenuous physical activity until they attended the laboratory at the University of Adelaide, Discipline of Medicine, Royal Adelaide Hospital, at ~0830 am. On arrival, a small-diameter (3.5 mm) catheter (total length: 100 cm, Dentsleeve International, Mui Scientific) was inserted into the stomach through an anesthetised nostril and allowed to pass into the duodenum by peristalsis. The infusion port of the catheter was located in the proximal small intestine 14.5 cm from the pylorus (194).

The protein solutions were prepared by dissolving whey protein hydrolysate powder (18.1% Hydrolysed Whey Protein 821, Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in varying amounts of saline and water to achieve the desired loads [i.e., 0.5, 1.5, and 3 kcal/min, which equates to 30, 90, and 180 kcal or 8, 24, and 48 g protein] and to ensure that they were iso-osmotic (640-680 mOsmol/L). The infusions were administered at a rate of 4 mL/min (240 mL over 60 min). The amino acid content of the hydrolysed (resembling partially digested protein) whey protein is presented in **Figure 6.1**.

Immediately before and at 15-min intervals during the intraduodenal infusion, blood samples (an intravenous cannula was positioned intravenously in the right forearm) for measurement of glucose, insulin, glucagon, ghrelin, GIP, GLP-1, PYY and amino acids were taken (0, 15, 30, 45 and 60 min). Blood samples were collected into ice-chilled ethylenediaminetetraacetic acid (EDTA) coated tubes. Plasma was obtained by centrifugation for 15 min at 3200 rpm at 4°C and stored at -80°C for further analysis.

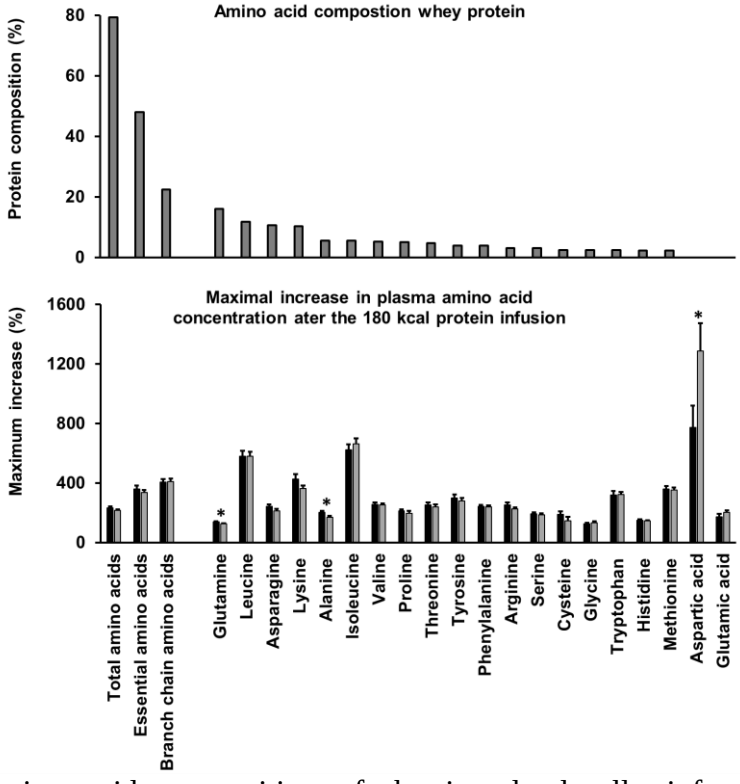


Figure 6.1: Amino acid composition of the intraduodenally infused whey protein hydrolysate (A) and increase of amino acid concentrations at 60 min during the 180-kcal

whey protein infusion as a percentage of baseline in healthy younger [n = 10 (n = 9 for histidine); black bars] and older (n = 10; grey bars) men (B), ranked in order of high to low amino acid content (g/100 g). Essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Branched-chain amino acids: isoleucine, leucine and valine.
Differences between older and younger men were determined using an independent sample t-test. Statistical significance was accepted at $P < 0.05$.
* $P < 0.05$ older compared to younger men.

Measurements of blood glucose and plasma gut hormone and amino acid concentrations

Blood glucose (millimoles per liter) was determined immediately after collection by the glucose oxidase method using a portable glucometer (Optium Xceed, Abbott Laboratories, Doncaster, VIC, Australia).

Total plasma insulin (milliunits per liter) was measured by Enzyme-linked immunosorbent *assay* (ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L. Intra- and inter-assay coefficients of variation were 3.1 and 9.4%. Homeostatic model assessment (HOMA) index at baseline was calculated according to the following formula: insulin concentration (microunits per liter) x glucose concentration (nanomoles per liter) / 22.5 (290).

Total plasma glucagon (picograms per milliliter) was measured by RIA (GL-32K; Millipore, Billerica, MA, USA). The minimum detectable limit was 20 pg/mL. The intra- and inter-assay coefficients of variation were 4.4 and 6.3%. The ratio of insulin to glucagon was calculated for each time point in each subject (291).

Plasma total ghrelin (picograms per milliliter) was measured using a radioimmunoassay (RIA) with modifications to the previously published method (292). The radiolabel was supplied by Perkin Elmer (NEX388; Boston, MA, USA). The standard and samples were incubated with the antibody and radiolabel for 3-4 days at 4^{o}C. The detection limit was 40 pg/mL. Intra- and inter-assay coefficients of variation were 5.0 and 12.8%.

Total plasma GIP (picomoles per liter) was measured by RIA with modifications to a previously published method (293). The standard curve was prepared in buffer rather than extracted charcoal stripped serum and the radio-iodinated label was supplied by Perkin Elmer (Boston, MA, USA). The minimum detectable limit was 2 pmol/L. The intra- and inter-assay coefficients of variance were 5.2 and 8.8%. GIP data are not available for one of the younger men.

Total plasma GLP-1 (picomoles per liter) was measured by RIA (GLPIT-36HK; Millipore, Billerica, MA, USA). The detection limit was 3 pmol/L. Intra - and inter -assay coefficients of variance were 6.4 and 9.5%.

Plasma total PYY (picomoles per liter) was measured using RIA (kindly donated by Dr. B Otto, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Munich, Germany) against human peptide YY (1-36) (Sigma-Aldrich, St Lois, MO, USA) and raised in rabbits. This antisera showed < 0.001% cross reactivity with human pancreatic polypeptide and 0.0025% cross reactivity with human neuropeptide Y. Standards (1.6-50 fmol/tube) or samples (200 µL plasma) were incubated in 200 µL assay buffer (50 mM $NaPO_4$, 10 mM EDTA, 2 g/L gelatin, 0.1 g/L Na-Azide, pH = 7.4) and a 1/12000 dilution of antisera for 24 hours. The standards and samples were further incubated with 10000 counts per minute tracer [Perkin Elmer (NEX3410; Boston, MA, USA)] for 24 hours. Separation of the antibody bound tracer from free tracer was by second antibody precipitation (i.e., 500 µL of 1/100 dilution of sheep anti-rabbit immunoglobulin in wash buffer comprising 50 mM Tris-base, 150 mM NaCl, 8% Polyethylene Glycol 6000, pH = 8.0 (Merck KGaA, Darmstadt, Germany), and 50 µL of normal rabbit serum diluted 1/50 in wash buffer), incubated 2 hours at room temperature then spun at 4000 rpm at 4˚C for at least 20 minutes, supernatants poured off and pellets counted in a gamma counter. The detection limit was 1.5 pmol/L. Intra- and inter-assay coefficients of variations were 8.4 and 13.7%.

Plasma free amino acid concentrations (mmol/L) of asparagine, aspartic acid, alanine, arginine, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, were analysed using precolumn derivatization with 6-aminoquinolyl-N hydroxysuccmimidyl carbamate (AQC) performed at the Australian Proteome Analysis's Facility established under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS). The maximum increase in plasma amino acid concentration of the highest protein load (180 kcal; 3 kcal/min for 60 min) at 60 min was calculated as a percentage of the average baseline concentration. The derivatives were separated and quantified by reversed-phase high-performance liquid chromatography (HPLC). The amino acids (with the exception of tryptophan) were detected by fluorescence, whereas tryptophan required UV detection. Before derivatization, 100 μL of plasma samples were diluted 1:1 with internal standard solution (Norvaline) and deproteinised by ultra-filtration through a membrane with 10 kDa nominal molecular weight cutoff (Ultrfree MC with PL-10 membrane, Millipore, MA, USA). Amino acids contained in the filtrate (100 μL) were labeled using the Waters AccQTag™ chemistry and analysed using a Waters Acquity™ UPLC system (Waters Corporation, MA, USA) (21). Histidine data are not available for one of the younger men.

Data analysis

Statistical analyses were performed using SPSS software (version 21; IBM, Armonk, NY, USA. Main effects of protein load and age, and their interaction effects on blood glucose and plasma hormone concentrations and plasma amino acid concentrations at baseline (fasting; 0 min), 15 min after starting the infusion, immediately before the meal (60 min), and net area under the curve (AUC; calculated from baseline to 60 min using the trapezoidal rule), were determined using a repeated-measures mixed-effect model, with protein load as

the within-subject factor and age as the between-subject factor, including baseline values at each treatment visit as a covariate. Post-hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when there were significant main or interaction effects. Within-subject correlations between energy intake [published previously (194)] and glucose, gut hormones and amino acid concentrations were determined by using a general linear model with fixed slope and random intercept (277). Assumptions of normality were verified for all outcomes before statistical analysis.

The original study (194) was powered to detect a suppression in energy intake by protein compared to control in 10 subjects per group. We calculated that 10 subjects per group would allow detection of differences in area under the curve (AUC) of the orexigenic hormone ghrelin and the anorexigenic hormone GLP-1 (294) within-subjects (protein compared to control) of 400 pg/mL and 9.1 pmol/L and between age groups (older compared to younger) of 1408 pg/mL and 20.5 pmol/L, respectively, with power equal to 0.8, alpha equal to 0.05 and 10% dropouts. Statistical significance was accepted at $P < 0.05$. Data are presented as mean ± SEM unless otherwise stated.

RESULTS

The study protocol was well tolerated by all subjects. Fasting concentrations of blood glucose ($P = 0.67$), and plasma insulin ($P = 0.50$), glucagon ($P = 0.61$), ratio of insulin to glucagon ($P = 0.29$), HOMA-IR ($P = 0.55$), ghrelin ($P = 0.68$), GIP ($P = 0.15$), GLP-1 ($P = 0.41$) and PYY ($P = 0.07$) were comparable in healthy younger and older men.

Glucose

15-min and AUC (0-60 min) glucose concentrations were not affected by protein load (15 min, AUC: $P = 0.62$, $P = 0.60$), age ($P = 0.64$, $P = 0.07$), or interaction of protein load by age ($P = 0.95$, $P = 0.23$; **Figure 6.2**).

Glucose concentrations at 60 min, immediately before the buffet meal, were higher in older than younger men (mean 60-min concentration: younger: 5.0 ± 0.1 mmol/L, older: 5.4 ± 0.1 mmol/L; effect of protein load: $P = 0.19$, effect of age: $P = 0.031$, interaction effect of protein load by age: $P = 0.003$). Interaction-effect post-hoc analyses revealed that 60-min glucose concentrations were lower after the 90-kcal and 180-kcal protein infusions compared to control in younger ($P = 0.036$ and $P = 0.037$) but not older men ($P > 0.05$) and, higher in older than younger men after the 90-kcal protein infusion ($P = 0.001$).

Insulin

Plasma insulin concentrations at 15 min were lower in older than younger men (mean 15-min concentration: younger: 9 ± 1 mU/L, older: 5 ± 1 mU/L; effect of protein load: $P < 0.001$, effect of age: $P = 0.003$, interaction effect of protein load by age: $P = 0.08$). Protein-load post-hoc analyses revealed that 15-min insulin concentrations were higher during all protein infusions compared to control (30 kcal: $P = 0.003$, 90 and 180 kcal: both $P < 0.001$). 60-min and AUC insulin concentrations protein-load dependently increased in younger and older men (mean 60 min concentration: younger: 13 ± 2 mU/L, older: 12 ± 2 mU/L; 60 min, AUC: effect of protein load: both $P < 0.001$; effect of age: $P = 0.97$, $P = 0.26$; interaction effect of protein load by age: both $P = 0.71$; **Figure 6.2**). Protein-load post-hoc analyses revealed that 60-min and AUC insulin concentrations were higher during the 90-kcal (60 min, AUC: both $P < 0.001$) and 180-kcal (both $P < 0.001$) protein infusions compared to control.

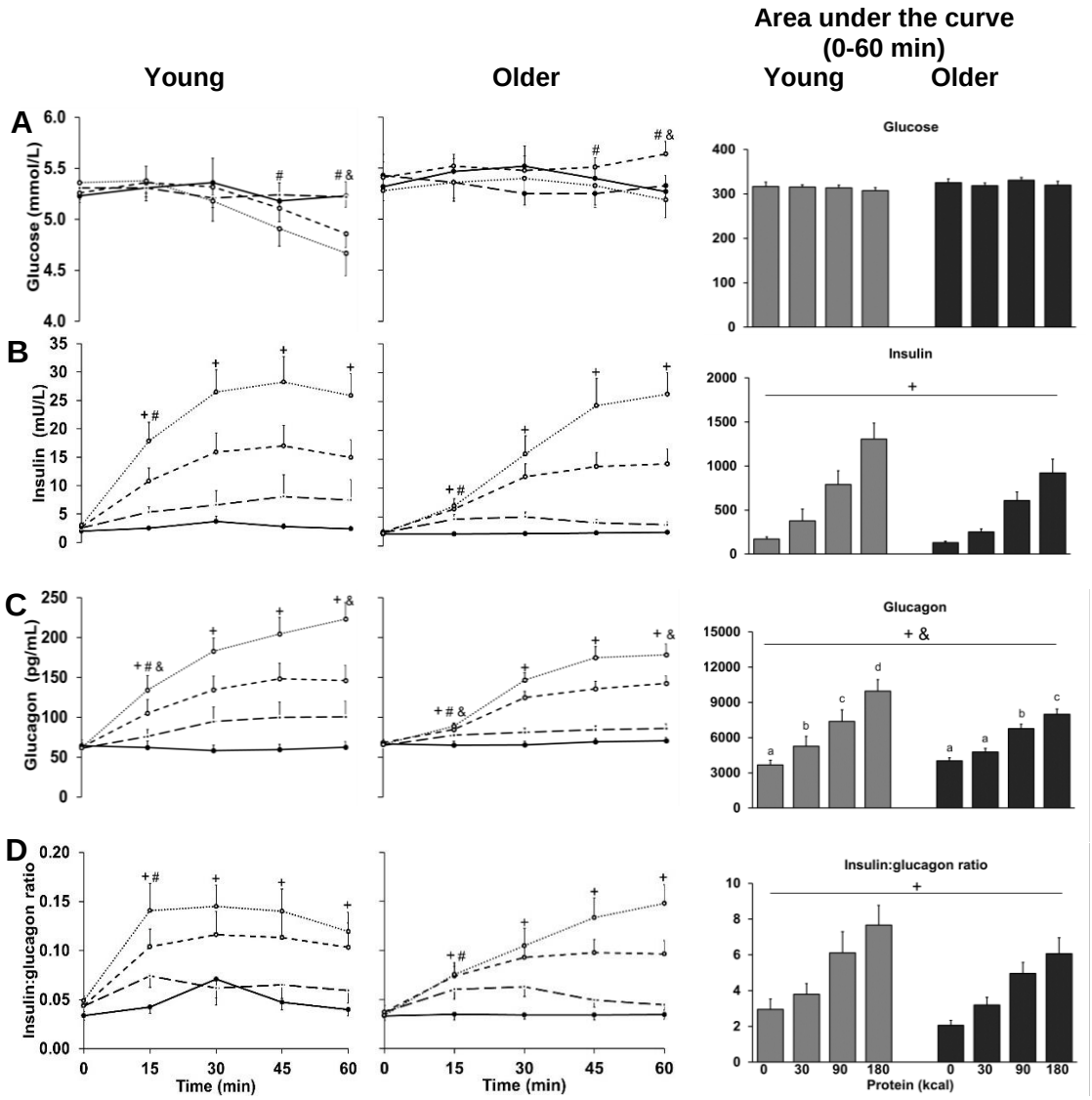


Figure 6.2: Mean (± SEM) and area under the curve concentrations of blood glucose (A), plasma insulin (B), glucagon (C), and ratio of insulin to glucagon (D) in healthy younger (n = 10) and older (n = 10) men during intraduodenal infusions of saline (control; solid line with closed circles) and whey-protein loads of 30 kcal (7.5 g protein; dashed line with closed circles), 90 kcal (22.5 g protein; dashed line with open circles) and 180 kcal (45 g protein; dotted line with open circles). Effect of protein load, age and interaction effect of protein load by age were determined using a mixed-effect model with baseline concentrations as a covariate and post-hoc Bonferroni correction. Statistical significance was accepted at $P < 0.05$.

\+ $P < 0.05$ Effect of protein load. Post-hoc tests: higher glucagon during 30 kcal protein infusion compared to control ($P = 0.004$); higher insulin, glucagon and ratio of insulin to glucagon during 90 kcal and 180 kcal protein infusions compared to control (insulin: both $P < 0.001$, glucagon: both $P < 0.001$, ratio of insulin to glucagon: $P < 0.001$ and $P = 0.002$).

\# $P < 0.05$ Effect of age.

\& $P < 0.05$ Interaction effect of protein load by age. ^{a,b,c,d} $P < 0.05$ Interaction effect post-hoc tests: a different letter indicates a difference between protein loads (AUC 0-60 min); higher glucose in older than younger men at 60 min during 90-kcal protein infusion ($P = 0.001$); lower glucagon in older than younger men at 15 min during 90- and 180-kcal protein infusions ($P = 0.048$ and $P = 0.008$); in younger and older men higher glucagon (AUC and 60 min) during 180-kcal protein infusion compared to 90-kcal (younger: both $P < 0.001$,

older: $P = 0.037$, $P = 0.020$), 30-kcal (younger: both $P < 0.001$, older: $P = 0.006$, $P = 0.001$) and control infusions (younger: both $P < 0.001$, older: both $P = 0.001$), and 90-kcal protein infusion compared to 30 kcal (younger: $P = 0.008$, $P = 0.004$, older: $P = 0.022$, $P = 0.001$) and control infusions (younger: both $P < 0.001$, older: both $P < 0.001$); in younger, but not older, men higher glucagon (AUC and 60 min) during 30-kcal protein infusion compared to control (younger: $P = 0.006$, $P = 0.028$).

Glucagon

Plasma glucagon concentrations at 15 min were lower in older than younger men (mean 15-min concentration: younger: 94 ± 12 pg/mL, older: 79 ± 4 pg/mL; effect of protein load: $P < 0.001$, effect of age: $P = 0.023$, interaction effect of protein load by age: $P = 0.021$). Interaction-effect post-hoc analyses revealed that 15-min glucagon concentrations were lower in older than younger men during the 90-kcal ($P = 0.048$) and 180-kcal ($P = 0.008$) protein infusions. In younger men, 15-min glucagon concentrations were higher during all protein infusions compared to control (30 kcal: $P = 0.001$, 90 and 180 kcal: both $P < 0.001$); in older men during the 30-kcal protein infusion ($P = 0.003$).

60-min and AUC glucagon concentrations protein-load dependently increased in younger and older men (mean 60-min concentration: younger 133 ± 14 pg/mL, older 119 ± 7 pg/mL; 60 min, AUC: effect of protein load: both $P < 0.001$, effect of age: $P = 0.42$, $P = 0.21$, interaction effect of protein load by age $P = 0.007$, $P = 0.030$; **Figure 6.2**). Interaction-effect post-hoc analyses revealed that 60-min and AUC glucagon concentrations were higher during all protein infusions compared to control in younger men (60-min, AUC: 30 kcal: $P = 0.028$, $P = 0.006$, 90 kcal: both $P < 0.001$, 180 kcal: both $P < 0.001$), and in older men during the 90-kcal and 180-kcal protein infusions compared to control (60-min, AUC: 30 kcal: $P = 0.47$, $P = 1.0$, 90 kcal: both $P < 0.001$, 180 kcal: both $P < 0.001$).

Ratio of insulin to glucagon

The ratio of insulin to glucagon at 15 min was lower in older than younger men (mean 15-min ratio: younger: 0.09 ± 0.01, older: 0.06 ± 0.01; effect of protein load: $P = 0.001$, effect of age: $P = 0.045$, interaction effect of protein load by age: $P = 0.39$). Protein-load post-hoc analyses revealed that the 15-min insulin to glucagon ratio was higher during all protein infusions compared to control (30 kcal: $P = 0.021$, 90 and 180 kcal: both $P = 0.003$).

The 60-min and AUC ratio of insulin to glucagon protein-load dependently increased in younger and older men (mean 60-min ratio: younger: 0.10 ± 0.02, older: 0.08 ± 0.01; 60 min, AUC: effect of protein load: both $P < 0.001$, effect of age: $P = 0.73$, $P = 0.55$, interaction effect of protein load by age: $P = 0.14$, $P = 0.69$; **Figure 6.2**). Protein-load post-hoc analyses revealed that the 60-min and AUC ratios of insulin to glucagon were higher during the 90-kcal (60-min, AUC: both $P = 0.001$) and 180-kcal ($P < 0.001$, $P = 0.002$) protein infusions compared to control.

Ghrelin

60-min ghrelin concentrations were higher in older than younger men (mean 60-min concentration: younger: 1235 ± 130 pg/mL, older: 1313 ± 156 pg/mL; 15 min, 60 min, AUC: effect of protein load: $P = 0.047$, $P < 0.001$, $P < 0.001$, effect of age: $P = 0.97$, $P = 0.029$, $P = 0.98$, interaction effect of protein load by age: $P = 0.84$, $P = 0.57$, $P = 0.41$; **Figure 6.3**). Protein-load post-hoc analyses revealed that 60-min and AUC ghrelin concentrations were lower during the 90-kcal (60 min, AUC: $P < 0.001$, $P = 0.006$) and 180-kcal ($P = 0.015$, $P = 0.002$) protein infusions compared to control.

GIP

15-min, 60-min and AUC GIP concentrations protein-load dependently increased in younger and older men (mean 60-min concentration: younger: 23 ± 1 pmol/L, older: 34 ± 3 pmol/L; 15 min, 60 min, AUC: effect of protein load all $P < 0.001$, effect of age $P = 0.59$, $P = 0.002$, $P = 0.011$, interaction effect of protein load by age $P = 0.20$, $P = 0.007$, $P = 0.06$; **Figure 6.3**). Interaction effect post-hoc tests revealed that 60-min GIP concentrations were higher in older than younger men during the 90-kcal ($P < 0.001$) and 180-kcal ($P = 0.013$) protein infusions. Protein-load post-hoc analyses revealed that 15-min, 60-min and AUC GIP concentrations were higher during all protein infusions compared to control (all $P < 0.001$).

GLP-1

15-min, 60-min and AUC GLP-1 concentrations protein-load dependently increased in younger and older men (mean 60-min concentration: younger: 37 ± 3 pmol/L, older: 31 ± 4 pmol/L; 15 min, 60 min, AUC: effect of protein load: $P = 0.013$, $P < 0.001$, $P < 0.001$, effect of age: $P = 0.30$, $P = 0.34$, $P = 0.38$, interaction effect of protein load by age: $P = 0.27$, $P = 0.41$, $P = 0.41$; **Figure 6.3**). Protein-load post-hoc analyses revealed that 15-min GLP-1

PYY

Plasma PYY concentrations at 15 min were lower in older than younger men (mean 15-min concentration: younger: 37 ± 4 pmol/L, older: 26 ± 1 pmol/L; effect of protein load: $P = 0.005$, effect of age: $P = 0.009$, interaction effect of protein load by age: $P = 0.49$). Protein-load post-hoc analyses revealed that 15-min PYY concentrations were higher during the 90-kcal protein infusion compared to control ($P = 0.005$).

60-min and AUC PYY concentrations protein-load dependently increased in younger and older men (mean 60-min concentration: younger: 41 ± 5 pmol/L, older: 36 ± 3 pmol/L; 60

min, AUC: effect of protein load: $P = 0.018$, $P = 0.042$, effect of age: both $P = 0.59$, interaction effect of protein load by age: $P = 0.49$, $P = 0.45$; **Figure 6.3**).

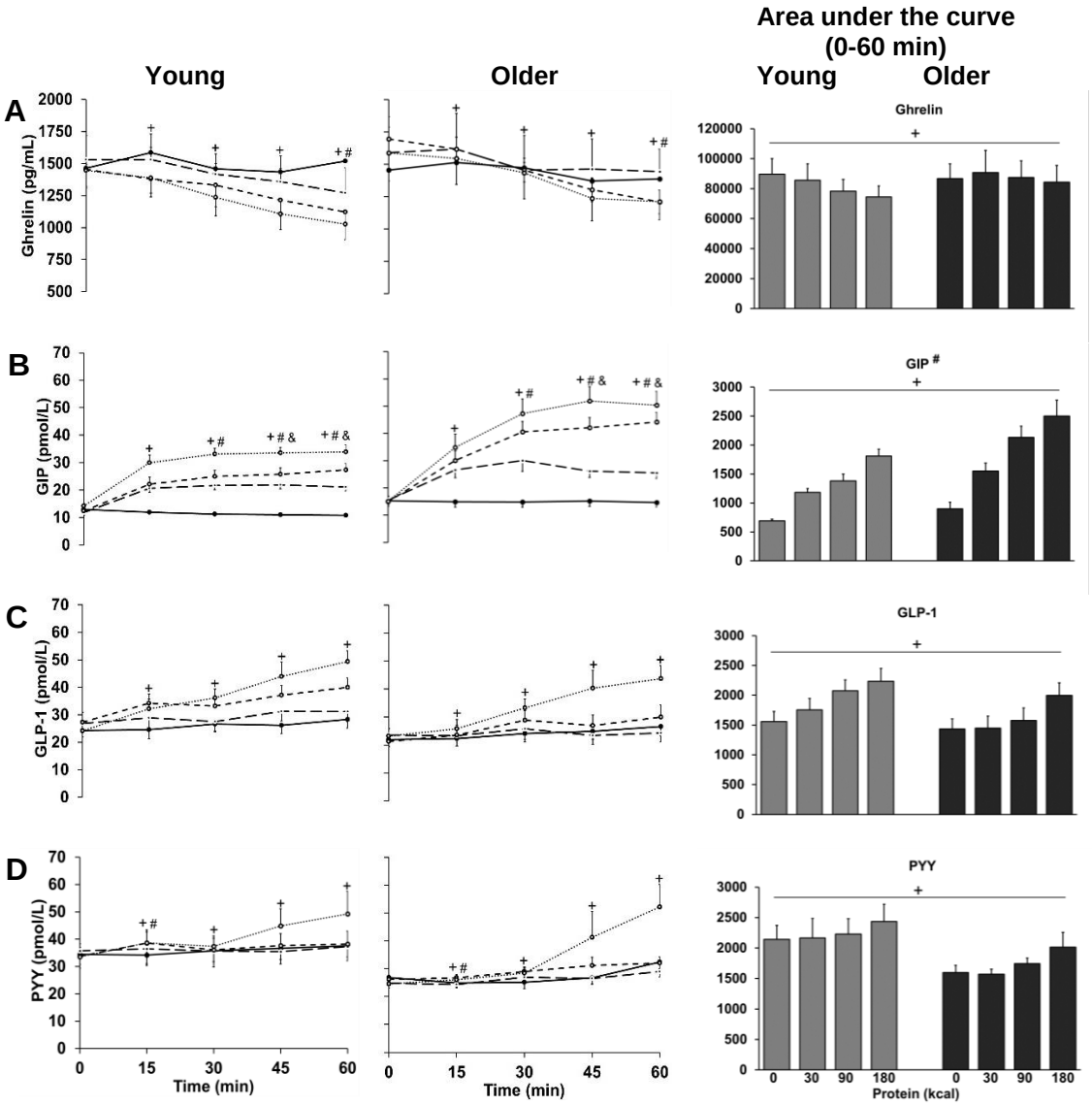


Figure 6.3: Mean (± SEM) and area under the curve concentrations of plasma ghrelin (A), GIP (B), GLP-1 (C) and PYY (D) in healthy younger (n = 10 for ghrelin and PYY; n = 9 for GIP and GLP-1) and older (n = 10) men during intraduodenal infusions of saline (control; solid line with closed circles) and whey protein loads of 30 kcal (7.5 g protein; dashed line with closed circles), 90 kcal (22.5 g protein; dashed line with open circles) or 180 kcal (45 g protein; dotted line with open circles). Effect of protein load, age and interaction effect of protein load by age were determined using a mixed-effect model with baseline concentrations as a covariate and post-hoc Bonferroni correction; statistical significance was accepted at $P < 0.05$.

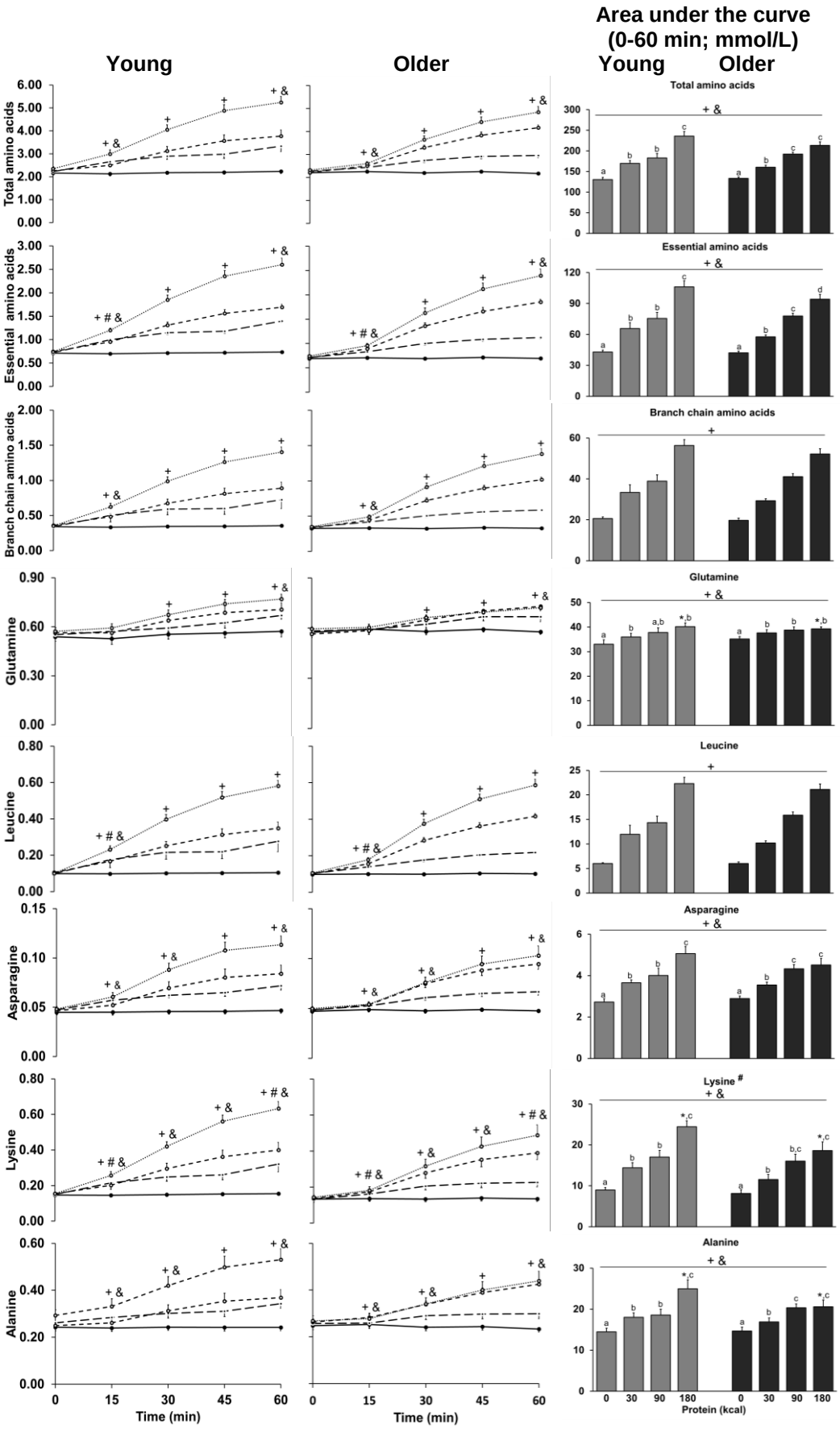
+ $P < 0.05$ Effect of protein load. Post-hoc tests: higher GIP (AUC 0-60 min) during 30-kcal protein infusion compared to control ($P < 0.001$); higher GIP during 30-kcal protein infusion compared to control ($P < 0.001$); lower ghrelin and higher GIP and GLP-1 during 90-kcal and 180-kcal protein infusions compared to control (ghrelin: $P = 0.006$, $P = 0.002$, GIP: both $P < 0.001$, GLP-1: $P = 0.034$, $P = 0.005$).

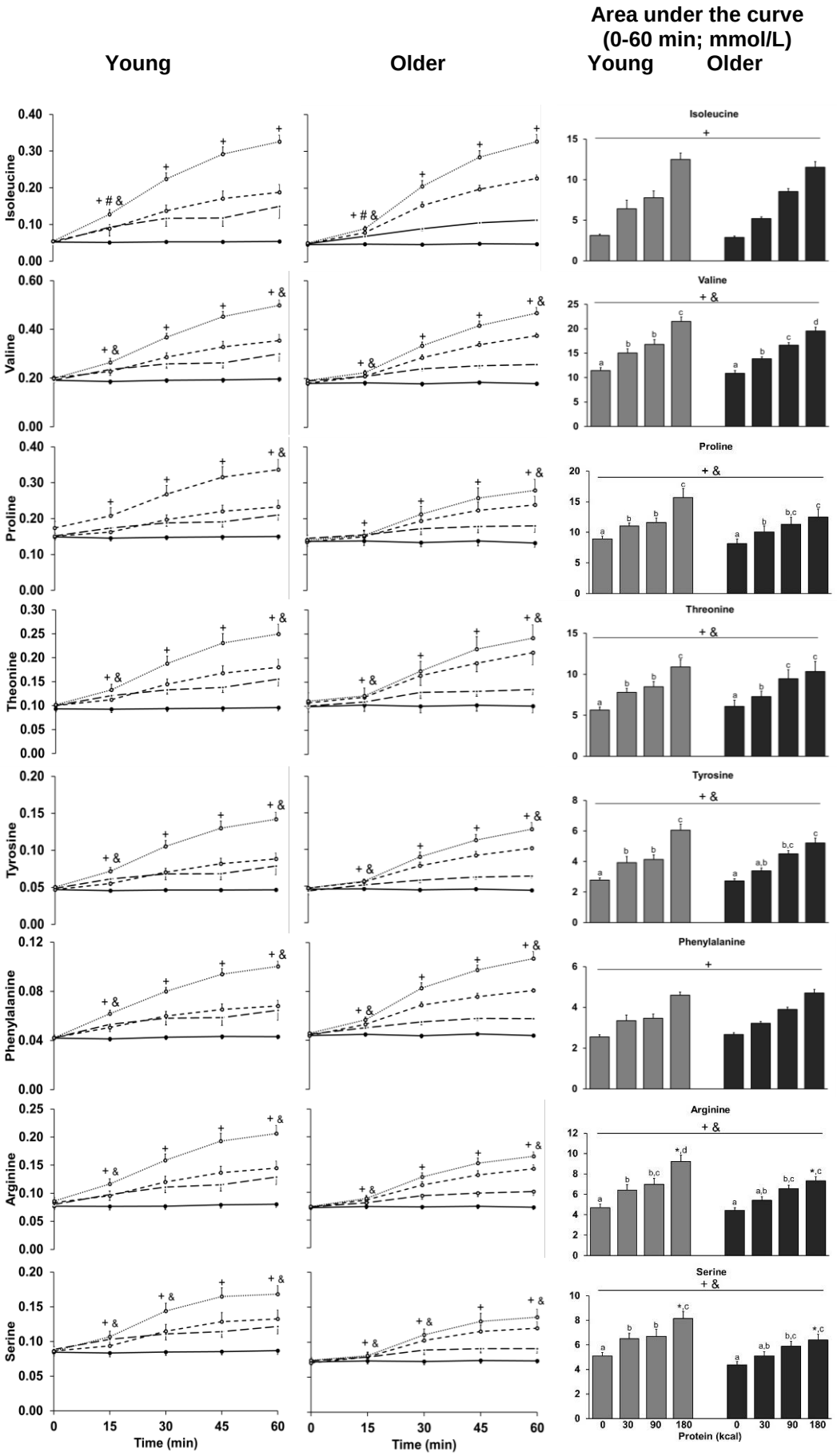
# $P < 0.05$ Effect of age.
& $P < 0.05$ Interaction effect of protein load by age. Post-hoc tests: higher GIP in older than young at 60 min during 90- and 180-kcal protein infusions ($P < 0.001$, $P = 0.013$).
concentrations were higher during the 180-kcal protein infusions compared to control ($P = 0.014$) and 60-min and AUC GLP-1 concentrations were higher during the 90-kcal (60-min, AUC: $P = 0.05$, $P = 0.034$) and 180-kcal ($P < 0.001$, $P = 0.005$) protein infusions compared to control.

Amino Acids

Fasting plasma total ($P = 0.97$) and essential amino acid ($P = 0.54$) concentrations were similar in older and younger men. Concentrations of cysteine ($P < 0.001$) and tryptophan ($P = 0.005$) were higher and of aspartic acid ($P < 0.001$), glutamic acid ($P = 0.001$) and serine ($P = 0.033$) lower in older than younger men.

15-min, 60-min and AUC plasma concentrations of total amino acids protein-load dependently increased during the infusions in younger and older men (15 min, 60 min, AUC: effect of protein load: all $P < 0.001$, effect of age: $P = 0.13$, $P = 0.37$, $P = 0.27$, interaction effect of protein load by age: $P = 0.024$, $P = 0.007$, $P = 0.016$). Essential amino acid concentrations at 15 min were lower in older than younger men (mean essential amino acid concentrations: younger: 1.0 ± 0.04, older: 0.8 ± 0.02; effect of protein load: both $P < 0.001$, effect of age: $P = 0.049$, interaction effect of protein load by age: $P = 0.024$). Post-hoc tests revealed that AUC concentrations of total ($P = 0.022$) and essential ($P = 0.014$) amino acids were lower during the 180-kcal protein infusion in older than younger men (**Figure 6.4**).


Young
Older
Area under the curve (0-60 min; mmol/L)
Young Older
Total amino acids
Essential amino acids
Branch chain amino acids
Glutamine
Leucine
Asparagine
Lysine #
Alanine
Time (min)
Protein (kcal)


Young
Older
Area under the curve (0-60 min; mmol/L)
Young
Older
Isoleucine
Valine
Proline
Threonine
Tyrosine
Phenylalanine
Arginine
Serine
Time (min)
Protein (kcal)

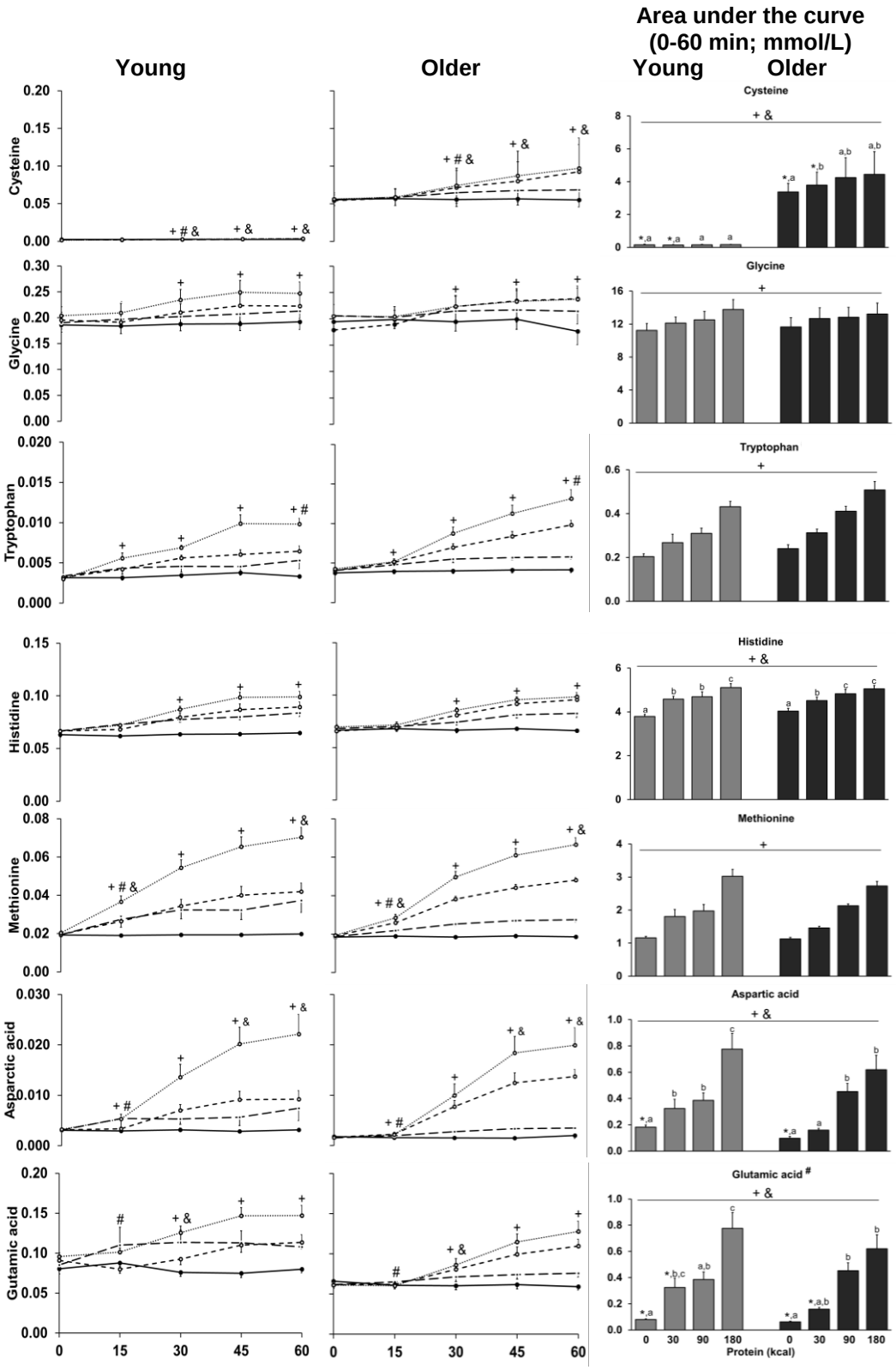


Figure 6.4: Mean (± SEM) and area under the curve concentrations of plasma total amino acids, essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), branched chain amino acids (isoleucine, leucine and valine) and individual amino acids ranked in order of highest to lowest prevalence of amino acids in the whey protein hydrolysate, in healthy younger (n = 10; n = 9 for histidine) and older (n = 10) men during intraduodenal infusions of saline (control; solid line with closed circles) and whey protein loads of 30 kcal (dashed line with closed circles), 90 kcal (dashed line with open circles) or 180 kcal (dotted line with open circles).

Main effect of protein load, age and interaction effects were determined using a mixed-effect model with baseline concentrations as a covariate and post-hoc Bonferroni correction; statistical significance was accepted at $P < 0.05$.
+ $P < 0.05$ Effect of protein load.
# $P < 0.05$ Effect of age.
& $P < 0.05$ Interaction effect of protein load by age.
* $P < 0.05$ Interaction effect post-hoc tests: older compared to younger men (AUC 0-60 min); ^{a,b,c,d} $P < 0.05$ a different letter indicates a difference between protein loads (AUC 0-60 min); at 15 min during 180-kcal protein infusion, older compared to younger men: lower total ($P = 0.022$), essential ($P = 0.014$), branched chain amino acids ($P = 0.017$), leucine ($P = 0.018$), asparagine ($P = 0.019$), lysine ($P = 0.011$), isoleucine ($P =0.019$), valine ($P = 0.018$), threonine ($P = 0.023$), tyrosine ($P = 0.026$), phenylalanine ($P = 0.028$), asparagine ($P =0.019$), serine ($P = 0.018$) and methionine ($P = 0.028$); at 60 min: lower glutamine ($P = 0.013$), lysine ($P = 0.038$), arginine ($P = 0.029$), and higher phenylalanine ($P = 0.014$) and aspartic acid ($P = 0.011$) during 90-kcal protein infusion, and cysteine during 30-kcal protein ($P = 0.010$) and control ($P = 0.003$) infusion.

Relationships of energy intake, gut hormones and amino acids

Energy intake (published previously (194); younger men: control: 1270 ± 150 kcal, 30 kcal: 1123 ± 151 kcal, 90 kcal: 1028 ± 163 kcal, and 180 kcal: 851 ± 161 kcal; older men: control: 1068 ± 93 kcal, 30 kcal: 1129 ± 91 kcal, 90 kcal: 1123 ± 98 kcal and 180 kcal: 899 ± 103 kcal) was positively related to plasma concentrations of ghrelin, and negatively related to insulin, glucagon, GIP, GLP-1, PYY (**Figure 6.5**) and amino acids (**Figure 6.6**). AUC concentrations of amino acids correlated positively with AUC concentrations of insulin, glucagon, ghrelin, GIP, GLP-1 and PYY, and negatively with ghrelin (**Table 6.1**).

GIP was, *within subjects*, related to GLP-1 ($r = 0.68$ $P < 0.001$); i.e. the greater the increase in plasma GIP concentrations the greater the increase in GLP-1. Ghrelin was, *within subjects*, inversely related to insulin ($r = -0.33$, $P = 0.010$); i.e. the greater the increase in plasma insulin concentrations the greater the inhibition of ghrelin production.

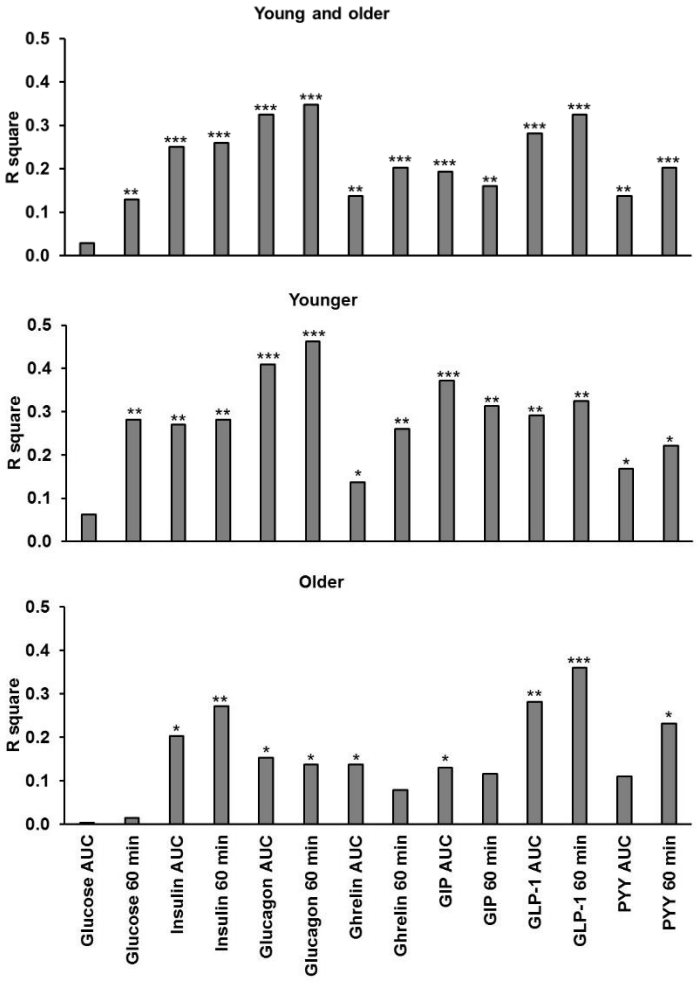


Figure 6.5: Within-subject relationships between energy intake [results of suppression of *ad libitum* energy intake after the intraduodenally infused whey protein loads have been published previously (194)] and area under the curve (AUC 0-60 min) of glucose, insulin, glucagon, ghrelin, GIP, GLP-1 and PYY concentrations in healthy older and younger men combined (n = 20; n = 19 for GIP and GLP-1), younger men (n = 10; n = 9 for GIP and GLP-1) and older men (n = 10). Within subject correlations were determined using a general linear model with fixed slope and random intercept. Statistical significance was accepted at $P < 0.05$.

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

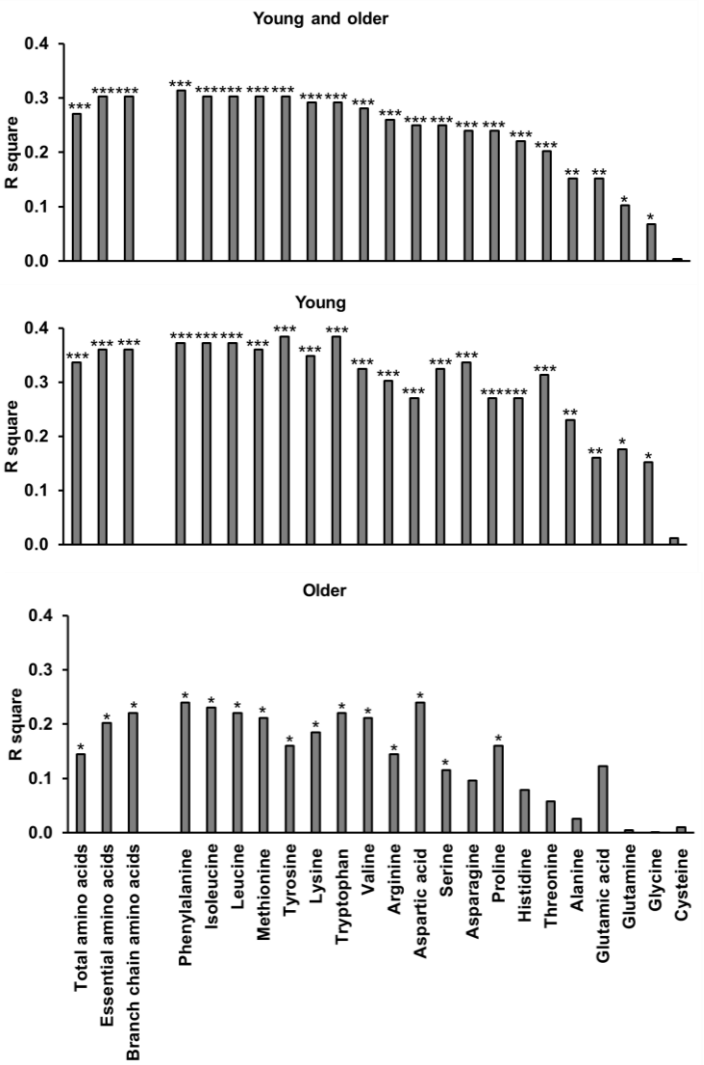


Figure 6.6: Within-subject relationships between energy intake [results of suppression of *ad libitum* energy intake after the intraduodenally infused whey protein loads have been published previously (194)] and area under the curve (AUC 0-60 min) of total, essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), branched chain amino acids (isoleucine, leucine and valine) and individual amino acids in healthy older and younger men combined (n = 20; n = 19 for histidine), younger men (n = 10; n = 9 for histidine) and older men (n = 10), ranked in order of the strongest to weakest R square in the combined group. Within subject correlations were determined using a general linear model with fixed slope and random intercept. Statistical significance was accepted at $P < 0.05$.
* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$

Table 6.1: Within-subject correlations between concentrations of total amino acids and glucose, insulin, glucagon, ghrelin, GIP, GLP-1 and PYY

	r	*P*
Glucose (mmol/L)	-0.12	0.35
Insulin (mU/L)	0.80	<0.001
Glucagon (pg/mL)	0.84	<0.001
Ghrelin (pg/mL)	-0.32	0.013
GIP (pmol/L)	0.82	<0.001
GLP-1 (pmol/L)	0.62	<0.001
PYY (pmol/L)	0.45	<0.001

r and *P* values of *within-subject* correlations between plasma concentrations of total amino acids (mmol/L; AUC) and concentrations (AUC) of blood glucose and plasma insulin, glucagon, ghrelin, GIP, GLP-1 and PYY in younger and older men. Within-subject correlations were determined by using a general linear model with fixed slope and random intercept. n = 20 (n = 19 for plasma concentrations of GIP and GLP-1).

DISCUSSION

The intraduodenal whey protein infusion, at rates lower than (0.5 kcal/min), comparable to (1.5 kcal/min), and at the upper end (3.0 kcal/min), of normal gastric energy emptying (273), dose-dependently suppressed plasma concentrations of ghrelin and stimulated concentrations of insulin, glucagon, GIP, GLP-1, PYY and amino acids in younger, as well as older, men. Plasma concentrations of the hormones insulin, glucagon, GLP-1 and PYY, which are secreted by the small intestine in response to the presence of nutrients, and largely act to suppress appetite and food intake, were increased to a comparable degree by protein infusions in younger and older men. GIP responses were greater in older than younger men. Consistent with their effects on appetite, these gut hormone concentrations were negatively correlated with energy intake at the subsequent *ad libitum* meal. Briefly, there was a dose-dependent suppression of energy intake by protein infusion (0, 30, 90, 180 kcal) in the

younger men and this was greater than the suppression in older men, which was only significant after the 180 kcal protein infusion (194). In contrast, plasma concentrations of ghrelin, which is mainly secreted by the stomach and acts to stimulate appetite and food intake (238) were suppressed by the protein infusions to a comparable degree in both age groups and were positively correlated to subsequent energy intake. Plasma concentrations of amino acids correlated negatively with energy intake at the *ad libitum* meal [published previously (194)], with tryptophan and tyrosine having the highest R square in younger men and lower R squares in older (range: 0.0 – 0.24) than younger men (range: 0.01 – 0.38).

While the younger and older men had comparable glucose, insulin and glucagon concentrations during the 60 min protein infusions, as assessed by the area under the curve method, insulin and glucagon concentrations increased more slowly in older than younger men (e.g., significantly lower concentrations at 15 min) and glucose concentrations immediately before the buffet meal (60 min) were lower during the 'higher' protein-load infusions (90 and 180 kcal) compared to control in young, but not older, men. We have reported that blood glucose and insulin concentrations were higher during intraduodenal glucose infusions in healthy older than younger men (44), which is likely related to reduced insulin sensitivity with aging. The slightly different responses in healthy older compared to younger men after whey protein administered directly into the small intestine confirms the effects of ageing on postprandial glycaemia in absence of insulin resistance or known glucose intolerance; both age groups had comparable HOMA-IR.

Healthy older and younger men had comparable AUC ghrelin concentrations during the protein infusions, consistent with responses to mixed-nutrient intake in some (88, 89) but not all previous studies (48, 52, 91). It has been suggested that aging-related changes in body composition (i.e., a decrease in lean mass and increase in fat mass) may act to decrease fasting (92) and postprandial (48) ghrelin concentrations, as body fat is negatively correlated to ghrelin concentrations (295) and tends to increase with aging. Other studies however have

found higher postprandial and fasting ghrelin concentrations in older than younger adults and impaired suppression of ghrelin after consumption of a mixed-nutrient meal in older than younger subjects (52, 91). The results of this study, where protein was infused directly into the small intestine, thereby bypassing and eliminating variations in the rate of gastric emptying, support the latter findings; plasma ghrelin levels were higher at the end of the protein infusions, immediately before the buffet meal, in older than younger men.

Plasma GIP concentrations increased rapidly during the protein infusions and these were higher in older than younger men, which may be related to differences in small intestinal transit of the whey protein. Previously, orally ingested (132, 135), but not intraduodenally infused (133), glucose evoked greater GIP responses in older than younger adults.

GLP-1 and PYY are mainly expressed more distally in the gastrointestinal tract (i.e., ileum and colon) and, when compared to GIP (expressed mainly in the duodenum and jejunum), had slower increases in plasma concentrations during the protein infusions. PYY concentrations particularly increased during the 180-kcal protein load, largely from 30 min onwards and more convincingly in the older men. Nevertheless older and younger men had comparable plasma AUC GLP-1 and PYY concentrations during the protein infusions, which is consistent with responses during intraduodenal infusions of lipid and glucose (53). Oral glucose (132) and mixed macronutrient (231) ingestion however are reported to increase GLP-1 concentrations more in older than younger women, again highlighting the age-related differences in hormone responses to nutrients depending on their route of delivery - older compared to younger adults have slightly slower gastric emptying (263).

The older compared to younger men had a slower increase in essential amino acids (lower concentrations at 15 min after starting the whey-protein infusions), particularly leucine, isoleucine and lysine, and lower AUC concentrations of total and essential amino acids were during the highest 180-kcal protein infusion. Our findings are consistent with previous reports that plasma amino acid concentrations peak later and remain elevated longer after

amino acid ingestion in older than younger adults, with AUC concentrations similar between age groups (17). The infused whey protein contained 12% leucine and 2.4% glycine, essential amino acids which are thought to play an important role in the modulation of skeletal muscle metabolism (296-298). The maximum increase in plasma amino acid concentration at the end of the protein infusion, compared to baseline, was comparable in younger and older men for most amino acids (with the exception that older men had higher maximum increase in aspartic acid and lower increases in alanine and glutamine than younger men). This is an important finding as elevated amino acid concentrations are a major determinant of muscle protein synthesis (23) and, due to suboptimal protein intake, may not always be high enough to have an optimal effect in older people. Studies utilizing stable isotope-labelled amino acids have shown that older adults have a reduced sensitivity of muscle protein synthesis to the ingestion of relatively small amounts (≤ 20 g) of whey protein compared to younger adults (23). These postprandial differences between the younger and old were however not evident after consumption of ample amounts of dietary protein (> 20g).

Plasma amino acids concentrations correlated positively with plasma concentrations of insulin, glucagon, GIP, GLP-1 and PYY and negatively with those of ghrelin. Interaction of dietary amino acids, oligopeptides and proteins in the gut induce the so-called ileal brake mechanism, including inhibition of proximal gastrointestinal motility, which stimulate the vagus nerve afferents to convey information to the nucleus of the solitary tract in the brainstem and thereby restrict food intake in the short term (299).

This study has several limitations, including the relatively small subject numbers. We studied only men, as they appear to have the greatest ability to respond to energy manipulation (46), so the findings warrant confirmation in women. Although the total loads of hydrolysed whey protein delivered (i.e., 8, 24 and 48 g) are representative of a snack or main meal, the observed findings may be different for other protein sources.

In summary, intraduodenal whey protein infusions resulted in load-dependent changes in gut hormones and amino acids in younger and older men, and these responses were related to subsequent *ad libitum* energy intake. Plasma concentrations of insulin, glucagon, GLP-1 and PYY were increased and ghrelin decreased to a comparable degree by the infusions in both age groups, while GIP responses were greater in older than younger men.

CHAPTER 7: LESSER SUPPRESSION OF ENERGY INTAKE BY ORALLY INGESTED WHEY PROTEIN IN HEALTHY OLDER MEN COMPARED WITH YOUNG CONTROLS

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Candidate	Caroline Giezenaar		
Contribution	Conception and design of the study, coordination, participant recruitment, data collection and interpretation, statistical analysis and drafting of the manuscript.		
Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	October 2017

Co-Author Contributions

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

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

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

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

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ABSTRACT

Background: Protein-rich supplements are used widely for the management of malnutrition in young and older people. Protein is the most satiating of the macronutrients in young. It is not known how the effects of oral protein ingestion on energy intake, appetite and gastric emptying are modified by age.

Objective: The aim of the study was to determine the suppression of energy intake by protein compared with control and underlying gastric-emptying and appetite responses of oral whey protein drinks in 8 healthy older men (69-80 years) compared to 8 young male controls (18-34 years).

Design: Subjects were studied on 3 occasions to determine the effects of protein loads of 30 g/120 kcal and 70 g/280 kcal compared to a flavoured water control-drink (0 g whey-protein) on energy intake *(ad libitum* buffet-meal), and gastric emptying (3D-ultrasonography) and appetite (0-180 min) in a randomised, double-blind, cross-over design.

Results: Energy intake was suppressed by the protein compared with control ($P = 0.034$). Suppression of energy intake by protein was less in older men (1 ± 5%) than young controls (15 ± 2%; $P = 0.008$). Cumulative energy intake (meal + drink) during the protein days compared with the control day increased more in older (18 ± 6%) men than young (1 ± 3%) controls ($P = 0.008$). Gastric emptying of all three drinks was slower in older men (50% gastric-emptying time: 68 ± 5 min) than young controls (36 ± 5 min; $P = 0.007$). Appetite decreased in young, whilst it increased in older ($P < 0.05$).

Conclusions: In summary, despite having slower gastric emptying, elderly men exhibited blunted protein-induced suppression of energy intake by whey protein compared with young controls, so that in the elderly protein ingestion increased overall energy intake more.

INTRODUCTION

The prevalence of malnutrition, both underweight/undernutrition and overweight/ overnutrition, has increased over recent decades in older adults (285). Both forms of malnutrition are associated with, and lead to, reduced functional capacity and decreased quality of life in the ageing population (6, 180, 190, 285). Healthy ageing is associated with a reduction of appetite and energy intake, termed 'the physiological anorexia of ageing' (6, 7). Low intake of energy, and protein, predisposes older people to weight loss, particularly loss of skeletal muscle (6, 190). A growing awareness of the prevalence and adverse effects of the major muscle loss that occurs during ageing, irrespective of body mass index (BMI), has led to the development of nutritional strategies designed specifically to preserve and/or restore skeletal muscle. Insufficient protein intake in the elderly is likely to exacerbate muscle loss by limiting muscle anabolism (300). When severe, muscle loss leads to sarcopenia in underweight and obese older individuals, which is strongly associated with adverse outcomes (184). Strategies designed to achieve an increase in muscle mass and function include exercise programs, especially resistance exercise. However, many older adults have co-morbidities and physical limitations that hamper their capacity to fully achieve the levels of exercise sufficient to fully protect the loss of muscle mass (301). As such, a 'common' strategy to (i) increase energy intake and body weight/lean mass in undernourished elderly (26, 27, 286), as well as to (ii) limit energy intake and preserve lean mass and promote fat loss during energy restriction in overweight and obese older adults (35), is the use of protein-enriched supplements. These supplements are usually high-energy drinks rich in protein. In particular whey protein, a major dairy protein source, which is rich in essential amino acids is often used. Despite the widespread use of such supplements by older people, information about their effects on energy intake is limited, and their 'optimal' composition unknown.

The rationale for using protein supplements in undernourished or obese sarcopenic older people is strengthened by evidence that ageing has only minimal inhibitory effects on the capacity to synthesise muscle protein acutely after protein ingestion (17, 21, 271). A recent consensus recommendation by the PROT-AGE study group (25) stated that dietary protein intake needs to be increased from ~0.8-1.0 g/kg body weight per day to ~1.2-1.5g/kg in older people (e.g., 90-112.5 g for 75 kg body weight) including a minimum of 25-30 g protein intake per meal. Older people however may have a significant protein portion waste of their meals (~23-68%) resulting in low protein intakes (~40-64 g/day) and, therefore, may require supplementation of up to 70 g protein/day (25, 302, 303). In young adults a protein intake of up to ~70 g is representative of intakes during a single meal (~ 250 g serving of lean steak). Accordingly, if older people can ingest sufficient protein throughout the day, it is likely to have positive anabolic effects. Protein, however, is also the most satiating of the macronutrients in young people, and high-protein energy restricted diets are used to promote weight loss in obese young adults (280).

In older, undernourished adults, the aim is, of course, to increase, rather than reduce, overall energy intake. The effects of dietary protein on energy intake and underlying gastrointestinal mechanisms in older people are largely unknown. We have recently demonstrated that administration of 30-kcal (7.5 g), 90-kcal (22.5 g) and 180-kcal (45 g) whey-protein loads directly into the small intestine suppressed subsequent energy intake less in older men than young controls (194). In addition, whereas cumulative energy intake (protein load plus *ad libitum* intake at subsequent buffet meal) was reduced by the intraduodenal protein infusions in young subjects, in the older subjects there was an increase in cumulative energy intake.

Variations in the rate of gastric emptying and gastric distension are important in the regulation of appetite and energy intake, particularly in the short-term after nutrient ingestion (42, 50, 70, 74). Gastric emptying is regulated primarily as a result of nutrient-mediated inhibitory feedback arising from the small intestine. Compared to young adults, in healthy

older people the perception of proximal gastric distension is reduced, and distension of the distal stomach, i.e., antral area, greater. Gastric emptying is probably slightly slower in older than young, particularly that of meals rich in carbohydrate and fat (42, 50, 74, 84). These differences would favour reductions in energy intake.

Older adults are less hungry and eat less than younger adults (41, 42, 44-46, 88, 190) and, due to lower body weights with disproportionally lower lean mass, require less energy to maintain their body weight (189). Therefore, a control condition containing no protein or energy is required to determine if young and older subjects differ in their susceptibility to further suppression of energy intake by ingestion of nutrients.

In this study we aimed to characterise the impact of ageing on feeding and gastric responses to orally ingested whey protein loads similar to (30 g), and higher than (70 g) the suggested protein intake per meal (25, 303) within a period of time (180 min) where these loads were expected to empty 'completely' from the stomach (50), compared with a control drink (0 g whey protein). We hypothesised that orally administered whey protein would slow gastric emptying and reduce voluntary energy intake and perceptions of appetite, in a load-related fashion, and these suppressive effects would be less in healthy older men than young controls.

SUBJECTS AND METHODS

Subjects

The study included 8 healthy young men [age (mean ± SEM): 25 ± 2 years (range: 18–34 years); body weight: 72 ± 3 kg (62–86 kg); height: 1.79 ± 0.02 m, BMI (in kg/m^2): 23 ± 1] and 8 healthy older men [age: 73 ± 1 years (69–80 years); body weight: 77 ± 4 kg (59–92 kg); height: 1.73 ± 0.02 m; BMI: 26 ± 1] who were recruited by advertisement. The body weight of the 2 groups did not differ significantly (P = 0.29). Height was less and,

accordingly, BMI higher in older men than young controls ($P = 0.045$). On the basis of our previous work (194), we calculated that 8 subjects per group would allow us to detect a difference in the suppression of energy intake by protein of 395 kcal with SD's of 316 kcal (young subjects) and 180 kcal (older subjects) (194) and in the 50% gastric emptying time of 80 min with SD's of 38 min (young subjects) and 63 min (older subjects) (50, 74, 82), between young and older subjects, with $\alpha = 0.05$ and power of 80%. Exclusion criteria were smoking, alcohol abuse, diabetes, gastrointestinal surgery (apart from uncomplicated appendectomy), significant gastrointestinal symptoms (pain, reflux, diarrhoea, or constipation) and use of medications known to potentially affect energy intake, appetite or gastrointestinal motor function. For older people, additional exclusion criteria were impaired cognitive function [score < 25 on Mini Mental State (274)], depression [score ≥ 11 on the Geriatric Depression Questionnaire (275)] and undernutrition [score < 24 on the Mini Nutritional Assessment (276)]. The Royal Adelaide Hospital Human Research Ethics Committee approved the study protocol. The study was registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number ACTRN12612000941864). All subjects provided written informed consent prior to their inclusion in the study.

Protocol

Subjects were studied on 3 occasions, separated by ≥ 3 days, to determine the effects of 2 oral whey protein loads (30 g / 120 kcal and 70 g / 280 kcal) and a flavoured water control-drink (~0 g whey protein / ~2 kcal) on energy intake, gastric emptying, perceptions of appetite and gastrointestinal symptoms in a randomised (using the method of randomly permuted blocks; www.randomization.com), double-blind, cross-over design.

Protein drinks (~450 mL) were prepared by dissolving whey protein isolate (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in varying volumes of demineralised water and diet lime cordial (Bickford's Australia Pty Ltd, South Australia) to achieve the desired loads. Drinks were prepared on the morning of each study by a research officer (PF) who was not involved in the data analysis. The drinks were served in a covered cup, so both the investigator and the subject were blinded to the treatment.

Subjects were provided with a standardised evening meal [beef lasagne (McCain Foods Pty Ltd, Wendouree, VIC, Australia), ~591 kcal] to consume on the night before each study day at ~19.00 h. They were instructed to fast overnight from solids and liquids and to refrain from strenuous physical activity until they attended the laboratory at the Discipline of Medicine, the University of Adelaide, Royal Adelaide Hospital, at ~08.30 h.

On arrival, subjects were seated in an upright position on a wooden chair, where they remained for the duration of the study. In each subject measurements of total gastric volume and perceptions of appetite and gastrointestinal symptoms were performed immediately before (during fasting; -5 min), and immediately after ingestion of the drink, and at 15-min intervals until 180 min, Subjects were instructed to consume the drink within 2 min. Gastric volume was acquired by 3-dimensional (3D) ultrasound images. Perceptions of appetite and gastrointestinal symptoms were assessed using validated visual analogue scales (VAS). At 180 min, subjects were presented with a standard, cold, buffet-style meal in excess of what they were expected to consume and instructed to eat freely for up to 30 min until comfortably full (180–210 min) (250, 251). The composition of the buffet-style meal is provided in **Table 4.1**.

Measurements

Energy intake

The amount eaten (g) was quantified by weighing the buffet meal before and after consumption. Energy intake (kcal) at the buffet meal and proportions of intake of protein, carbohydrate and fat were calculated using commercially available software (Foodworks; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake was calculated both as the intake at the buffet meal and as the cumulative energy intake, defined as the sum of energy intake at the buffet meal and the energy content of the preload drink. Absolute (kcal) and percentage suppression/change (expressed as % of energy intake of the control day) of energy intake at the buffet meal by a given protein load compared to control was calculated.

Gastric emptying

Total gastric volume was assessed by 3D ultrasonography, a method that has been validated against the 'gold standard' scintigraphy for measurement of the emptying of liquids from the stomach (68). A Logiq™ 9 ultrasound system (GE Healthcare Technologies, Australia) with TruScan Architecture [built-in magnetically sensored 3D positioning and orientation measurement (POM)] including a 3D sensor, attached to a 3.5C broad spectrum 2.5-4 MHz convex transducer, and a transmitter, placed at the level of the stomach immediately behind the subject were used. As the transmitter produces a spatially varying magnetic field that is distorted by conductive metals, all metal objects were removed from the patient to minimise interference during image acquisition. The stomach was scanned by a continuous translational movement along its long axis (~10 s). During each scan subjects were instructed to sit still and hold their breath at the end of inspiration. If gastric contractions were observed, the acquisition was paused until the contraction wave had passed. The raw data (original scan planes) were transferred for 3D reconstructions and volume estimation using EchoPAC

- 3D software (GE Vingmed Sound, Horten, Norway). Gastric retentions were calculated as total gastric volumes minus baseline 'empty' gastric volume at each time point expressed as percentage of the maximal gastric volume (100%), ie, ~450 mL volume of the ingested drink. When ultrasound images lacked sufficient clarity to determine the volume of the stomach, data were imputed by linear interpolation. The quality of ultrasound stomach images was insufficient to determine gastric emptying in all three conditions in one older subject, and this subject was, therefore, excluded from the analysis. The time at which 50% of the preload drink had emptied from the stomach (50% gastric emptying time; T50; min) and 'complete' emptying time (T100; min) of the drink, defined as the time when the residual volume of the drink in the stomach was ≤ 5%, was calculated for all conditions. Complete emptying time was set to 180 min when the residual volume at 180 min was ≤ 5%. Rate of gastric emptying was calculated as mean of rates of emptying during each 15-min interval respectively of the early (0-45 min) and late (45-180 min) phase and total (0-180 min) time period.

Perceptions of appetite and gastrointestinal symptoms

Perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating were rated using a visual analogue scale (VAS) questionnaire (47). The questionnaire consisted of 100-mm horizontal lines, where 0 represented that the sensation was 'not felt at all' and 100 represented that the sensation was 'felt the greatest'. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation at the specified time points. Baseline fasting ratings were calculated as mean of the three study days. One young and one older subject did not comply with the guidelines of the VAS questionnaires on one or more study days and were excluded from the analyses.

Data analysis

Statistical analyses were performed using SPSS software (version 21; IBM, Armonk, NY, USA). Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA. Relations of energy intake with the rate of gastric emptying (kcal/min) and appetite were evaluated by between- and within-subject correlations (277, 278). Statistical significance was accepted at $P < 0.05$. All data are presented as means ± SEMs.

RESULTS

The study protocol was well tolerated by all subjects.

Energy intake

Energy intake at the buffet meal was suppressed by whey protein compared with control (mean of young and older: there was a decrease in energy intake of 134 ± 38 kcal after the 30 g (120 kcal) and 105 ± 49 kcal after the 70 g (280 kcal) protein load; main effect of protein-load $P = 0.034$; **Figure 7.1**). The main effect of age ($P = 0.27$) and the interaction effect of age x protein-load ($P = 0.06$) were not significant.

Suppression of energy intake by whey protein compared to control was less in older men than young controls (young compared with older: 15 ± 2% compared with 1 ± 5%; main effect of age; $P = 0.008$; in young men 17 ± 3% by the 120-kcal and 12 ± 3% by the 280-kcal protein load compared to control [$P < 0.05$] and in older men 2 ± 5% and 0 ± 8% [$P > 0.05$]; **Figure 7.2**). The main effect of protein-load ($P = 0.61$) and the interaction effect of age x protein-load ($P = 0.68$) were not significant.

Cumulative energy intake during the protein days compared with the control day increased more in older men than young controls (young compared with older: 1 ± 3% compared with 18 ± 6%; main effect of age $P = 0.008$) and increased more during the 70 g than the 30 g

protein day (30 compared with 70 g protein load: 1 ± 4% compared with 19 ± 6%; main effect of protein-load $P = 0.011$). The interaction age x protein-load was not significant ($P = 0.68$).

Macronutrient proportions at the buffet meal did not differ between the age groups at baseline, nor on any of the other study days, and were not affected by either of the protein treatments ($P > 0.05$).

Cumulative energy intake (i.e. energy intake at the buffet meal plus energy content of the drink) during the protein day compared with the control day was age and protein-load dependent. The main effects of age ($P = 0.008$) and protein-load ($P = 0.011$) for cumulative energy intake during the protein days compared with the control day were significant; the interaction age x protein-load was not significant ($P = 0.68$).

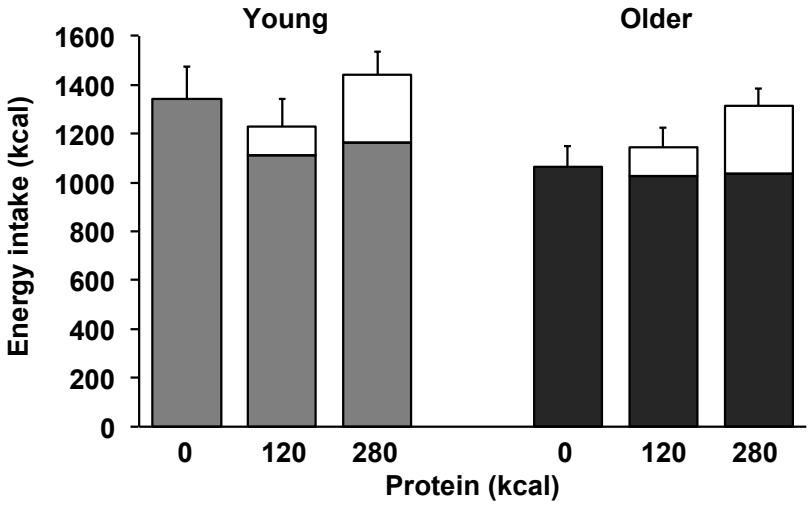

Figure 7.1: Mean (± SEM) energy intake (kcal) in young (grey shading; n = 8) and older (black shading; n = 8) subjects after drinks (open bars) containing water (control) and whey protein loads of 30 g and 70 g. Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.
The protein-drinks suppressed subsequent energy intake at the buffet meal compared with control. The main effect of protein-load for energy intake was significant ($P = 0.034$); the main effect of age ($P = 0.27$) and the interaction effect of age x protein-load ($P = 0.06$) were not significant.

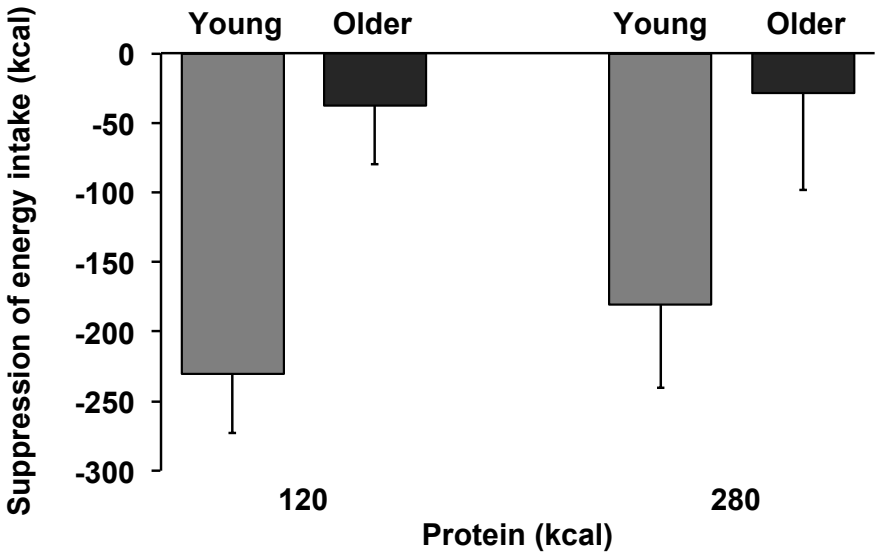

Figure 7.2: Mean (± SEM) suppression of energy intake at the buffet meal (kcal) in young (grey shading; n = 8) and older (black shading; n = 8) subjects after whey protein loads of 30 g and 70 g compared to after control (0 g whey protein). Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.
Suppression of energy intake by protein was less in older than young subjects. The main effect of age for suppression of energy intake was significant ($P = 0.008$); the main effect of protein-load ($P = 0.61$) and the interaction effect of age x protein-load ($P = 0.68$) were not significant.

Gastric emptying

Gastric emptying parameters are detailed in **Table 7.1**. Baseline gastric volumes were not different between young and older men (31 ± 6 mL compared with 39 ± 3 mL; $P = 0.28$). The control drink (water) emptied in a non-linear pattern, whereas the pattern of the 280-kcal protein drink was more linear, particularly in the older men - age appears to affect the *initial* rate of gastric emptying (**Figure 7.3**). After ingestion of all 3 study drinks gastric emptying was slower in older men than young controls, with T50 times (mean of 3 study days young compared with older of 36 ± 5 min compared with 68 ± 5 min; main effect of age $P = 0.007$), equating to an emptying rate of energy of (1.0 ± 0.0 kcal/min in the young subjects compared to 0.8 ± 0.0 kcal/min in the older subjects on the two protein days (effect of age $P = 0.022$), and higher measures of gastric retention (main effect of age $P = 0.003$).

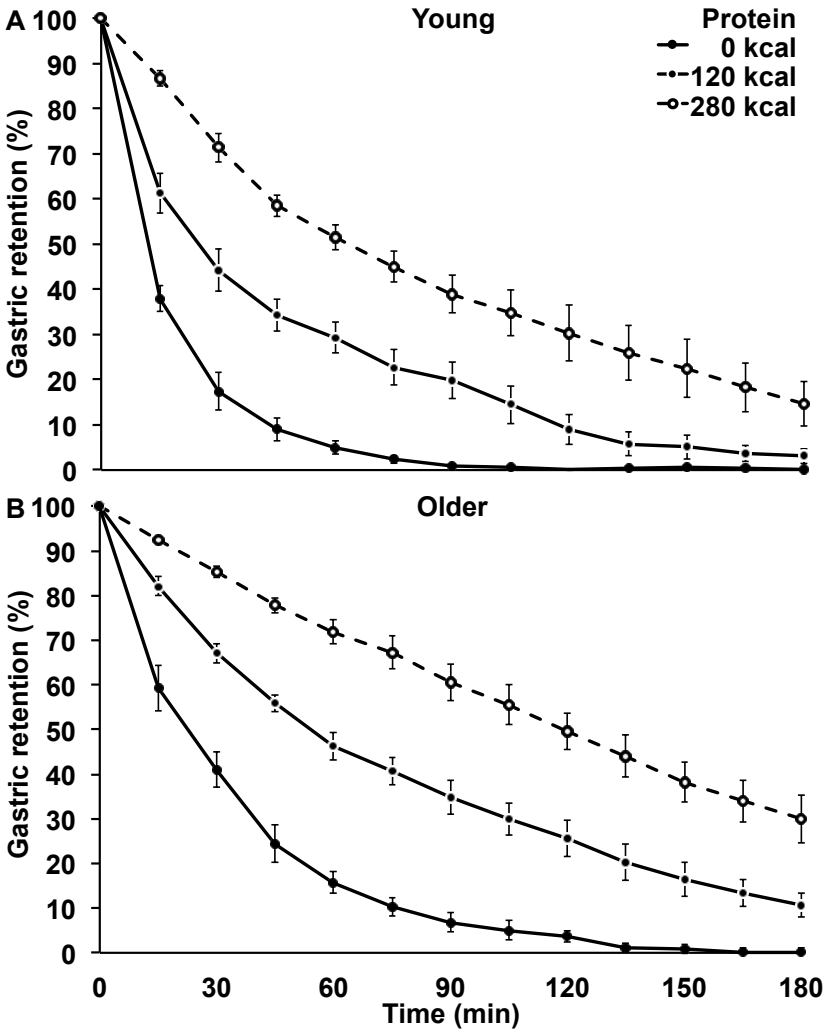

Figure 7.3: Mean (± SEM) gastric retention (%) in young (n = 8) and older (n = 7) subjects after drinks containing water (control) and whey protein loads of 30 g or 70 g. Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.
Gastric emptying of the water and protein drinks was slower in older than young men. The main effects of age and protein-load for the 50% gastric emptying time (T50; min; $P = 0.007$; $P < 0.001$) and gastric retention (%; area under the curve; $P = 0.003$; $P < 0.001$) were significant; the age x protein-load interactions for T50 ($P = 0.08$) and gastric retention ($P = 0.22$) were not significant.

Ingestion of the protein drinks resulted in a dose-dependent slowing of gastric emptying, of comparable magnitude in both age groups, with T50 more than a doubling from the control to the 120-kcal day (mean of all men; 17 ± 2 to 41 ± 5 min), with a further similar increase from the 120-kcal to the 180-kcal day (mean of all men; 41 ± 5 to 96 ± 11 min; main effect of protein-load; $P < 0.001$). Consequently rates of energy emptying (kcal/min) did not differ

between the 120-kcal and 280-kcal days. The age x protein-load interactions for T50 (P = 0.08) and gastric retention (P = 0.22) were not significant.

Table 7.1: Gastric emptying rate, T50, T100 and gastric retention (area under the curve; 0-180 min) of water (control) and protein drinks in young and older men[1]

	Young men (n = 8)			Older men (n = 7)		
	0 g	30 g	70 g	0 g	30 g	70 g
T50 (min)[2,3]	12±1[4,5]	25±4[4,5]	72±13[4,5]	23±3[4,5]	59±5[4,5]	123±13[4,5]
T100 (min)[2,6]	60±7[4,5]	126±14[4,5]	171±6[5]	109±13[4,5]	174±6[4,5]	174±6
Amount emptied at 180 min (%)[7]	100±0	98±1	86±5	99±0	89±2	70±5
Gastric retention (%)[3]	173±10	337±31	591±45	271±24	544±32	807±39
Rate of gastric emptying (0-180 min; kcal/min)[7]		0.7±0.0	1.3±0.1		0.6±0.0	1.1±0.1
Early phase of rate of gastric emptying (0-45 min; kcal/min)[6,7]		1.8±0.3[4,5]	2.6±0.2[4,5]		1.2±0.0[4]	1.4±0.1[4]
Late phase of rate of gastric emptying (45-180 min; kcal/min)[7,8]		0.3±0.0	0.9±0.1		0.4±0.0	1.0±0.1

[1]All values are means ± SEMs. Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.

[2]50% emptying time (T50; min). Complete emptying time (T100; min) of the drink was defined as the time when the residual volume of the drink in the stomach was ≤ 5% and T100 was set to 180 min when the residual volume at 180 min was ≥ 5%.
[3]The main effects of age and protein-load for gastric retention (AUC from baseline to 180 min; $P = 0.003$; $P < 0.001$) and T50 ($P = 0.007$; $P < 0.001$) were significant. The age (young, older) x protein-load (0, 120, 280 kcal) interaction for gastric retention (AUC from baseline to 180 min; $P = 0.22$) and T50 ($P = 0.08$) was not significant.
[4]$P < 0.05$; between age groups
[5]$P < 0.05$; between protein conditions
[6]The age x protein-load interaction and main effects of age and protein-load for T100 ($P = 0.048$, $P = 0.027$; $P < 0.001$) and early phase rate of gastric emptying (0-45 min; $P = 0.003$, $P = 0.001$; $P = 0.001$) were significant.
[7]Rate of gastric emptying was calculated as mean of rates of emptying during each 15-min interval respectively of the early (0-45 min) and late (45-180 min) phase and total (0-180 min) time period.
[8]The main effect of protein-load for the amount emptied at 180 min (%; $P < 0.001$), rate of gastric emptying (0-180min; $P < 0.001$) and late phase of rate of gastric emptying (45-180 min; $P < 0.001$) were significant. The age x protein-load interaction and main effect of age for the amount emptied at 180 min (%; $P = 0.17$; $P = 0.06$), rate of gastric emptying (0-180 min; $P = 0.14$; $P = 0.08$) and late phase of rate of gastric emptying (45-180 min; $P = 0.87$; $P = 0.23$) were not significant.

Perceptions of appetite and gastrointestinal symptoms

Appetite

Baseline ratings of desire to eat (young compared with older; 49 ± 8 compared with 32 ± 6 mm; $P = 0.11$), prospective food consumption (54 ± 8 compared with 43 ± 5 mm; $P = 0.25$), hunger (43 ± 8 compared with 34 ± 6 mm; $P = 0.37$) and fullness (17 ± 4 compared with 7 ± 3 mm; $P = 0.09$) were not statistically different between young and older men. Ratings of desire to eat, prospective food consumption, and hunger differed from baseline over time (0-180 min) during all study days in both age groups ($P < 0.05$); fullness increased from baseline in young ($P < 0.05$), but not in older ($P > 0.05$), subjects (**Figure 7.4**).

The main effect of age for AUC ratings of desire to eat (mean of 3 study days; young: a decrease of 647 ± 910 mm from baseline; older: an increase of 1027 ± 458 mm from baseline; $P = 0.024$) and prospective food consumption (mean of 3 study days; young: a decrease of 1616 ± 706 mmm from baseline; older: an increase of 119 ± 370 mm from baseline; $P =$

0.021) was significant, while the main effect of age for ratings of hunger (P = 0.48) and fullness (P = 0. 59) was not. The main effect of protein-load and the age x protein-load interaction for ratings of desire to eat, prospective food consumption, hunger and fullness were not significant (**Table 7.2**).

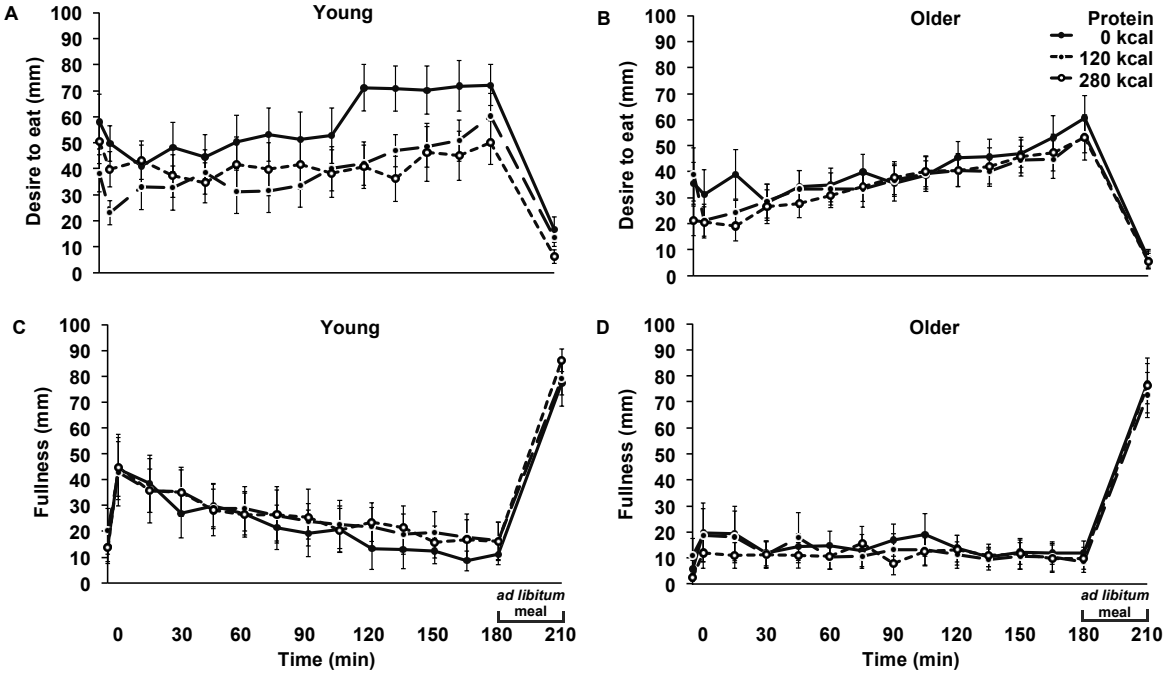

Figure 7.4: Mean (± SEM) visual analogue scores (mm; 0-180 min) of desire to eat (A and B) and fullness (C and D) in young (n = 7) and older (n = 7) subjects after whey protein loads of 30 g and 70 g and a control drink (water), and after the *ad libitum* buffet meal (210 min). Time (0-180 min) effects were determined by using repeated-measures ANOVA. Ratings of desire to eat changed from baseline during all study days in both age groups (P < 0.05); fullness was increased from baseline in young (P < 0.05), but not in older (P > 0.05), subjects.

Nausea and bloating

Baseline ratings of nausea (young compared with older; 13 ± 4 compared with 3 ± 1 mm, P = 0.040) were lower in older men than young controls, while ratings of bloating were not (8 ± 4 compared with 3 ± 1 mm, P = 0.29). Ratings of bloating increased in young (P < 0.05), but not in older (P > 0.05), subjects (**Figure 7.5**). The main effects of age and protein-load and age x protein-load interaction for ratings of nausea and bloating were not significant (**Table 7.2**).

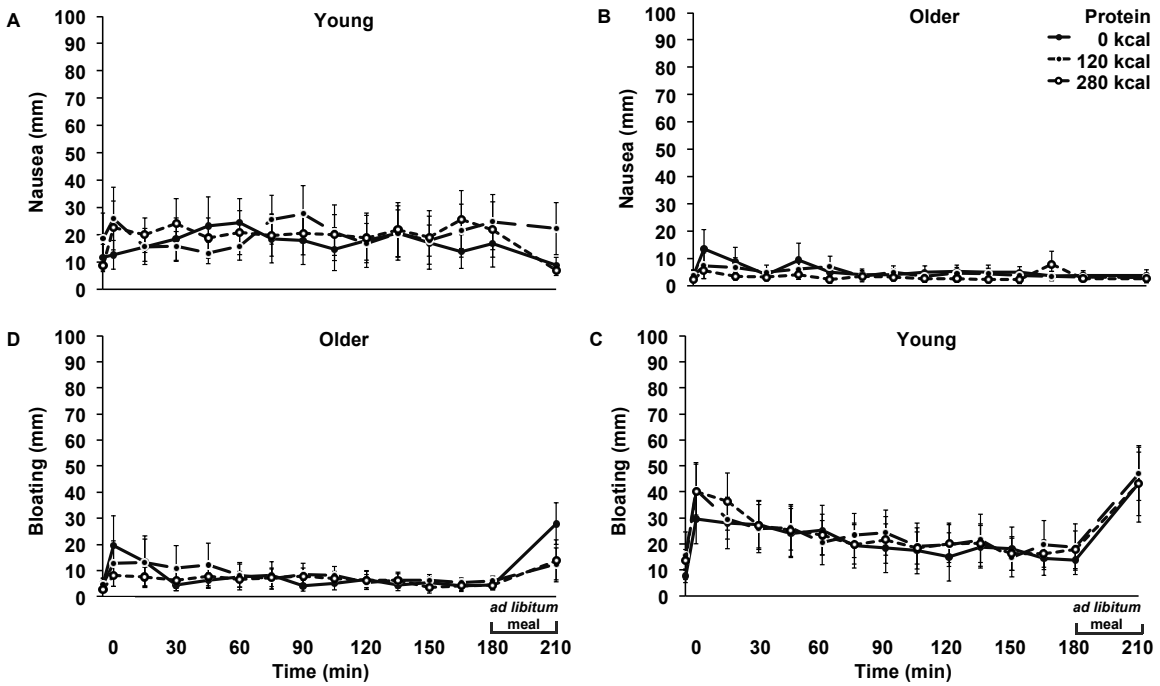

Figure 7.5: Mean (± SEM) visual analogue scores (0-180 min; mm) of nausea (A and B) and bloating (C and D) in young (n = 7) and older (n = 7) subjects after whey protein loads of 30 g and 70 g and a control drink (water), and after the *ad libitum* buffet meal (210 min). Time effects (0-180 min) were determined by using repeated-measures ANOVA.
Ratings of nausea were not different from baseline in either age group ($P > 0.05$); ratings of bloating were increased from baseline in young ($P < 0.05$), but not in older ($P > 0.05$), subjects.

Relations between energy intake with gastric emptying and perceptions of appetite

Energy intake (kcal) at the buffet meal was, *within subjects*, inversely related to gastric retention (AUC from baseline to 180 min; %) in young ($r = -0.54$, $P = 0.026$), but not older ($r = -0.16$, $P = 0.57$), men (all men; $r = -0.35$, $P = 0.05$); i.e., the slower the study drink emptied from the stomach (0-180 min) within a young subject - 70 g < 30 g < 0 g - the lower the subsequent energy intake (180-210 min).

Table 7.2: Hunger, desire to eat, prospective food consumption, fullness, nausea and bloating (area under the curve) after water (control) and protein drinks (0-180 min) in young and older men[1]

	Young men (n = 7)				Older men (n = 7)				
	0 g	30 g	70 g	Mean change by protein compared with control	0 g	30 g	70 g	Mean change by protein compared with control	*P*[2]
Desire to eat (mm)[3]	-86±485	186±835	-1534±1181	-588±860	933±678	-400±1311	2454±690	94±421	0.490
Prospective food consumption (mm)[3]	231±876	-1422±1082	-1811±1228	-1848±643	257±638	-793±846	1030±1095	-138±921	0.154
Hunger (mm)	1376±945	28±582	-553±1168	-1638±1065	167±719	-173±1804	2030±1405	761±862	0.105
Fullness (mm)	1453±1756	1521±602	2366±1086	490±1502	1670±697	275±1525	1655±790	-705±1007	0.521
Nausea (mm)	1021±1093	274±1717	2059±1160	145±1210	334±308	175±181	204±126	-145±209	0.817
Bloating (mm)	2186±1328	1311±2023	1581±1331	-739±913	342±376	618±475	595±366	265±166	0.301

[1]All values are means ± SEMs. Main age and protein-load effects and interaction effects were determined by using repeated-measures ANOVA.
[2] Age effect of mean change by protein (30 g and 70 g) compared with control (0 g) (ANOVA).

[3] The main effect of age for ratings of desire to eat (P = 0.024) and prospective food consumption (P = 0.021) was significant. The age (young, older) x protein-load (0, 120, 280 kcal) interaction for ratings of desire to eat (P = 0.10), prospective food consumption (P = 0.60), hunger (P = 0.33), fullness (P = 0.83), nausea (P = 0.47) and bloating (P = 0.34) was not significant. The main effect of age for ratings of hunger (P = 0.48), fullness (P = 0. 59), nausea (P = 0.19) and bloating (P = 0.34) was not significant. The main effect of protein-load for ratings of desire to eat (P = 0.46), prospective food consumption (P = 0.42), hunger (P = 0.74), fullness (P = 0. 64), nausea (P = 0.67) and bloating (P = 0.54) was not significant.

Suppression of energy intake at the buffet meal by protein compared to control was, *between subjects,* directly related to suppression of prospective food consumption by protein compared to control (r = 0.60, P = 0.024) in all men. There was no association between fullness ratings at 180 min (i.e., just before the buffet meal) and energy intake, in the young (r = 0.04, P = 0.92), older (r = 0.35, P = 0.40) or combined subjects (r = 0.18, P = 0.50). Suppression of energy intake by protein was, irrespective of age, directly related to energy intake during the control day (young: r = 0.88, P = 0.004; older: r = 0.71, P = 0.048; combined subjects: r = 0.82, $P < 0.001$). The age effect on the suppression of energy intake at the buffet meal by protein was still significant (P = 0.001) taking energy intake during the control day into account as a covariate.

DISCUSSION

This study examined the influence of ageing on the acute effects of oral whey protein consumption on suppression of energy intake, appetite and gastric emptying. The major finding was that suppression of energy by protein was less in healthy older men (1%) than young controls (15%), so that the cumulative energy intake (buffet meal plus preload drink) was increased more by the protein ingestion in older men (18%) than young controls (1%). These observations are consistent with our recent finding that the suppression of subsequent energy intake by intraduodenal infusions of whey protein was less in healthy older men (~1%) than young controls (~19%) (194). They are also consistent with reports of reduced responsiveness in older people to the suppressive effects of oral mixed macronutrient meals on energy intake (46, 63), and extend these to show that these age differences also apply when protein is ingested on its own.

The oral whey protein consumptions decreased the ratings of desire to eat and prospective food consumption when compared to the baseline, after overnight fasting, ratings in young,

whilst they were increased in the healthy older males. The ratings of prospective food consumption reflected subsequent energy intake in both age groups. The greater the reduction in these ratings after protein consumption the greater the reduction in subsequent energy intake. Thus, while older people are less hungry and eat less than younger adults (41, 42, 44-46, 88, 190), they appear to also be less susceptible to further suppression of appetite and eating behaviour by ingestion of energy and nutrients, including protein.

The finding of an age-related reduction in the satiating effects of protein is potentially important. In young adults protein is the most satiating macronutrient when ingested orally (280), and there is evidence that high protein diets promote satiety and facilitate deliberate weight loss during energy restriction diets in overweight, younger adults (281). While beneficial in *those* circumstances, protein-enriched nutritional supplements given to older people for management of undernutrition, could have unintended adverse effects, if the satiating effects of protein are undiminished, or increased, by age. The use of high-protein supplements by older people for this purpose is widespread and increasing. This is partly in response to greater awareness of the prevalence of undernutrition and sarcopenia in older people, and evidence that protein supplementation may increase both muscle mass and function (20, 271). The age-related reduction in suppression of appetite and feeding responses to oral protein, and to protein directly infused into the small intestine (194), suggest that if timing and preparation are optimised, it may be possible to give sufficient protein to older people to preserve, or increase muscle mass and function, without suppressing energy intake. The optimum composition for nutritional supplements for management of undernutrition in older people is not known. The nutritional supplements are probably best given in liquid form between meals (60) as suppression of energy intake by energy-containing beverages are less when compared to iso-energetic solid loads (304).

In the young men, suppression of energy intake by the 70 g whey protein was comparable with the 30 g, suggesting that there may be a threshold of maximum suppression in energy

intake. This observation is consistent with comparable suppression of energy intake by protein rich meals 3 hours after whey protein ingestion of 15 and 30 g (305) and 4 hours after oral protein intakes of 24, 44 and 80 g (306).

Cumulative energy intake was increased most by the highest protein dose (70 g); a substantial increase of 19% or 175 kcal. Although only a limited number of studies have examined the effects of state of nutrition in older people on the regulation of appetite, there is persuasive evidence of substantial differences between undernourished and healthy older people, which may potentially be an outcome of and/or contribute to the undernourished state. In particular, suppression of energy intake by a mixed nutrient preload was less in undernourished older women when compared to healthy older women (48). These findings raises the possibility that appropriately designed protein supplements might even act to increase overall energy intake in undernourished people by meaningful amounts.

The regulation of appetite and energy intake is dependent on the precise co-ordination of interrelated 'intragastric' and 'small intestinal' sensory and motor mechanisms, including variations in gastric emptying (on average 1-4 kcal/min) (261) and gastric distension (70). Gastric emptying of ingested nutrients results in a relatively constant rate of energy delivery of the ingested nutrient load from the stomach to the duodenum. Slower gastric emptying results in greater distension of the stomach at a given time after food ingestion. This can, in turn, lead to greater fullness and a consequent reduction in subsequent energy intake. The suppressive effect of gastric distension on energy intake has led to successful attempts to reduce energy intake and induce weight loss in obese people by implanting gastric balloons (307). While slower gastric emptying prolongs retention of food in the stomach favouring satiation, it also delays the onset of powerful satiety signals initiated by the interaction of nutrients with the small intestine (295).

In the present study, gastric emptying of the water and both protein drinks was slower in older men than young controls, consistent with results of previous studies (74, 78, 82). The

water drink emptied slower in older men than young controls, which implies an intragastric etiology. There was dose-dependent slowing of gastric emptying by protein to a similar degree in both age groups, with 50% gastric emptying time more than doubling from the control to the 30 g protein day, and from the 30 g to the 70 g day. The older subjects had a slower gastric emptying of the protein drinks than the young subjects (0.8 kcal compared with 1.0 kcal/min on average over 180 min), especially during the *initial* phase of emptying. Therefore the older men had greater intragastric volumes at all time points between protein ingestion and the buffet meal than the young men, despite the latter, fullness increased from baseline in young but not in older and the protein-induced suppression of subsequent energy intake was less in the older men compared with the young controls. This finding, and the lack of an association between fullness ratings at the start of the buffet meal and energy intake at that meal, suggests that age-related slowing of gastric emptying rate is not a major mediator of the age-related differences in protein-induced satiety at three hours after ingestion. It should, however, be recognised that to evaluate intragastric factors, the buffet meal has to be given much earlier. At the start of the meal, more than 85% of all drinks had emptied from the stomach, except the 70 g protein drink in the older subjects. Also all drinks, except the 70 g protein drink in the older subjects, emptied in a non-linear pattern, so once gastric emptying has started both intragastric volume and small-intestinal feedback were relevant. In older people, however, the perception of proximal gastric distension is diminished (84), which may explain why their slower gastric emptying had little if any suppressive effect on subsequent energy intake in this study. More likely, age-related differences in the satiating effects of orally ingested protein are mediated predominantly by mechanisms activated after the protein passes into the small intestine, e.g., gut hormone secretion (cholecystokinin, glucagon-like peptide-1, peptide tyrosine tyrosine and gastric inhibitory peptide) (114) and gut motility (269), rather than intragastric mechanisms, or by central mechanisms of amino acids, e.g., leucine, which is abundant in whey acts directly in

the central nervous system, to reduce intake. This is consistent with our previous finding of greater suppression of energy intake by protein in young than older men when protein is infused directly into the small intestine, thus removing any influence of differences in gastric emptying (194), implying that both intragastric and small intestinal mechanisms act to suppress energy intake are attenuated in healthy elderly. Further studies are needed to determine the nature of these mechanisms.

This study has several limitations. The subject numbers were relatively small. We studied only men, as they appear to have the greatest ability to regulate energy intake in response to energy manipulation (46). The results do not, therefore, necessarily apply to the effects of ageing in women. Further studies are needed to determine this, and also the effects of protein when administered as part of a mixed macronutrient supplement in both undernourished and obese older people. Nevertheless, our findings support the use of protein supplements to increase energy intake in older people.

The significant finding was that despite having slower gastric emptying, older men exhibited blunted protein-induced suppression of energy intake by oral whey protein compared with young controls, so that in the older men compared with the young controls protein ingestion increased overall energy intake more. The observations support the use of protein supplements in undernourished older people, but suggest that their use as a strategy to decrease energy intake in older obese individuals may not be effective. Future studies are needed to characterise the effects of protein and carbohydrate and fat in isolation and as a mixture, both in a liquid and solid form, in malnourished, i.e., underweight and obese, elderly and, thereby, provide comprehensive insights into the underlying mechanisms, and lead to improved, evidence-based, strategies for the use of supplements to increase energy intake in older undernourished individuals or to decrease energy intake as part of a weight-loss diet strategy in obese elderly.

CHAPTER 8: EFFECTS OF TIMING OF WHEY-PROTEIN INTAKE ON APPETITE AND ENERGY INTAKE IN HEALTHY OLDER MEN

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Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	October 2017

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

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

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

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ABSTRACT

Background: Protein-rich supplements are used widely to prevent and manage malnutrition in older adults. We previously showed that 30 g whey-protein ingestion, three hours before a buffet meal, suppressed energy intake in young, but not in older, men. Information about the impact of the timing of ingestion of protein drinks on the suppression of energy intake in older adults is lacking.

Objective: The aim of the study was to determine the effect of the timing of whey-protein ingestion on appetite and subsequent *ad libitum* energy intake in healthy older men.

Design: In a single blind, randomised design, sixteen older men were studied on five occasions, on which they consumed a whey-protein drink (30 g/ 120 kcal, 140 mL) three, two, one hour(s), or immediately before a buffet meal, from which *ad libitum* energy intake was quantified, and iso-palatable non-caloric drinks (~1 kcal) at the remaining time-points. On the control day, non-caloric drinks were ingested at all time points. Perceptions of appetite and gastrointestinal symptoms were determined, by visual analogue scales, throughout the study days.

Results: There was no effect of the timing of protein ingestion on perceptions of appetite and gastrointestinal symptoms ($P > 0.05$) or energy intake at the buffet meal (3 h: 888 ± 49 kcal, 2 h: 879 ± 56 kcal, 1 h: 909 ± 47 kcal, 0 h: 892 ± 51 kcal, control: 930 ± 49 kcal, $P = 0.94$). Total energy intake (i.e., preload + test meal) was higher on the protein days compared to control (82 ± 24 kcal increase, $P = 0.003$).

Conclusions: In older men ingestion of 30 g protein increased total energy intake, irrespective of the time of intake in relation to the meal. These observations support the use of 'pure' whey-protein drinks to increase overall protein and energy intake in older adults at risk of undernutrition.

INTRODUCTION

The prevalence of undernutrition in the ageing population, which is associated with reduced functional capacity and decreased quality of life, has increased over recent decades (6, 180, 190, 285). A growing awareness of the prevalence, and substantial adverse effects, of the major muscle loss that occurs during ageing has stimulated the development of nutritional strategies designed specifically to preserve and/or restore skeletal muscle mass and function. A 'common' strategy is the use of supplements, which are usually high-energy drinks rich in whey protein (26, 27, 35, 286). Older people ingest protein in the range of ~40-66 g/day, less than the recommended minimum of 30 g protein intake per meal (i.e., ≥ 90 g/day)(25, 308). Despite the widespread use of protein-rich drinks, information about their effects on energy intake in older adults is largely lacking.

In young adults protein is the most satiating of the macronutrients(280) and the timing of intake affects the suppression of subsequent energy intake. For example, in young women less food is consumed during an *ad libitum* meal when a preload is administered 30 min, compared to intakes at 60 or 120 min, before a meal(309). Also a recent systematic review indicated that, in young and middle-aged adults (18-65 years), i) *ad libitum* energy intake at a meal is suppressed most when a mixed-macronutrient preload is given no more than 30 min before the meal, ii) whereas an inter-meal interval of 30-120 min suppresses energy intake modestly and, iii) an inter-meal interval of 120 min or more is likely to increase total energy intake (i.e., preload + test meal), compared to a control day(310).

To our knowledge only one study has evaluated the effect of the timing of supplements on appetite (i.e., inter-meal interval) and subsequent *ad libitum* energy intake in older individuals. Wilson *et al.* reported that total energy intake was higher when a mixed macronutrient preload (300 kcal) was consumed 60 min or more before a meal, compared to when the preload was given directly before, the meal in a group of younger (23-35 years,

BMI: 20-25 kg/m^2) and older adults (70-85 years, BMI: 21-24 kg/m^2) combined(60). A limitation of this study was that the potential effect of the timing of supplement intake could only be analysed as main time-effect (younger and older adults combined) due to the specificity of the study design [i.e., three-factor interaction between age, preload-type (water, high-protein, high-fat, high-carbohydrate) and time (≥ 60 min compared to directly before the meal)](60). Accordingly, whether the timing of ingestion of a protein supplement influences energy intake in older subjects is not known. Despite this; based on the above findings, recommendations have been made that supplements are best given between meals with a substantial time gap between the supplement and the next meal, to maximise overall protein and energy intake (60, 311-314). We recently reported that administration of both a 30 g- and 70 g-protein drink (~450 mL) three hours before a subsequent buffet meal, suppressed *ad libitum* energy intake substantially in younger (25 ± 2 years; suppression of energy intake after a protein load of 30 g compared to control: 17 ± 3%; 70 g: 12 ± 3%), but not in older (73 ± 1 years; 30 g: 2 ± 5%; 70 g: 0 ± 8%), men (263). These marked differences in suppression of energy intake by protein is not surprising in view of the 'anorexia of ageing' (6) - healthy ageing is associated with a reduction in appetite and food intake (315). The aim of this study was to determine the effects of the timing of whey-protein intake on appetite and energy intake in healthy older men. Given the lesser suppression of energy intake by protein in older than younger people, we hypothesised that the timing of protein-supplement administration can be much more liberal in older adults so that they can be given closer to meals without a substantial suppressive effect on subsequent *ad libitum* energy intake.

SUBJECTS AND METHODS

Subjects

The study included 16 Caucasian older men [age: Mean ± SEM: 76 ± 1 years (range: 66 – 85 years); body weight: 81 ± 2 kg (67 – 94 kg); height: 1.75 ± 0.01 m (1.65 – 1.85 m); body mass index (BMI): 27 ± 1 kg/m^2 (22 – 31 kg/m^2)] who were recruited by advertisement. Exclusion criteria were smoking, consuming > 10 alcoholic drinks per week, diabetes, gastrointestinal surgery (apart from uncomplicated appendectomy), significant gastrointestinal symptoms (abdominal pain, gastro-oesophageal reflux, diarrhoea, or constipation), use of medications known to potentially affect appetite, food intake, or gastrointestinal motor function, impaired cognitive function [score < 25 on Mini Mental State(274)], depression [score ≥ 11 on the Geriatric Depression Questionnaire(275)] and undernutrition [score < 24 on the Mini Nutritional Assessment(276)].

The Royal Adelaide Hospital Human Research Ethics Committee approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki. The study was registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number ACTRN12615000070538). All subjects provided written informed consent prior to their inclusion.

Protocol

In randomised order (using the method of randomly permuted blocks; www.randomization.com), each subject was studied on five occasions, each separated by 3-14 days. On the protein study days an oral whey-protein load (30 g/ 120 kcal) was ingested at either three (P3), two (P2), one (P1) hour(s), or immediately before (P0) a buffet meal, and iso-palatable, non-caloric drinks (~1 kcal) were consumed at the remaining time points. On the remaining control day, non-caloric drinks were ingested at all time points; i.e., at

three, two, one hour(s), and immediately before the meal. Subjects were blinded to the treatment order. Drinks were served in a covered cup and matched for taste by using diet lime cordial, as per our previous protocol (263). Protein drinks (30 g/ 120 kcal, 140 mL) were prepared by dissolving whey-protein isolate (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in 70 mL of water and 50 mL of diet lime cordial (Bickford's Australia Pty Ltd, Salisbury South, SA, Australia). Control drinks were made up of 90 mL of water and 50 mL of diet lime cordial.

Subjects were provided with a standardised evening meal [beef lasagne (McCain Foods Pty Ltd, Wendouree, VIC, Australia), ~591 kcal] to consume on the night before each study day at 07.00 pm. They were instructed to fast overnight from solids and liquids and to refrain from strenuous physical activity and alcohol for the 24 hours before they attended the laboratory at the Discipline of Medicine, the University of Adelaide, Royal Adelaide Hospital, at 08.30 am.

Perceptions of appetite and gastrointestinal symptoms were determined immediately before (during fasting; 0 min), after ingestion of each drink (+5 min), and at 30-min intervals until 180 min by validated visual analogue scales (VAS), i.e., 0, 5, 30, 60, 65, 90, 120, 125, 150, 180, 185 min (47). Subjects were instructed to consume each drink within ~2 min. At 185 min, subjects were presented with a standard, cold, buffet meal, in excess of what they were expected to consume, and instructed to eat freely for up to 30 min until comfortably full (185-215 min; ~12.00-12.30 pm)(263). The composition of the buffet meal is presented in **Table 4.1**.

Measurements

Energy intake

The amount eaten (g) was quantified by weighing the buffet meal before and after consumption. Energy intake (kcal) at the buffet meal and proportions of protein, carbohydrate and fat (energy-percent) were calculated using commercially available software (Foodworks; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake was calculated both as intake at the buffet meal and as the total energy intake; defined as the sum of energy intake at the buffet meal and the energy content of the whey-protein preload drink (263). Absolute (kcal) and percentage suppression of energy intake at the buffet meal (expressed as % of energy intake of the control day) for a given time of protein consumption compared to control were calculated.

Habitual energy intake was assessed after the initial screening visit by a food diary maintained for three successive days, either three weekdays or two weekdays and a weekend day, and was calculated using commercially available software (Foodworks; Xyris Software Pty Ltd, Spring Hill, QLD, Australia).

Perceptions of appetite and gastrointestinal symptoms

Perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating were rated using a visual analogue scale (47). The questionnaire consisted of 100-mm horizontal lines, where 0 represented that the sensation was ‘not felt at all’ and 100 represented that the sensation was ‘felt the greatest’. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation at the specified time points.

Data analysis

Statistical analyses were performed using SPSS software (version 21; IBM, Armonk, NY, USA). On the basis of our previous work (194, 263) we calculated that 16 subjects would allow for a detectable difference of 169 kcal of suppression of energy intake compared with control between the timing conditions of the whey-protein ingestions, i.e., at three, two, one hour(s) or immediately before the buffet-style meal. Main time effects were determined by using repeated-measures ANOVA and post-hoc Bonferroni corrections. Time effects of perceptions of appetite and gastrointestinal symptoms were determined with repeated measures ANOVA. Comparisons of perceptions of appetite and gastrointestinal function between baseline and 185 min, and before and after drink consumption, were determined with paired two-tailed student t-tests. Relations of energy intake with perceptions of appetite were evaluated by within-subject correlations(277). Statistical significance was accepted at $P < 0.05$. Area under the curves (AUCs) were calculated by using the trapezoidal rule. All data are presented as means ± SEMs.

RESULTS

The study protocol was well tolerated by all subjects. Habitual energy intake, assessed by 3-day food diaries, was 2,406 ± 177 kcal, with proportions of protein, carbohydrate and fat of 18 ± 1, 41 ± 2 and 35 ± 1 energy-percent. Habitual energy intake correlated positively with energy intake during the control day (r = 0.608; $P = 0.012$).

Energy intake

Energy intake at the buffet meal (**Figure 8.1**) did not differ between study days (protein load at three, two, one hour(s), or just before the meal and control day; P3: 888 ± 49 kcal, P2: 879 ± 56 kcal, P1: 909 ± 47 kcal, P0: 892 ± 51 kcal, control: 930 ± 49 kcal; $P = 0.73$). Total

energy intake during the four protein days was greater compared with the control day (average increase of 82 ± 24 kcal *ad libitum* energy intake after protein drink compared to control, 10 ± 3%; $P = 0.004$).

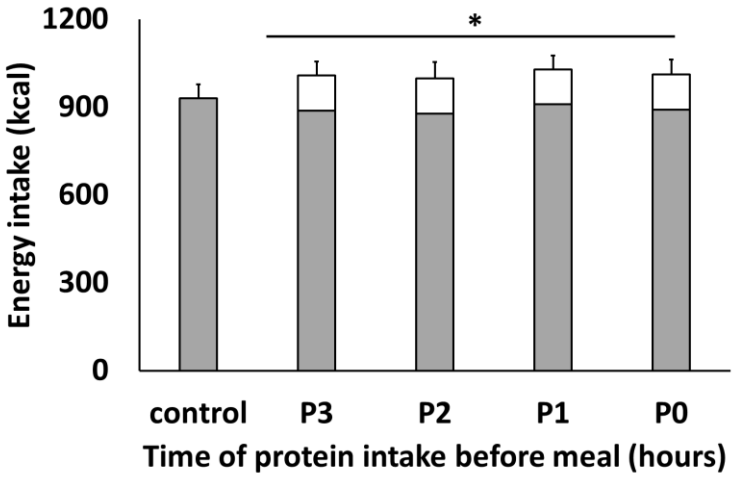

Figure 8.1: Mean (± SEM) *ad libitum* energy intake (kcal) in older men (n = 16) after drinks (open bars) during a control study day (no protein) and on protein study days with administration of a 30 g whey-protein drink three (P3), two (P2), one (P1) hour(s), or immediately before (P0) a buffet meal.
The time effect was determined by repeated-measures ANOVA ($P > 0.05$, energy intake).
* $P = 0.004$ Mean total (preload plus test meal) energy intake protein days compared to control day (ANOVA).

Perceptions of appetite and gastrointestinal symptoms

Baseline and AUC perceptions of hunger (48 ± 8 mm; 8546 ± 1336 mm), desire to eat (41 ± 7 mm; 8083 ± 1149 mm), prospective food consumption (47 ± 6 mm; 9048 ± 1046 mm), fullness (6 ± 2 mm; 2458 ± 630 mm), nausea (6 ± 2 mm; 1252 ± 319 mm) and bloating (5 ± 2 mm; 1520 ± 335 mm) were not different between study days ($P > 0.05$).

Perceptions of hunger ($P = 0.035$), desire to eat ($P = 0.002$), prospective food consumption ($P = 0.040$), fullness ($P = 0.001$) and bloating ($P = 0.010$), but not nausea (all $P = 0.22$), changed over time from baseline (**Figure 8.2**). Perceptions of desire to eat (10 ± 5 mm; $P = 0.047$), fullness (14 ± 4 mm; $P = 0.005$) and bloating (7 ± 2 mm; $P = 0.009$), but not hunger (4 ± 5 mm; $P = 0.43$), prospective food consumption (7 ± 4 mm; $P = 0.13$) and nausea (2 ±

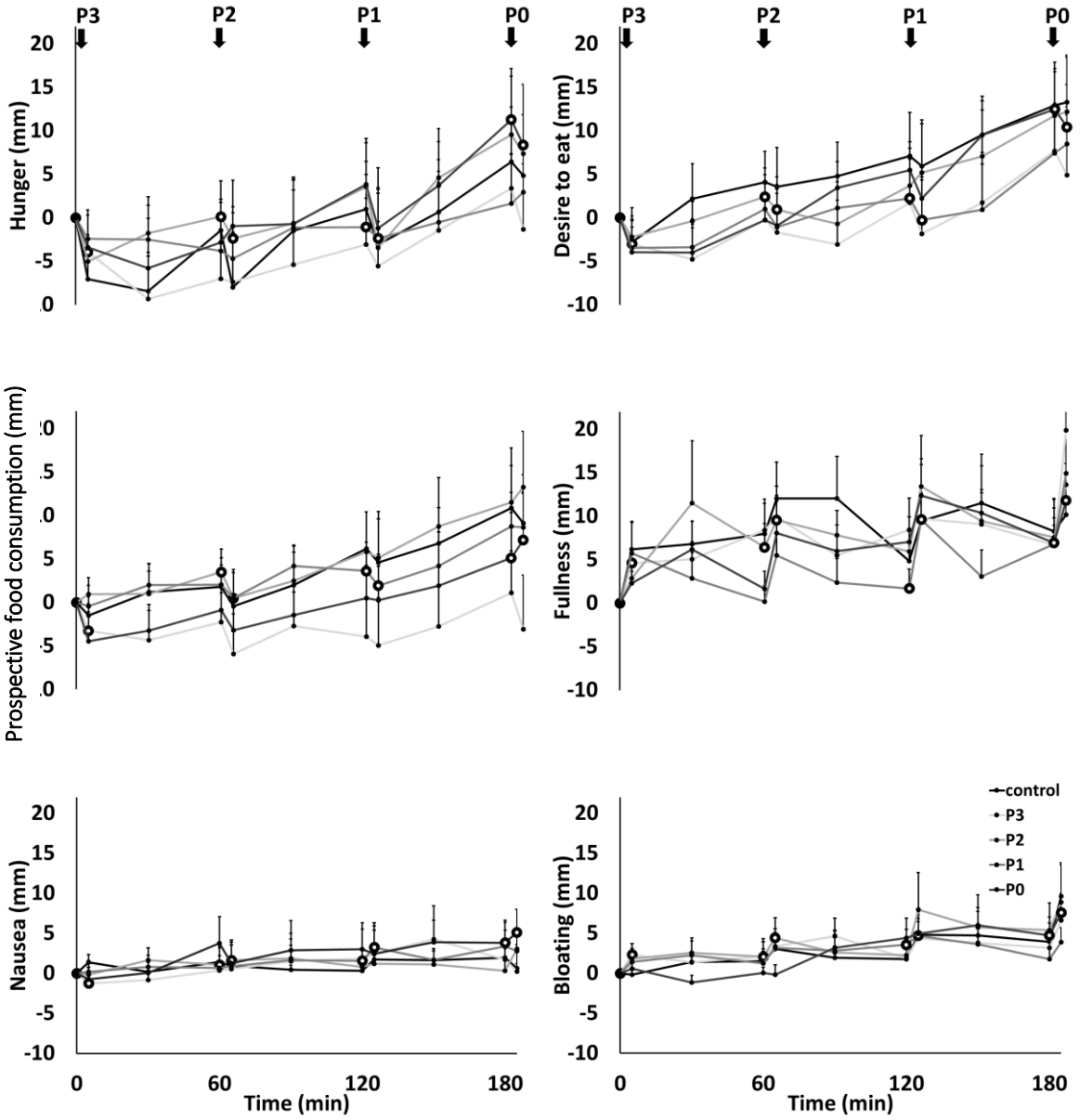

Figure 8.2: Mean (± SEM) visual analogue score (VAS) rating of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating in older men (n = 16) during the control study day (black line) and during the protein study days with consumption of a 30 g whey-protein drink three (P3), two (P2), one (P1) hour(s), or just before (P0) a buffet meal. The time points of protein administration are indicated with a larger round open marker (O). The time effect was determined by repeated-measures ANOVA ($P > 0.05$, area under the curve for hunger, desire to eat, prospective food consumption, fullness, nausea and bloating).

2 mm; $P = 0.16$), were higher after the final drink immediately before the meal (185 min) compared to baseline. Both drinks; the 30 g whey-protein and the iso-palatable control drink, induced comparable reductions, 5 min post compared to pre drink ingestion, in hunger (protein drink: 3 ± 1 mm reduction, $P = 0.030$; control drink: 3 ± 1 mm reduction, $P = 0.038$;

protein drink compared to control drink $P = 0.60$), and increases in fullness (protein: 5 ± 2 mm increase, $P = 0.015$; control: 5 ± 1 mm increase, $P = 0.001$; protein compared to control $P = 0.99$) and bloating (protein: 2 ± 1 mm increase, $P = 0.024$; control: 2 ± 1 mm increase, $P = 0.029$; protein compared to control $P = 0.99$). There were no changes, in desire to eat (protein: 2 ± 2 mm reduction, $P = 0.23$; control: 2 ± 1 mm reduction, $P = 0.17$; protein compared to control $P = 0.46$), prospective food consumption (protein: 1 ± 1 mm reduction, $P = 0.26$; control: 1 ± 1 mm reduction, $P = 0.20$; protein compared to control $P = 0.97$) or nausea (protein 1 ± 1 mm increase, $P = 0.63$; control 0 ± 0 mm, $P = 0.84$; protein compared to control $P = 0.61$).

DISCUSSION

This study evaluated the acute effects of the timing of consumption of a 30 g whey-protein preload on energy intake, appetite and gastrointestinal symptoms in healthy older men. The protein drink did not suppress *ad libitum* energy intake when administered three, two, one hour(s), or just before a meal. However, there was a comparable effect of the whey-protein preload to increase total (i.e., preload plus test meal) energy intake by ~10% compared to the control study day in the older men, irrespective of the timing of its ingestion. These observations support the use of 'pure' whey protein to increase overall protein and energy intake in older adults at risk of undernutrition.

We have previously shown that an identical 30 g whey-protein drink suppresses energy intake in young people when it is given 180 min before a meal (263). The lack of suppression of energy intake in older adults is likely to be associated with a reduced responsiveness to the suppressive effects of nutrients on appetite and energy intake (46, 63, 194, 263).

The 30 g protein dose used in this study has the capacity to increase body weight, particularly when ingested several times per day. It is also, in the range reported to have favourable

effects on muscle mass (23) and, therefore, often recommended as an appropriate dose for supplementation in older people (25, 316). Lower amounts of protein may not reach the threshold for postprandial muscle protein accretion (23, 25). Our observations support the use of protein supplements in older people to preserve or increase skeletal muscle mass and function without suppressing energy intake at the following meal. Accordingly the timing of ingestion of protein or protein-rich supplements by older people relative to usual meals may not be important, as previously proposed (60, 311, 312), so that they can even be taken just before the usual meal without suppressing subsequent food intake.

Studies have shown that frail, older people who performed resistance exercise and consumed supplemental dietary protein for 24 weeks had significant muscle gain, together with increased muscle strength and performance (317). In older people, administration of protein immediately after (resistance) exercise results in increased muscle hypertrophy compared to administration of protein two hours after completion of the training bout (318). Given the outcomes of this study it is likely that timing of protein supplements related to physical exercise is much more important than the timing related to food intake.

This study has limitations which should be appreciated. The subject numbers were relatively small, although the results seem to be clear-cut. Only one dose (30 g/ 120 kcal) of whey protein was used, and the effect may not apply to other macronutrients (i.e., fat and carbohydrate). The timing of the whey-protein drink varied in relation to the buffet meal (three, two, one hour(s), or immediately before the meal), but timing of assessment of *ad libitum* energy intake from ingestion of the first drink was identical in all study days, i.e., three hours thereafter. Also a consequence of the study design, the protein study days (i.e., 30 g whey-protein drinks of 140 mL administered three, two, one hour(s), or just before the meal) contained three control study drinks for blinding the timing of protein ingestion (total volume of 560 mL in both control and protein study days), therefore, the volume effect of the drinks should be appreciated. In older people, however, the perception of proximal

gastric distension is diminished (84). Also we did not determine gastrointestinal mechanisms (e.g., gastric emptying and gut hormones).

In conclusion, in healthy older subjects, a 30 g whey-protein supplement has no suppressive effect on *ad libitum* energy intake within three hours of administration, and increases total energy intake (preload plus test meal), irrespective of the time of intake before the meal. This supports the use of 'pure' whey supplements to increase overall protein and energy intake in older adults at risk of undernutrition.

CHAPTER 9: EFFECTS OF RANDOMISED WHEY PROTEIN LOADS ON ENERGY INTAKE, APPETITE, GASTRIC EMPTYING AND PLASMA GUT HORMONE CONCENTRATIONS IN OLDER MEN AND WOMEN

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Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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ABSTRACT

Background: Protein- and energy-rich supplements are used widely for the management of malnutrition in the elderly. Information about the effects of protein on energy intake and related gastrointestinal mechanisms and whether these differ between men and women is limited.

Objective: The aim of the study was to determine the effects of whey protein on energy intake, appetite, gastric emptying and gut hormones in healthy older men and women.

Design: Eight older women and eight older men (mean ± SEM; age 72 ± 1years and BMI 25 ± 1 kg/m^2) were studied on three occasions in which they received protein loads of 30 g (120 kcal) or 70 g (280 kcal), or a flavoured-water control-drink (0 kcal). At regular intervals over 180 min, appetite (visual analogue scales), gastric emptying (3D-ultrasonography), blood glucose and plasma gut hormone concentrations (insulin, glucagon, ghrelin, CCK, GIP, GLP-1 and PYY) were measured and *ad libitum* energy intake was quantified from a buffet meal (180-210 min - energy intake, appetite and gastric emptying in the men have been published previously).

Results: Energy intake at the buffet meal was ~80% higher in older men than older women ($P < 0.001$). Energy intake was not suppressed by protein compared to control in men or women ($P > 0.05$). There was no effect of gender on gastric emptying, appetite and gastrointestinal symptoms, glucose or gut hormones ($P > 0.05$). There was a protein load-dependent slowing of gastric emptying, increase in concentrations of insulin, glucagon, CCK, GIP, GLP-1 and PYY, and increase in total energy intake (drink + meal, 12% increase with 30 g, 32% increase with 70 g, $P < 0.001$). Energy intake at the buffet meal was inversely related to stomach volume and the area under the curve of hormone concentrations ($P < 0.05$).

Conclusions: In older men and women whey protein drinks, compared to control, load-dependently slowed gastric emptying, and altered gut hormone secretion, but had no suppressive effect on subsequent *ad libitum* energy intake.

INTRODUCTION

Over recent decades, the prevalence of malnutrition, both under-nutrition and obesity, has increased in older men and women (285, 319). A growing awareness of the prevalence and adverse effects of the major muscle loss that occurs during ageing, irrespective of body mass index (BMI, kg/m^2), e.g. reduced functional capacity and decreased quality of life (6, 190, 285), has led to the development of nutritional strategies designed specifically to preserve and/or restore skeletal muscle mass and function. A 'common' strategy is the use of supplements which are usually high-energy drinks rich in whey protein (26, 27, 35, 286, 320).

Despite this increasing use of protein-rich drinks, information about their effects on energy intake and underlying gastrointestinal mechanisms in older men and, particularly, older women, is lacking. In young adults protein is the most satiating of the three macronutrients (280). After a mixed macronutrient preload young women, when compared to young men, appear to compensate less in the subsequent meal resulting in a higher total (i.e. meal plus preload) energy intake (321, 322). Variations in gut hormone secretion and/or action [e.g., ghrelin, cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide/ gastric inhibiting polypeptide (GIP), glucagon-like polypeptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY)], as well as the rate of gastric emptying and gastric distension are likely to play a role in the regulation of appetite and energy intake in younger adults, particularly in the short-term after nutrient ingestion (70, 112-114, 234, 237, 288).

Compared to young adults healthy older people exhibit decreased taste and food palatability, are less hungry and more full during fasting and postprandial states, and consume less food and energy, including protein (315). This has been termed 'the physiological anorexia of ageing' (6, 190). Healthy ageing is associated with a reduced responsiveness to the suppressive effects of nutrients on appetite and energy intake (46, 63, 194, 263). We have

recently demonstrated that acute administration of 30 g (120 kcal), and 70 g (280 kcal) oral whey protein loads suppressed subsequent energy intake by 12-17% in young, without suppression in healthy older, men (263). Accordingly, protein ingestion increased total energy intake (protein plus subsequent *ad libitum* energy intake) compared to control in older men more than in the young men.

In this study we aimed to further characterise the feeding and gut (hormone) responses to orally ingested whey protein loads in older people, by studying older women as well as men. We hypothesised that orally administered whey protein would have a load-related effects on gastric emptying and plasma gut hormone concentrations (insulin, glucagon, ghrelin, CCK, GIP, GLP-1, PYY) in healthy older men and women, suppress of subsequent *ad libitum* energy intake less and, therefore, result in a greater increase in overall energy intake (protein drink plus meal intake) compared to control in older women than older men (previously published data of energy intake, appetite and gastric emptying).

SUBJECTS AND METHODS

Subjects

The study included 8 older men [age: Mean ± SEM: 73 ± 1 years; body weight: 77 ± 4 kg; height: 1.73 ± 0.02 m; BMI: 26 ± 1 kg/m^2; the men were included in our previous study comparing energy intake, appetite and gastric emptying between young and older men (263)] and 8 older women (70 ± 1 years; 63 ± 2 kg; 1.58 ± 0.02 m; 25 ± 1 kg/m^2) who were recruited by advertisement. There were no differences in either age (P = 0.14) or BMI (P = 0.70) between the males and females. On the basis of our previous work (194), with an observed within-subjects SD of 181 kcal and upper 60% confidence limit of 234 kcal, we calculated that 8 subjects per group would allow detection of a within-groups difference between treatments of 271 kcal (n = 8 older women), and a between genders difference of 353 kcal

(n = 8 older women compared with n = 8 older men), with power equal to 0.8 and alpha equal to 0.05.

Exclusion criteria were smoking, alcohol abuse, use of illicit substances, diabetes, gallbladder or pancreatic disease, gastrointestinal surgery (apart from uncomplicated appendectomy), gastrointestinal symptoms (abdominal pain, gastro-oesophageal reflux, diarrhoea, or constipation), use of medications known to potentially affect energy intake, appetite or gastrointestinal motor function, impaired cognitive function [score < 25 on Mini Mental State (274)], depression [score ≥ 11 on the Geriatric Depression Questionnaire (275)] and undernutrition [score < 24 on the Mini Nutritional Assessment (276)], being lactose intolerant or having food allergies, low ferritin levels or blood donation in the 12 weeks prior to the study days, and failing to comprehend the study protocol. The Royal Adelaide Hospital Human Research Ethics Committee approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki. The study was registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number ACTRN12612000941864). All subjects provided written informed consent prior to their inclusion.

Protocol

The protocol was identical to that of our previous study comparing young and older men and the results (i.e. energy intake, appetite and gastric emptying) in the older men have been published previously (263). Each subject was studied on 3 occasions, separated by 3-14 days, to determine the effects of two oral whey protein loads (30 g/ 120 kcal and 70 g/ 280 kcal) and a flavoured water control-drink (~0 g protein) on energy intake, gastric emptying, perceptions of appetite and gastrointestinal symptoms, in a randomised [using the method of

randomly permuted blocks; www.randomization.com (16 subjects randomised in 1 block with random permutations)], double-blind, cross-over design.

Protein drinks were served in a covered cup and prepared by dissolving whey protein isolate (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in varying volumes of demineralised water and diet lime cordial (Bickford's Australia Pty Ltd, Salisbury South, SA, Australia) to achieve the desired loads [i.e. 30 g whey protein (volume of the powder: 19 mL) in 335 mL distilled water and 85 mL cordial (2.5 kcal/100mL), 70 g whey protein (volume of the powder: 45 mL) in 280 mL water and 100 mL cordial, the control drink consisted of 359 mL distilled water and 90 mL cordial]. Sodium chloride in the amount of 0.3 g and 1.2 g was added to the 30 g and control drinks, respectively, to match the osmolality (88 mOsm/L) with the 70 g drink. To ensure that all ingredients were dissolved evenly throughout and to minimise the layer of foam on top of the solution, the drinks were stirred continuously at low speed on a stirring plate. The volume of each drink was measured before serving and the recorded volumes differed modestly (i.e. control: 450 mL; 30 g protein: 439 mL; and 70 g protein: 425 mL). All drinks were provided to participants in a covered cup, so that both the investigator conducting the data collection and the participants were blinded to the test drinks. The drinks were prepared by a research assistant who was not involved in data analysis of the study results.

Subjects were provided with a standardised evening meal [beef lasagne (McCain Foods Pty Ltd, Wendouree, VIC, Australia), ~591 kcal] to consume on the night before each study day at ~19.00 h. They were instructed to fast overnight from solids and liquids and to refrain from strenuous physical activity until they attended the laboratory at the Discipline of Medicine, The University of Adelaide, Royal Adelaide Hospital, at ~08.30 h.

On arrival, subjects were seated in an upright position on a wooden chair, where they remained for the duration of the study, and an intravenous cannula for blood taking was inserted. In each subject, measurements of total gastric volume and perceptions of appetite

and gastrointestinal symptoms were performed immediately before (during fasting; 0 min), and immediately after, ingestion of the drink, and at 15-min intervals until 180 min. Subjects were instructed to consume the drink within 2 min. Gastric volume was measured by 3-dimensional (3D) ultrasonography (263). Perceptions of appetite and gastrointestinal symptoms were assessed using validated visual analogue scales (VAS) and blood samples were collected for the measurements of gut hormones. At 180 min, each subject was presented with a standard, cold, buffet-style meal in excess of what they were expected to consume (total energy content of 2,457 kcal; 19% protein, 50% carbohydrates, 31% fat) for 30 min (180-210 min) until comfortably full, in a room by themselves to limit external distractions (257). The buffet-style meal consisted of palatable food items including sliced bread, cheese, ham and chicken, fruits, yoghurt, custard, margarine, mayonnaise, iced coffee, orange juice, fruit salad and water (263). The composition of the buffet meal is presented in **Table 4.1**.

Measurements

Energy intake

The amount eaten at the buffet meal (g) was quantified by weighing the food before and after consumption. Energy intake (kcal) at the buffet meal and proportions of protein, carbohydrate and fat were calculated using commercially available software (Foodworks version 8; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake was calculated both as intake at the buffet meal and as the cumulative energy intake, defined as the sum of energy intake at the buffet meal and the energy content of the preload drink. Absolute (kcal) and percentage suppression/ change energy intake at the buffet meal (expressed as % of energy intake of the control day) by a given protein load compared to control were calculated.

Perceptions of appetite and gastrointestinal symptoms

Perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating were rated using a visual analogue scale (VAS) questionnaire at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210 min (47). The questionnaire consisted of 100-mm horizontal lines, where 0 represented that the sensation was 'not felt at all' and 100 represented that the sensation was 'felt the greatest'. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation at the specified time points.

Gastric emptying

Total gastric volume was measured by a Logiq™ 9 ultrasound system (GE Healthcare Technologies, Australia) with TruScan Architecture [built-in magnetically sensored 3D positioning and orientation measurement (POM)] including a 3D sensor, attached to a 3.5C broad spectrum 2.5-4 MHz convex transducer, and a transmitter, placed at the level of the stomach immediately behind the subject at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 min (68). As the transmitter produces a spatially varying magnetic field that is distorted by conductive metals, all metal objects were removed from the subject to minimise interference during image acquisition. The stomach was scanned by a continuous translational movement along its long axis (~10 s). During each scan the subject was instructed to sit still and hold their breath at the end of inspiration. If gastric contractions were observed, the acquisition was paused until the contraction wave had passed. The raw data (original scan planes) were transferred for 3D reconstructions and volume estimation using EchoPAC - 3D software (GE Vingmed Sound, Horten, Norway). Gastric retention was calculated as total gastric volume minus baseline 'fasting' gastric volume at each time point expressed as percentage of the maximal gastric volume (100%), i.e. volume of the ingested drink. When ultrasound images lacked sufficient clarity to determine the volume of the stomach, data were imputed by linear interpolation. In one male subject the quality of

ultrasound stomach images was insufficient to determine gastric emptying in all three conditions, and data of gastric emptying data of this subject were, therefore, excluded from the analysis. The time at which 50% of the preload drink had emptied from the stomach (50% gastric emptying time; T50; min) was calculated for all conditions. Rate of gastric emptying was calculated as the mean of rates of emptying (kcal/min) during each 15-min interval respectively of the early (0-60 min) and late (60-180 min) phase and total (0-180 min) time period.

Blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP, GLP-1 and PYY

Blood samples were collected, at 0, 5, 15, 30, 45, 60, 90, 120, 150, 180 min, into ice-chilled, EDTA-coated tubes. No inhibitors were added (264). Plasma was obtained by centrifugation for 15 min at 3200 rpm at 4°C and samples were stored at -80°C for further analysis of hormone concentrations.

Blood glucose (millimoles per liter) was determined immediately after collection by the glucose oxidase method using a portable glucometer (Optium Xceed, Abbott Laboratories, Australia). Intra- and inter-assay coefficients of variation were 3.2% and 10.8%, respectively.

Total plasma insulin (milliunits per liter) was measured by enzyme-linked immunosorbent assay (ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L. Intra- and inter-assay coefficients of variation were 3.0% and 8.7%, respectively.

Total plasma glucagon (picograms per milliliter) was measured by radioimmunoassay (RIA) (GL-32K; Millipore, Billerica, MA, USA). The minimum detectable limit was 20 pg/mL. The intra- and inter-assay coefficients of variance were 4.3 and 7.1%. The ratio of insulin to glucagon was calculated for each time point in each subject. Homeostatic model assessment (HOMA) index at baseline was calculated according to the following formula: insulin

concentration (microunits per liter) x glucose concentration (nanomoles per liter) / 22.5 (290).

Total plasma ghrelin (picograms per milliliter) was measured using a RIA with some modifications to a published method (323). The radiolabel was supplied by Perkin Elmer (Boston, MA, USA; NEX388). The standard and samples were incubated with the antibody and radiolabel for 3-4 days at 4ºC. The detection limit was 40 pg/mL. Intra- and inter-assay coefficients of variation were 6.7 and 12.1%, respectively.

Plasma CCK-8 (picomoles per liter) was measured by RIA using an adaption of a previous method (324). Samples were extracted in 66% ethanol; extracts were dried down and re-suspended in assay buffer (50 mmol phosphate/L phosphate, 10 mmol EDTA/L, 2 g gelatin/L, pH = 7.4). Standards were prepared using synthetic sulphated CCK-8 (Sigma Chemical, St Louis, MO, USA), antibody (C2581, Lot 105H4852, Sigma Chemical) was added at a working dilution of 1/17,500 and sulphated CCK-8 ^{125}I-labeled with Bolton and Hunter reagent (Perkin Elmer, Boston, MA, USA) was used as tracer. Incubation was for 7 days at 4˚C. The antibody bound fraction was separated by the addition of dextran-coated charcoal containing gelatin (0.015 g gelatin, 0.09 g dextran, 0.15 g charcoal in 30 mL assay buffer) and the radioactivity determined in the supernatants following centrifugation. The detection limit was 1 pmol/L. The intra- and inter-assay coefficients of variation were 5.4% and 13.9%, respectively.

Total plasma GIP (picomoles per liter) was measured by RIA (293). The standard curve was prepared in buffer, rather than extracted charcoal stripped serum and the radio-iodinated label was supplied by Perkin Elmer (Boston, MA, USA). The minimum detectable limit was 2 pmol/L. The intra- and inter-assay coefficients of variation were 3.9% and 9%, respectively.

Total plasma GLP-1 (picomoles per liter) was measured by RIA (GLPIT-36HK; Millipore, Billerica, MA, USA). The detection limit was 3 pmol/L. Intra - and inter -assay coefficients of variation were 6.3% and 10.3%, respectively.

Total plasma PYY (picomoles per liter) was measured using RIA using antisera (kindly donated by Dr. B Otto, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Munich, Germany) against human peptide YY (1-36) (Sigma-Aldrich, St Lois, MO, USA) and raised in rabbits. This antisera showed < 0.001% cross reactivity with human pancreatic polypeptide or sulphated CCK-8 and 0.0025% cross reactivity with human neuropeptide Y. Standards (1.6-50 fmol/tube) or samples (200 µL plasma) were incubated in 200 µL assay buffer (50 mM $NaPO_4$, 10 mM EDTA, 2 g/L gelatin, 0.1 g/L Na-Azide, pH = 7.4) and a 1/12000 dilution of antisera for 24 hours. The standards and samples were further incubated with 10000 counts/min tracer [Perkin Elmer (Boston, MA, USA; NEX3410)] for 24 hours. Separation of the antibody bound tracer from free tracer was by second antibody precipitation (i.e. 500 µL of 1/100 dilution of sheep anti-rabbit immunoglobulin in wash buffer comprising 50 mM Tris-base, 150 mM NaCl, 8% polyethylene glycol 6000 pH = 8.0 and 50 µL of normal rabbit serum diluted 1/50 in wash buffer), incubated 2 hours at room temperature centrifuged at 4000 x g for at least 20 min at 4˚C, supernatants poured off and pellets counted in a gamma counter. The detection limit was 1.5 pmol/L. Intra- and inter-assay coefficients of variations were 8.7% and 18.2%, respectively.

Data analysis

Statistical analyses were performed using SPSS software (version 21; IBM, Armonk, NY, USA). Main effects of gender and protein load, and their interaction effects on energy intake and gastric emptying were determined using repeated measures ANOVA, with protein load as the within-subject factor, and gender as the between-subject factor. Main effects of gender

and protein load and their interaction effects on perceptions of appetite and gastrointestinal symptoms, blood glucose and plasma hormone concentrations, were determined using a repeated measures mixed-effect model, with protein load as the within-subject factor and gender as the between-subject factor, including baseline values at each treatment visit as a covariate. Post-hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when there were significant main or interaction effects.

Within-subject correlations were determined by using a general linear model with fixed slope and random intercept (277). Areas under the curve (AUC) were calculated from baseline to 180 min, using the trapezoidal rule. Assumptions of normality were verified for all outcomes before statistical analysis. Statistical significance was accepted at $P < 0.05$. All data are presented as means ± SEMs.

RESULTS

The study protocol was well tolerated by all subjects.

Energy intake

Energy intake at the buffet meal (**Figure 9.1**) was approximately 80% higher in older men than older women (mean energy intake of 3 study days in men: 1042 ± 69 kcal and women: 584 ± 61 kcal; main effect of gender $P < 0.001$, main effect of protein load $P = 0.34$, interaction effect of gender by protein load $P = 0.81$). Energy intake at the buffet meal did not differ between study days [preload drink containing 0 g (control), 30 g or 70 g protein], with no significant suppression of energy intake compared to that on the control day by either protein load [mean suppression of 45 ± 23 kcal or 5 ± 3% after the 30 g (120 kcal) or 70 g (280 kcal) protein drinks compared to control; main effect of gender $P = 0.62$, main effect of protein load $P = 0.83$, interaction effect of gender by protein load $P = 0.67$]. There was a

dose-dependent effect of the protein load on total (preload drink + meal) energy intake [mean total energy intake of men and women control: 843 ± 77 kcal, 30 g protein: 923 ± 75 kcal (12% increase), 70 g protein: 1073 ± 78 kcal (32% increase), mean total energy intake of 3 study days in men: 1175 ± 69 kcal and women: 717 ± 61 kcal; main effect of gender $P < 0.001$, main effect of protein load $P < 0.001$, interaction effect of gender by protein load $P = 0.81$]. Macronutrient preferences during the buffet meal did not differ between either men or women, or study visits (mean macronutrient composition of the buffet meal; protein: 20 ± 1%, fat: 28 ± 1%, carbohydrates 53 ± 1%; $P > 0.05$).

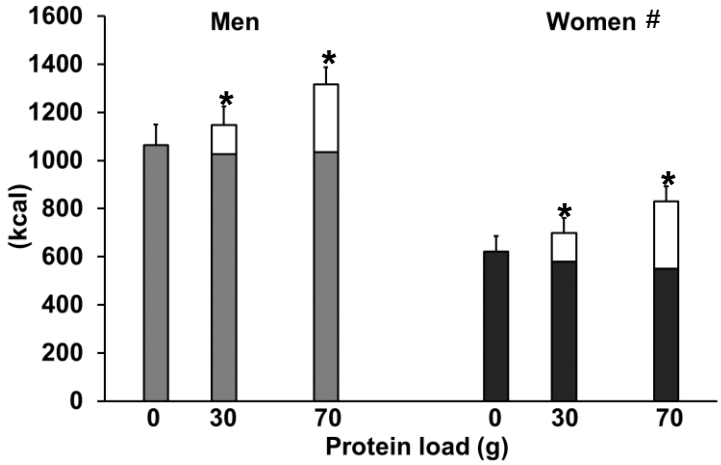

Figure 9.1: Mean (± SEM) energy intake at the buffet meal (kcal) in older men (energy intake at the buffet meal in grey shading; n = 8) and women (energy intake at the buffet meal in black shading; n = 8) after drinks (energy intake of the drink as the white part of each bar) containing flavoured water (control) and whey protein loads of 30 g (120 kcal) and 70 g (280 kcal). Main gender and protein load effects and interaction effects were determined by using repeated-measures ANOVA and post-hoc Bonferroni correction.
# $P < 0.001$ Main effect of gender: energy intake at the buffet meal was higher in older men than older women (main effect of protein load $P = 0.34$, interaction effect of gender by protein load $P = 0.81$).
* $P < 0.001$ Main effects of gender and protein load: total energy intake (preload drink + meal) was higher in older men than older women, and total energy intake was higher after the 30 g (9.5% increase) and 70 g (27% increase) protein load compared to control (interaction effect of gender by protein load $P = 0.81$).
Suppression of energy intake by protein (30 g and 70 g) compared to control (main effect of gender $P = 0.62$, main effect of protein load $P = 0.83$, interaction effect of gender by protein load $P = 0.67$).

Perceptions of appetite and gastrointestinal symptoms

Baseline perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating were not different between men and women or study days ($P > 0.05$, **Figure 9.2**). There was an interaction effect of gender by protein load for prospective food consumption (effect of gender $P = 0.50$, effect of protein load $P = 0.10$, interaction effect of gender by protein load $P = 0.015$) - the post-hoc analysis revealed that women have higher perceptions of prospective food consumption during control compared to the 70 g protein condition ($P = 0.018$). The main effects of gender and protein load and the interaction effect of gender by protein load for hunger, desire to eat, fullness, nausea and bloating (AUC 0-180 min), as well as the gender and treatment effect of prospective food consumption were not significant ($P > 0.05$).

Gastric emptying

Gastric emptying parameters are detailed in **Table 9.1**. Baseline gastric volumes were not different between men and women (39 ± 3 mL and 34 ± 5 mL, $P = 0.45$) or study days ($P = 0.76$). The control drink (water), as well as the 30 g protein drink, emptied in an overall non-linear pattern, whereas the pattern of emptying of the 70 g protein drink was linear (**Figure 9.3**).

There was a dose-dependent effect of the whey protein load to slow gastric emptying [T50 for mean of men and women control: 23 ± 2 min, 30 g: 65 ± 7 min, 70 g: 130 ± 10 min; effect of gender T50 $P = 0.41$, effect of protein load T50 $P < 0.001$, interaction effect of gender by protein load T50 $P = 0.77$; effect of gender AUC 0-180 min $P = 0.22$ AUC 0-60 min (early phase) $P = 0.27$ AUC 60-180 min (late phase) $P = 0.24$, effect of protein load AUC 0-180 min $P < 0.001$ AUC 0-60 min $P < 0.001$ AUC 60-180 min $P < 0.001$, interaction effect of gender by protein load AUC 0-180 min $P = 0.58$ AUC 0-60 min $P = 0.43$ AUC 60-

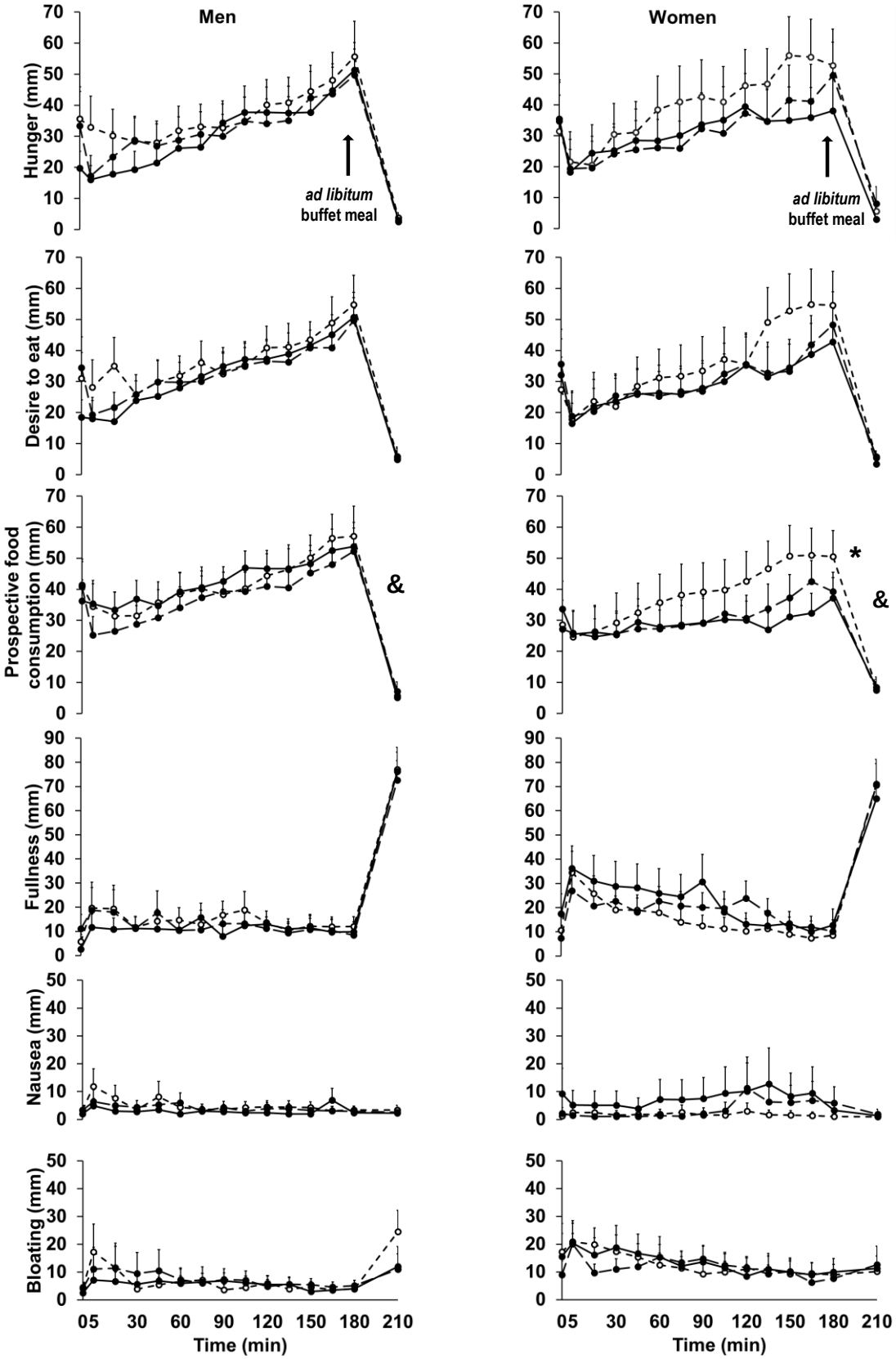

Figure 9.2: Mean (± SEM) visual analogue score (VAS) of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating in older men (n = 8) and women (n = 8) after drinks containing flavoured water (control; dotted line with open circles) and whey protein loads of 30 g (dashed line with closed circles) or 70 g (solid line with closed circles). Main effect of gender and protein load and interaction effects were determined using a

mixed-effect model with baseline concentrations as a covariate and post-hoc Bonferroni correction.
$P > 0.05$ main effect of gender and protein load and interaction effect of gender by protein load for visual analogue scales of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating (AUC 0-180 min).
$^{\&}$ $P < 0.05$ Interaction effect of gender by protein load
* $P < 0.05$ 70 g protein compared with the control (in women, perceptions of prospective food consumption were higher after the 70 g protein drink compared to control).

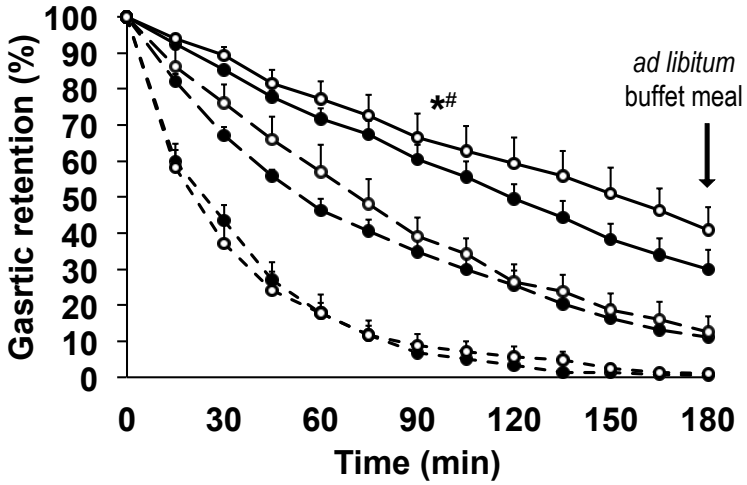

Figure 9.3: Mean (± SEM) gastric retention (%) in older men (n = 7; closed circles) and women (n = 8; open circles) after drinks containing flavoured water (control; dotted line) and whey protein loads of 30 g (dashed line) or 70 g (solid line). Main gender and protein load effects and interaction effects were determined by using repeated-measures ANOVA.
* $P < 0.001$ main effect of protein load for 50% gastric-emptying time (main effect of gender $P = 0.41$; interaction effect of gender by protein load $P = 0.77$).
$^{\#}$ $P < 0.001$ main effect of protein load AUC 0-180 min, 0-60 min (early phase) and 60-180 min (late phase) (main effect of gender AUC 0-180 min $P = 0.22$ AUC 0-60 min $P = 0.27$ AUC 60-180 min $P = 0.24$, interaction effect of gender by protein load AUC 0-180 min $P = 0.58$ AUC 0-60 min $P = 0.43$ AUC 60-180 min $P = 0.46$).

180 min $P = 0.46$], with no difference in rate of gastric emptying between men and women [mean rate of gastric emptying of men and women total phase 0-180 min: 30 g: 0.6 ± 0.02 kcal/min, (range: 0.4 - 0.7 kcal/min); 70 g: 1.0 ± 0.07 kcal/min (range: 0.5 - 1.4 kcal/min); early phase 0-60 min (when drinks were still emptying): 30 g: 1.0 ± 0.09 kcal/min, (range: 0.2 - 1.5 kcal/min); 70 g: 1.2 ± 0.1 kcal/min (range: 0.2 - 2.2 kcal/min); late phase 60-180 min: 30 g: 0.4 ± 0.03 kcal/min (range: 0.3 - 0.8kcal/min); 70 g: 0.9 ± 0.07 kcal/min (range: 0.3 - 1.3 kcal/min); effect of gender $P = 0.29$, effect of protein load $P < 0.001$, interaction

Table 9.1: Gastric emptying of water (control) and protein drinks in older men and women.

	Older men (n = 7)			Older women (n = 8)			*P* value		
	0 g	30 g	70 g	0 g	30 g	70 g	protein load	gender	interaction
50% emptying time (T50; min)	23±3	59±5	123±13	23±3	70±13	136±16	< 0.001	0.41	0.77
Rate of gastric emptying (kcal/min) [1]		0.6±0.0	1.1±0.1		0.6±0.0	0.9±0.1	< 0.001	0.29	0.25
Early phase rate of gastric emptying (0-60 min; kcal/min) [1]		1.1±0.1	1.3±0.1		0.9±0.2	1.1±0.2	0.08	0.25	0.86
Late phase rate of gastric emptying (60-180 min; kcal/min) [1]		0.4±0.0	1.0±0.1		0.5±0.1	0.8±0.1	< 0.001	0.67	0.12
Amount emptied at 60 min (%)	84±2	54±3	28±3	82±5	43±8	23±5	< 0.001	0.19	0.67
Amount emptied at 180 min (%)	99±0	89±2	70±5	99±1	87±4	59±6	< 0.001	0.29	0.29

All values are mean ± SEM. Main gender and protein load effects and interaction effects were determined by using repeated-measures ANOVA.
[1]Rate of gastric emptying was calculated as mean of rates of emptying during each 15-min interval respectively of the early (0-60 min) and late (60-180 min) phase and total (0-180 min) time period

effect of gender by protein load $P = 0.25$]. By 180 min, the 30 g protein drink had 'complete' gastric emptying (90% or more) in seven subjects, a further seven subjects showed emptying of ~85% and one subject of ~60%; whilst the 70 g protein was emptied from the stomach by ~85% or more in only three subjects.

Glucose and gut hormones

Baseline concentrations of blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP, GLP-1, PYY, as well as HOMA-IR and the ratio of insulin to glucagon were not significantly different between men and women or study visits ($P > 0.05$, **Figures 9.4 and 9.5**).

The main effect of gender was not significant for concentrations (AUC 0-180 min) of glucose ($P = 0.70$) glucagon ($P = 0.94$), ghrelin ($P = 0.35$), CCK ($P = 0.16$), GIP ($P = 0.18$), or GLP-1 ($P = 0.55$). Older women showed higher plasma concentrations of insulin (main effect of gender; $P = 0.040$) and PYY ($P = 0.037$) and an increased ratio of insulin to glucagon ($P = 0.008$) compared to older men.

The main effect of protein load was significant for concentrations (AUC 0-180 min) of insulin, glucagon, ghrelin, CCK, GIP, GLP-1, PYY, and ratio of insulin to glucagon (all $P < 0.001$), but not significant for glucose ($P = 0.36$).

The interaction effect of gender by protein load was significant for concentrations (AUC 0-180 min) of ratio of insulin to glucagon ($P = 0.018$), but not significant for glucose ($P = 0.44$), insulin ($P = 0.081$), glucagon ($P = 0.45$), ghrelin ($P = 0.26$), CCK ($P = 0.18$), GLP-1 ($P = 0.60$) and PYY ($P = 0.45$). Post-hoc analyses revealed that the ratio of insulin to glucagon was higher in women than men after the 30 g ($P = 0.034$) and 70 g ($P = 0.006$) protein drink.

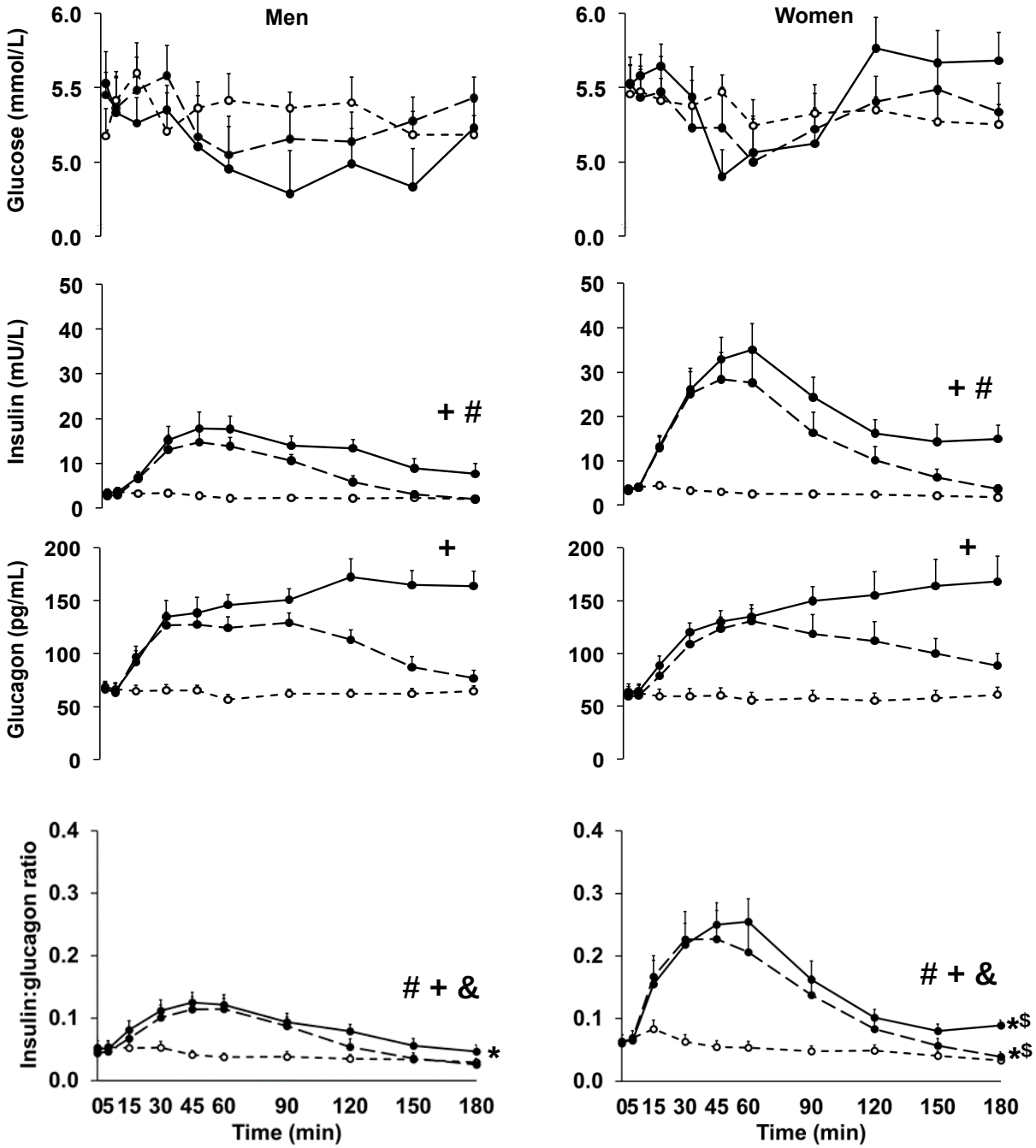

Figure 9.4: Mean (± SEM) concentrations (AUC 0-180 min) of blood glucose and plasma insulin and glucagon and ratio of insulin to glucagon in older men (n = 8) and women (n = 8) after drinks containing flavoured water (control; dotted line with open circles) and whey protein loads of 30 g (dashed line with closed circles) or 70 g (solid line with closed circles). Main effect of gender and protein load and interaction effects were determined using a mixed-effect model with baseline concentrations as a covariate and post-hoc Bonferroni correction.

[#] $P = 0.034$ Main effect of gender (plasma insulin and the ratio of insulin to glucagon concentrations were higher in older women than older men).

[+] $P < 0.001$ Main effect of protein load (plasma insulin, glucagon and the ratio of insulin to glucagon concentrations were protein load dependent).

[&] $P < 0.05$ Interaction effect of gender by protein load (Post-hoc tests: [$] $P < 0.05$ plasma insulin and the ratio of insulin to glucagon concentrations were higher in women than men after both the 30 g and 70 g protein load; * $P < 0.05$ in women the ratio of insulin to glucagon

was higher after both the 30 g and 70 g protein load compared control, in men the ratio of insulin to glucagon was higher after the 30 g protein drink compared to control).

In women, the ratio of insulin to glucagon was higher after both the 70 g and 30 g protein drink compared to control (all $P < 0.001$). In men, the ratio of insulin to glucagon was higher after the 70 g protein drink compared to control ($P = 0.002$).

In many cases the protein load-effects were time dependent. The difference (earlier return to baseline after 30 g vs. 70 g protein intake) in late phase responses (> ~90-120 min) of ghrelin in men, CCK in women, and glucagon and GIP in men and women between both protein loads (**Figures 9.4 and 9.5**) may be related to gastric emptying being completed earlier after the 30 g than 70 g protein load (**Figure 9.3 and Table 9.1**).

Relationships between energy intake, appetite, gastric emptying and gut hormones

Energy intake (kcal) at the buffet meal was, *within subjects*, inversely related to gastric emptying (T50 and gastric retention AUC 0-180 min, early phase AUC 0-60 min and late phase AUC 60-180 min) and gastric volume at 180 min in women (**Table 9.2**); i.e. the slower the drink emptied from the stomach within a subject – 70 g < 30 g < 0 g – the lower the subsequent energy intake (180-210 min).

Energy intake (kcal) at the buffet meal was, *within subjects*, positively related to plasma concentrations (AUC 0-180 min) of ghrelin and inversely related to plasma concentrations of PYY in older men and women combined and to insulin, glucagon, CCK, GIP and PYY in older women (e.g. the greater the increase in plasma concentrations of insulin, glucagon, CCK GIP and PYY within a subject – 70 g > 30 g > 0 g – the lower the subsequent energy

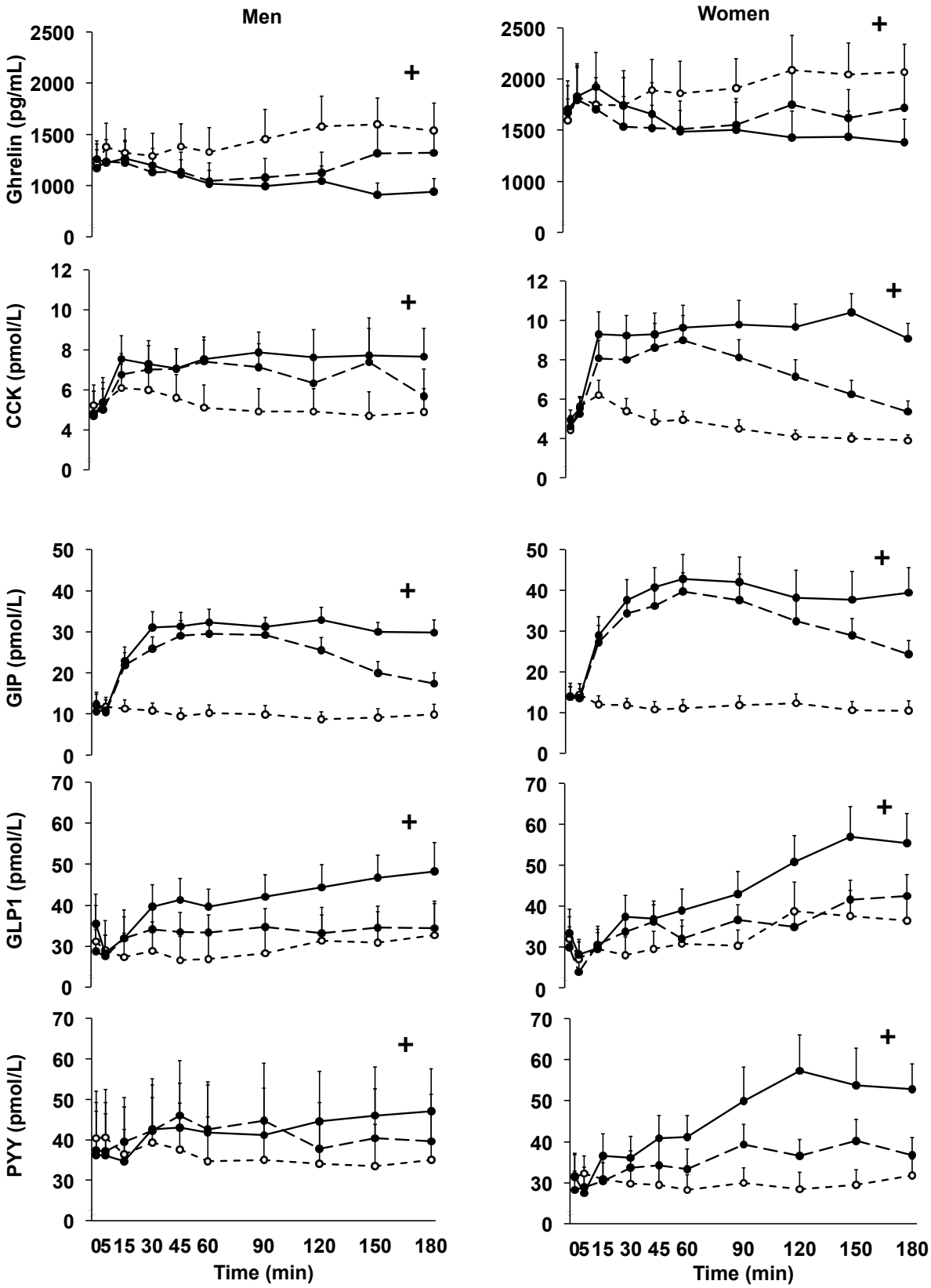

Figure 9.5: Mean (± SEM) plasma concentrations (AUC 0-180 min) of ghrelin, CCK, GIP, GLP-1 and PYY in older men (n = 8) and women (n = 8) after drinks containing flavoured water (control; dotted line with open circles) and whey protein loads of 30 g (dashed line with closed circles) or 70 g (solid line with closed circles). Main effects of gender and protein load and interaction effects were determined using a mixed-effect model with baseline

concentrations as a covariate. Main effects of gender and interaction effects of gender by protein load of the gut hormones were not significant.
$^{+}$ $P < 0.001$ Main effect of protein load.

intake. Energy intake was also, *within subjects*, related to plasma hormone concentrations before the meal (180 min) in older women (insulin r = -0.50 $P = 0.041$, ghrelin r = 0.65 $P = 0.005$, CCK r = -0.54 $P = 0.025$, GIP r = -0.49 $P = 0.048$, PYY r = -0.73 $P = 0.001$).

Gastric emptying (gastric retention AUC 0-180 min) was, *within subjects*, related to plasma insulin, glucagon, CCK, GIP, GLP-1 and PPY concentrations (AUC 0-180 min) and inversely related to ghrelin concentrations as well as perceptions of appetite and gastrointestinal symptoms (**Table 9.3**); i.e. the higher the plasma hormone concentrations of insulin, glucagon, CCK, GIP, GLP-1 and PPY the slower the rate of gastric emptying within a subject and the lower the concentrations of ghrelin and feelings of hunger.

Hunger and prospective food consumption were, *within subjects*, inversely related to CCK concentrations and desire to eat was inversely related with CCK and GIP concentrations. Fullness correlated positively to CCK concentrations and nausea correlated positively with glucose and negatively to ghrelin concentrations (**Table 9.4**).

GIP was, *within subjects*, related to GLP-1 (r = 0.52 $P = 0.002$); i.e. the greater the increase in plasma GIP concentrations the greater the increase in GLP-1. Ghrelin was, *within subjects*, inversely related to insulin (r = -0.59, $P < 0.001$); i.e. the greater the increase in plasma insulin concentrations the greater the inhibition of ghrelin production.

DISCUSSION

This study examined the acute effects of oral whey protein consumption on energy intake, appetite, gastric emptying and plasma gut hormone concentrations in older women, as well as men. The protein drinks did not suppress subsequent *ad libitum* food intake in either gender. Consequently, there was a dose-dependent effect of the whey protein drinks (30, 70

Table 9.2: Within-subject correlations between energy intake at the buffet meal and perceptions of appetite and gastrointestinal symptoms, gastric emptying and concentrations of blood glucose and plasma gut hormones in older men and women.

	Within-subject correlations					
	Older men (n = 8)[1]		Older women (n = 8)		Combined	
	r	*P*	r	*P*	r	*P*
Hunger	0.43	0.11	0.29	0.30	0.35	0.06
Desire to eat	0.23	0.42	-0.43	0.11	-0.07	0.71
Prospective food consumption	0.11	0.70	0.22	0.43	0.14	0.47
Fullness	0.10	0.72	0.29	0.29	0.16	0.42
Nausea	0.14	0.62	-0.40	0.14	-0.09	0.66
Bloating	-0.05	0.85	0.00	1.00	-0.01	0.94
50% emptying time (T50)	-0.10	0.71	-0.66	0.004	-0.29	0.11
Gastric retention AUC 0-180	-0.15	0.59	-0.61	0.01	-0.30	0.10
Gastric retention AUC 0-60	-0.16	0.57	-0.59	0.01	-0.30	0.10
Gastric retention AUC 60-180	-0.15	0.60	-0.59	0.01	-0.29	0.11
Gastric retention at 180 min (before buffet meal)	-0.36	0.19	-0.62	0.008	-0.41	0.021
Glucose	-0.10	0.71	0.26	0.31	0.04	0.84
Insulin	-0.10	0.70	-0.64	0.006	-0.29	0.10
Glucagon	-0.10	0.70	-0.57	0.016	-0.24	0.17
Ghrelin	0.33	0.19	0.43	0.08	0.35	0.045

CCK	-0.11	0.66	-0.52	0.031	-0.25	0.16
GIP	-0.18	0.49	-0.54	0.027	-0.28	0.11
GLP-1	-0.42	0.10	0.13	0.63	-0.05	0.77
PYY	-0.43	0.08	-0.69	0.002	-0.45	0.009

r and *P* values of *within-subject* correlations between energy intake at the buffet meal (kcal) and visual analogue scale (mm; AUC 0-180 min) perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating (mm; AUC 0-180 min), gastric emptying half time (T50), gastric retention (%; AUC 0-180 min), gastric retention at 180 min and concentrations (AUC 0-180 min) of blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL), ghrelin (pg/mL), CCK (pmol/L), GIP (pmol/L), GLP-1 (pmol/L) and PYY (pmol/L) in older men and women. Within-subject correlations were determined by using a general linear model with fixed slope and random intercept. [1]n = 7 for gastric retention in the older men.

g protein) to increase total energy intake (preload drink + meal) compared to the control drink in both men and women. In both genders, protein caused a load-dependent slowing of gastric emptying, and increase in plasma concentrations of insulin, glucagon, ghrelin, CCK, GIP, GLP-1 and PYY. As the protein doses used are in the range reported to have favourable effects on muscle mass (13), our observations of comparable effects of protein on appetite and underlying gastrointestinal mechanisms in men and women, support the use of protein supplements in older people to preserve, or increase skeletal muscle mass and function without suppressing energy intake.

Total energy intake was increased most by the highest protein dose [70 g (280 kcal)] compared to the control day; a substantial increase of 32% or 230 kcal, compared with an increase of 12% or 80 kcal after the 30 g (120 kcal) protein load. These observations are consistent with evidence of reduced suppression of energy intake by nutrient ingestion in older, compared with young, men (46, 63, 194, 263), and extend these findings to older women. Importantly, these observations indicate that doses of protein that have been shown to be sufficient to cause dietary protein muscle deposition, can be ingested by both older women and men, without suppressing appetite or overall energy intake. In older, when

compared to younger adults, the sensitivity of muscle protein synthesis to the ingestion of small amounts (≤ 20 g) of whey protein may be reduced (23). However, these postprandial differences between the young and old are, not evident after consumption of ample amounts of dietary protein (> ~35 g). Moreover, administration of protein supplements in older people may increase total energy intake (supplement plus subsequent meal), as was observed in this study. This contrasts to the effects of protein in younger adults - identical whey drinks given according to the same study protocol produced a significant ~15% suppression of *ad libitum* food intake compared to control in our previous study (263). It should be appreciated that the long-term effects of protein supplements on energy intake in older adults are unknown and reported effects on muscle mass and function are inconsistent (325-328).

Appetite and energy intake are dependent on the precise co-ordination of interrelated 'gastric' and 'small intestinal' mechanisms triggered by the interaction with the nutrients ingested. We, and others, have shown that healthy ageing is associated with modest slowing of gastric emptying of both solids and liquids, although the rate of emptying generally remains within the normal range for young subjects (i.e. ~1 – 4 kcal/min) (50, 74, 82, 263). Slower gastric emptying results in greater distension of the stomach at any given time after ingestion of a meal. This can, in turn, lead to greater fullness and, at least in young adults, a consequent reduction in subsequent energy intake. In the present study there was a marked, dose-dependent, slowing of gastric emptying by the protein drinks, with the 50% gastric emptying time more than doubling from control to 30 g protein day, and again from the 30 g to 70 g protein day. The rate of gastric emptying of the protein drinks in both men and women (~1 kcal/min) was apparently at the lower end of the normal range. Gastric emptying of the 30 g protein drink slowed further after the early phase (~0.5 kcal/min) when on average ~48% of the drink was emptied after 60 min; whereas the 70 g protein drink continued being emptied at ~1 kcal/min after ~25% had emptied on average after 60 min.

There was no significant difference between emptying rates in older men and women. Controversy exists regarding the gender-related difference in gastric emptying. Previously gastric emptying, determined by scintigraphy, has been found to be modestly slower in young and middle aged lean, but not obese (329), women than men (83, 330, 331), but not in all studies (80). Bennink *et al.* also observed slower gastric emptying of a solid, but not liquid, test meal in lean healthy young women compared to men (332). We have reported that energy intake at a buffet meal is associated negatively ($r = -0.90$, $P < 0.001$) with antral area (distal stomach) immediately before the meal in young and older subjects who received mixed macronutrient drinks (0, 250, and 750 kcal) (42). Although the negative association between gastric retention and energy intake in the female (but not male) subjects in this study is consistent with some suppression of energy intake by gastric distension, the finding that the marked protein-induced slowing of gastric emptying after ingestion of the protein drinks was not associated with suppression of subsequent energy intake, suggests that this effect, if present, is probably minor in elderly people. This would be consistent with the finding of Rayner *et al.* that the perception of gastric distension is diminished in healthy older people (84).

Gastric emptying was, *within subjects*, related to plasma gut hormone concentrations –higher plasma concentrations of insulin, glucagon, CCK, GIP, GLP-1 and PYY correlated with lower plasma ghrelin concentrations and perceptions of hunger, and slower gastric emptying of the protein drink (70 g < 30 g < 0 g). There was an immediate load-dependent increase in the plasma hormone concentrations of CCK and GIP (both mainly produced in the duodenum and proximal jejunum) reaching a plateau from 15-30 min onwards, whereas the hormones that are produced more distally in the gut; GLP-1 and PYY (GLP-1 mainly produced in the ileum and PYY in the ileum and colon) showed a more constant increase. Gastric emptying was completed earlier after the 30 g than the 70 g protein load, which resulted in a time-dependent response (earlier return to baseline after 30 g vs. 70 g protein

intake) in plasma concentrations of CCK in women, and glucagon and GIP in men and women after 30 g vs. 70 g protein load. Healthy older, when compared to young people, have higher postprandial concentrations of CCK, GIP, GLP-1 and PYY, which may contribute to slowing of gastric emptying (42, 48, 52, 132). The latter may in part be related to impairment of clearance, including GIP and GLP-1 inactivation by dipeptidyl peptidase IV (DPP-IV) and renal processes (132).

Healthy ageing is characterised by impaired glucose tolerance or insulin resistance (132, 239). The latter may reflect increased adiposity and reduction in the secretion of, or pancreatic beta cell sensitivity to (176), the incretins GLP-1 and GIP (132, 134). Insulin peaked between 30 and 60 min and returned to baseline after 180 min, and this effect was greater in older women than older men. Glucagon increased almost concurrently in older men and women, before decreasing again after 90 min after the 30 g, whereas it stayed elevated after the 70 g protein load.

Our study has several limitations which should be recognised. The number of subjects was relatively small and, therefore, the study may be underpowered for secondary outcomes, including perceptions of appetite and gastrointestinal symptoms and the change in gastric emptying after the 70 g whey protein load in older women than older men. Energy intake was assessed three hours after protein intakes at a buffet meal, and not during the remainder of the day - accordingly, potential compensating changes in energy intake after lunch were not evaluated. While the drinks were matched for taste and osmolality, we did not assess the subject's perceptions of taste, pleasantness and/or palatability of the drinks. As a consequence of the study design the protein preload drinks were iso-caloric for both older men and women. Older women are expected to have lower energy requirements when compared to the older men, and the drinks given to female group in this study could therefore be judged to be 'larger' than those given to the male group when considered in relation to energy requirements. Blood glucose was measured by a glucometer, and blood samples of

glucagon and GLP-1 were collected without protease inhibitors, which could be considered to be less than optimal, however, the results appeared clear-cut with significant changes in both glucagon and GLP-1 in response to the protein loads in the direction expected.

In summary, ingestion of protein drinks at doses previously shown to suppress energy intake in young men, had no effect on *ad libitum* energy intake in either older women or men, three hours after consumption. Consequently, consumption of protein drinks lead to an increase in the total energy intake.

Table 9.3: Within-subject correlations between gastric retention and perceptions of appetite and gastrointestinal symptoms and concentrations of blood glucose and plasma gut hormones in older men and women.

	Older men (n = 7)		Older women (n = 8)		Combined	
	r	*P*	r	*P*	r	*P*
Hunger	0.00	0.99	-0.44	0.08	-0.63	0.12
Desire to eat	-0.09	0.76	-0.52	0.031	-0.36	0.044
Prospective food consumption	0.14	0.62	-0.65	0.005	-0.34	0.06
Fullness	-0.45	0.09	0.51	0.004	0.20	0.29
Nausea	0.11	0.70	0.14	0.58	0.12	0.54
Bloating	0.21	0.46	0.07	0.79	0.09	0.63
Glucose	-0.55	0.033	-0.23	0.38	-0.35	0.053
Insulin	0.90	<0.001	0.89	<0.001	0.87	<0.001
Glucagon	0.94	<0.001	0.96	<0.001	0.94	<0.001
Ghrelin	-0.73	0.020	-0.57	0.017	-0.64	<0.001
CCK	0.78	0.001	0.92	<0.001	0.87	<0.001
GIP	0.92	<0.001	0.85	<0.001	0.87	<0.001
GLP-1	0.76	0.001	0.75	0.001	0.75	<0.001
PYY	0.72	0.002	0.85	<0.001	0.80	<0.001

r and *P* values of *within-subject* correlations between gastric retention (%; AUC 0-180 min) and visual analogue scale (mm, AUC 0-180 min) perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating, and concentrations (AUC 0-180) of blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL) ghrelin (pg/mL), CCK (pmol/L), GIP (pmol/L), GLP-1 (pmol/L) and PYY (pmol/L) in older men and women. Within-subject correlations were determined by using a general linear model with fixed slope and random intercept.

Table 9.4: Within-subject correlations between perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating, and concentrations of blood glucose and plasma gut hormones in older men and women (n = 16).

	Hunger		Desire to eat		Prospective food consumption		Fullness		Nausea		Bloating	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Glucose	0.09	0.63	0.01	0.97	-0.09	0.63	0.14	0.44	0.37	0.047	-0.22	0.23
Insulin	-0.31	0.09	-0.35	0.06	-0.36	0.05	0.29	0.12	0.09	0.63	0.09	0.63
Glucagon	-0.27	0.14	-0.32	0.09	-0.30	0.10	-0.06	0.75	0.10	0.58	0.10	0.59
Ghrelin	0.18	0.33	0.12	0.50	0.10	0.57	-0.14	0.44	-0.37	0.048	-0.02	0.90
CCK	-0.41	0.029	-0.50	0.009	-0.56	0.004	0.38	0.043	0.18	0.32	0.11	0.56
GIP	-0.35	0.06	-0.37	0.047	-0.34	0.07	0.21	0.25	0.13	0.46	0.08	0.68
GLP-1	-0.22	0.23	-0.27	0.149	-0.25	0.17	0.04	0.83	0.05	0.77	0.06	0.75
PYY	-0.12	0.53	-0.19	0.30	-0.30	0.10	0.15	0.40	0.04	0.83	0.16	0.39

r and *P* values of *within-subject* correlations between perceptions of appetite and gastrointestinal symptoms (AUC 0-180 min) and concentrations (AUC 0-180 min) of blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL), ghrelin (pg/mL), CCK (pmol/L), GIP (pmol/L), GLP-1 (pmol/L) and PYY (pmol/L) in older men and women. Within-subject correlations were determined by using a general linear model with fixed slope and random intercept.

CHAPTER 10: EFFECT OF GENDER ON THE ACUTE EFFECTS OF WHEY PROTEIN INTAKE ON ENERGY INTAKE, APPETITE, GASTRIC EMPTYING AND GUT HORMONE RESPONSES IN HEALTHY YOUNG ADULTS

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Contribution	Conception and design of the study, coordination, participant recruitment, data collection and interpretation, statistical analysis and drafting of the manuscript.		
Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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

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Contribution	Conception and design of the study, data interpretation, statistical analysis, drafting of the manuscript, and overall responsibility for the study.		
Signature		Date	October 2017

ABSTRACT

Background: Protein supplements, usually drinks rich in whey protein, are used widely for weight loss purposes in overweight adults. Information comparing the effects of whey protein on appetite and energy intake in men and women is limited.

Objective: To compare the acute effects of whey-protein intake on energy intake, appetite, gastric emptying and gut hormones in healthy young men and women.

Design: Gastric emptying (3D-ultrasonography), blood glucose and plasma insulin, glucagon, ghrelin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) concentrations (0-180min), appetite (visual analogue scales), and *ad libitum* energy intake from a buffet meal (180-210min) were determined after ingestion of 30 g (120 kcal) or 70 g (280 kcal) whey protein, or a flavoured-water control drink (~2 kcal) in 8 healthy young men (25 ± 2y, 72 ± 3kg, 23 ± 1 kg/m^2) and 8 women (23 ± 1y, 64 ± 2kg, 24 ± 0.4 kg/m^2).

Results: There was a protein-load effect on gastric emptying, blood glucose, plasma insulin, glucagon, ghrelin, CCK, GIP and GLP-1 concentrations, and perceptions of hunger, desire to eat and prospective food consumption ($P < 0.05$). *Ad libitum* energy intake (average decrease of 206 ± 39kcal [15 $\pm$ 2%] for men and of 46 ± 54kcal [0 $\pm$ 26%] for women for the mean of the intakes after the 30 g and 70 g whey-protein loads) and hunger were suppressed more by the whey-protein ingestion compared to control in men than women ($P < 0.05$). Consequently, total energy intake (from protein drink plus buffet meal) was greater in women than men ($P < 0.05$). The drinks emptied more slowly, and plasma glucagon, CCK and GLP-1 responses were less, in women than men ($P < 0.05$).

Conclusion: The acute effects of whey protein ingestion on appetite, energy intake, gastric emptying and gut hormone responses are influenced by gender in healthy young adults.

INTRODUCTION

Supplements and diets high in protein content, particularly whey protein, are used frequently for weight loss purposes, in both men and women, based on the rationale that ingestion of protein has a muscle sparing effect and greater satiating effects than dietary carbohydrate and fat (287, 333). As such, many high protein diets have been developed and recommended to aid weight loss; well-known versions include the Atkins Diet, South Beach Diet, Zone Diet and Stillman Diet. Our recent studies in healthy young men have shown that whey protein, ingested either orally, or infused intraduodenally, suppresses *ad libitum* energy intake at a subsequent meal, in excess of the caloric content of the protein load, so that total energy intake (protein plus meal) is less after intake of protein than after a non-caloric control (114, 334). When infused intraduodenally, whey protein increases pyloric and decreases antral and duodenal motility, factors important in the regulation of gastric emptying (114, 334). Oral whey protein ingestion load-dependently slows gastric emptying and increases plasma insulin, glucagon, ghrelin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) concentrations in healthy young men (334). These effects on gastrointestinal mechanisms are associated with the suppression of appetite and energy intake (114, 334).

It has been reported that women, when compared to men, exhibit lower compensation of energy intake, but comparable perceptions of appetite, after ingestion of liquid and semi-liquid caloric preloads (321, 322). For example, after a milk or fruit drink preload, women compared to men compensate less in subsequent energy intake i.e. on average 50% in women versus 107% in men when compared to a control day, resulting in an increase in total energy intake (drink plus meal) in women (322). It has also been reported that after mixed-nutrient meals women compared to men have slower gastric emptying (83, 330-332) and lower plasma glucagon (335), CCK (336) and GLP-1 concentrations (335). It is not known whether

gender modulates the acute effects of whey protein, administered in loads representative of a small to large meal (30–70 g, e.g. ~100–250 g serving of lean steak), to suppress energy intake and, if so, what the underlying gastrointestinal mechanisms are.

The aim of the study was to determine the comparative load-dependent effects of 30 g and 70 g whey protein intake on *ad libitum* energy intake at a buffet meal, as well as perceptions of appetite and gastrointestinal symptoms, gastric emptying, blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP and GLP-1 concentrations in healthy young men and women. We hypothesised that women compared to men would have less suppression of energy intake, slower gastric emptying and lower gut hormone responses after whey protein ingestion.

SUBJECTS AND METHODS

Subjects

Participants were recruited by advertisement. The study included 8 young men [mean ± SEM: age: 25 ± 2 years; body weight: 72 ± 3 kg; height: 1.79 ± 0.02 m; body mass index (BMI): 23 ± 1 kg/m^2 - the men were included in our previous study relating to energy intake, gastric emptying and perceptions of appetite and gastrointestinal symptoms in healthy older compared to younger men (263)] and 8 young age- (P = 0.60) and BMI-matched (P = 0.24) women [23 ± 1 years; 64 ± 2 kg; 1.64 ± 0.02 m; 24 ± 0.4 kg/m^2].

All participants were non-smokers, unrestrained eaters [score ≤ 12 on the eating restraint component of the Three-Factor Eating Questionnaire (337)]. Further exclusion criteria were gastrointestinal surgery (apart from uncomplicated appendectomy), gastrointestinal symptoms (abdominal pain, gastro-esophageal reflux, diarrhea, or constipation), use of medications known to potentially affect energy intake, appetite or gastrointestinal motor function, diabetes, gallbladder or pancreatic disease, lactose intolerance or food allergies,

consumption of a vegetarian diet, alcohol abuse, use of illicit substances, low ferritin levels or blood donation in the 12 weeks prior to the study, failure to comprehend the study protocol and, in women, lactation, pregnancy or the use of hormonal contraceptives. The Royal Adelaide Hospital Human Research Ethics Committee approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki and registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au, registration number ACTRN12611000706976). All participants provided written informed consent prior to their inclusion.

Protocol

Subjects were studied on 3 occasions, separated by 3-14 days, to determine the comparative effects of two oral whey protein loads; 30 g (120 kcal) and 70 g (280 kcal), and a flavoured water control-drink (~2 kcal) on energy intake, perceptions of appetite and gastrointestinal symptoms, gastric emptying, blood glucose and plasma gut hormone concentrations in a randomised [www.randomization.com (16 subjects with random permutations)], double-blind, cross-over design. In women, study days were scheduled during the follicular phase of their menstrual cycle (i.e. the first 13 days of the cycle) to minimise the potential effect of fluctuations in hormones on gastric emptying and energy intake at the buffet meal (338).

Protein drinks were prepared by dissolving whey protein isolate (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) in varying volumes of demineralised water and diet lime cordial (Bickford's Australia Pty Ltd, Salisbury South, SA, Australia) to achieve the desired loads [i.e. 30 g whey protein (volume of the powder: 19 mL) in 335 mL distilled water and 85 mL cordial (2.5 kcal/100 mL), 70 g whey protein (volume of the powder: 45 mL) in 280 mL water and 100 mL cordial, the control drink consisted of 359

mL distilled water and 90 mL cordial]. Sodium chloride in the amount of 0.3 g and 1.2 g was added to the 30 g and control drinks, respectively, to match the osmolarity (88 mOsm/L) with the 70 g drink. To ensure that all ingredients were dissolved evenly throughout and to minimise the layer of foam on top of the solution, the drinks were all stirred continuously at low speed on a stirring plate. The volume of each drink was measured before serving and the recorded volumes differed modestly (i.e. control: 450 mL; 30 g protein: 439 mL; and 70 g protein: 425 mL). All drinks were provided to subjects in a covered cup, so that both the investigator conducting the data collection and the subject were blinded to the test drinks. The drinks were prepared by a research assistant who was not involved in analysis of the study results.

Subjects were provided with a standardised evening meal [beef lasagna (McCain Foods Pty Ltd, Wendouree, VIC, Australia), ~591 kcal] to consume on the night before each study day at ~19.00 h. They were instructed to fast overnight from solids and liquids and refrain from strenuous physical activity until they attended the laboratory at ~08.30h.

On arrival, subjects were seated in an upright position on a wooden chair, where they remained for the duration of the study, and an intravenous cannula for blood sampling was inserted. In each subject, measurements of total gastric volume and perceptions of appetite and gastrointestinal symptoms were performed before (during fasting; 0 min) and immediately after ingestion of the drink, and at 15-min intervals until 180 min (47). Subjects were instructed to consume the drink within 2 minutes. Gastric volume was measured by 3-dimensional (3D) ultrasonography (263). Perceptions of appetite and gastrointestinal symptoms were assessed using validated visual analogue scales (VAS) and blood samples were collected for the measurement of blood glucose and plasma gut hormone concentrations. At 180 min, subjects were presented, in a room by themselves to limit external distractions, with a standard, cold, buffet meal in excess of what they are expected to consume (total energy content of 2,457 kcal; 19% protein, 50% carbohydrates,

31% fat) over 30 min (257). The buffet meal consisted of palatable food items including sliced bread, cheese, ham and chicken, fruits, yoghurt, custard, margarine, mayonnaise, iced coffee, orange juice, fruit salad and water (263).

Measurements

Energy intake

The amount eaten at the buffet meal (g) was quantified by weighing the food before and after consumption. Energy intake (kcal) at the buffet meal and proportions of protein, carbohydrate and fat were calculated using commercially available software (Foodworks version 8; Xyris Software Pty Ltd, Spring Hill, QLD, Australia). Energy intake was calculated both as intake at the buffet meal and as total energy intake, defined as the sum of energy intake at the buffet meal and the energy content of the drink. Absolute change (kcal) and percentage suppression (expressed as % of energy intake of the control day) of energy intake at the buffet meal by a given protein load compared to control were calculated (263).

Perceptions of appetite and gastrointestinal symptoms

Perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating were rated using a visual analogue scale (VAS) questionnaire at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210 min (47). The questionnaire consisted of 100-mm horizontal lines, where 0 represented that the sensation was ‘not felt at all’ and 100 represented that the sensation was ‘felt the greatest’. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation.

Gastric emptying

Total gastric volume was measured by a Logiq™ 9 ultrasound system (GE Healthcare Technologies, Sydney, NSW, Australia) with TruScan Architecture [built-in magnetically sensored 3D positioning and orientation measurement (POM)] including a 3D sensor, attached to a 3.5C broad spectrum 2.5-4 MHz convex transducer, and a transmitter, placed at the level of the stomach immediately behind the subject at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 min (263). As the transmitter produces a spatially varying magnetic field that is distorted by conductive metals, all metal objects were removed from the subject to minimise interference during image acquisition. The stomach was scanned by a continuous translational movement along its long axis (~10 s). During each scan the subject was instructed to sit still and hold their breath at the end of inspiration. If gastric contractions were observed, the acquisition was paused until the contraction wave had passed. The raw data (original scan planes) were transferred for 3D reconstructions and volume estimation using EchoPAC - 3D software (GE Vingmed Sound, Horten, Norway). Gastric retention (early phase: 0-60 min and late phase: 60-180 min; %) was calculated as total gastric volume minus baseline 'fasting' gastric volume at each time point expressed as percentage of the maximal gastric volume (100%), i.e. volume of the ingested drink. When ultrasound images lacked sufficient clarity to determine the volume of the stomach, data were imputed by linear interpolation. The time at which 50% of the preload drink had emptied from the stomach (50% gastric emptying time; T50; min) and 'complete' gastric emptying time (100% gastric emptying time; T100; min), defined as the time when the residual volume of the drink in the stomach was ≤5%, was calculated for all conditions. Complete emptying time was set to 180 min when the residual volume at 180 min was >5% (263). The overall rate of gastric emptying was calculated as the mean of rates of emptying (kcal/min) during each 15-min interval respectively of the early phase (0-60 min), late phase (60 min until complete

emptying time per individual) and total time period (0 min until complete emptying time per individual).

Blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP, GLP-1 and PYY

Blood samples were collected, at 0, 5, 15, 30, 45, 60, 90, 120, 150, 180 min, into ice-chilled, ethylenediaminetetraacetic acid (EDTA) coated tubes. No inhibitors were added (264). Blood glucose concentrations (millimoles per liter) were determined immediately after collection by the glucose oxidase method using a portable glucometer (Optium Xceed, Abbott Laboratories, Doncaster, VIC, Australia). Plasma was obtained by centrifugation for 15 min at 3200 rpm at 4°C and samples were stored at -80°C for further analysis of hormone concentrations (339).

Plasma total insulin concentrations (milliunits per liter) were determined by enzyme-linked immunosorbent assay (ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden). The detection limit was 1.0 milliunits per liter. Intra- and inter-assay coefficients of variation were 3.0 and 8.7%. Homeostatic model assessment (HOMA) index was calculated according to the following formula: insulin concentration at baseline (microunits per liter) x glucose concentration at baseline (nanomoles per liter) / 22.5 (290).

Plasma total glucagon concentrations (picograms per milliliter) were determined by radioimmunoassay (RIA; GL-32K; Millipore, Billerica, MA, USA). The detection limit was 20 picograms per milliliter. Intra- and inter-assay coefficients of variance were 4.3% and 7.1%.

Plasma total ghrelin concentrations (picograms per milliliter) were determined by RIA (Perkin Elmer, Boston, MA, USA; NEX388) (292, 340, 341). The standard and samples were incubated with the antibody and radiolabel for 3-4 days at 4^{o}C. The detection limit was 40 pg/mL. Intra- and inter-assay coefficients of variation were 6.7% and 12.1%.

Plasma CCK-8 (picomoles per liter) were determined by RIA (324, 341). Samples were extracted in 66% ethanol, extracts were dried down and re-suspended in assay buffer (50 mM phosphate, 10 mM EDTA, 2 g/L gelatin, pH = 7.4). Standards were prepared using synthetic sulphated CCK-8 (Sigma Chemical, St Louis, MO, USA) and an antibody (C2581, Lot 105H4852, Sigma Chemical) was added at a working dilution of 1/17,500 and sulphated CCK-8 ^{125}I-labeled with Bolton and Hunter reagent (Perkin Elmer, Boston, MA, USA). Incubation was for 7 days at 4˚C. The antibody bound fraction was separated by the addition of dextran-coated charcoal containing gelatin (0.015 g gelatin, 0.09 g dextran, 0.15 g charcoal in 30 mL assay buffer). The radioactivity was determined in the supernatants following centrifugation with a detection limit of 1 picomoles per liter. Intra- and inter-assay coefficients of variation were 5.4% and 13.9%.

Plasma total GIP concentrations (picomoles per liter) were measured by RIA (Perkin Elmer, Boston, MA, USA) (293). The standard curve was prepared in buffer, rather than extracted charcoal stripped serum. The detection limit was 2 picomoles per liter. Intra- and inter-assay coefficients of variance were 3.9% and 9%.

Plasma total GLP-1 concentrations (picomoles per liter) were measured by RIA (GLPIT-36HK; Millipore, Billerica, MA, USA). The detection limit was 3 pmol/L. Intra - and inter -assay coefficients of variance were 6.3% and 10.3%.

Data analysis

On the basis of our previous work (194), with an observed within-subjects standard deviation (SD) of 181 kcal, we estimated an SD using the upper 60% confidence limit of 234 kcal and calculated that 8 subjects per group would allow detection of a within-groups (n = 8) difference between treatments of 271 kcal and a between groups difference of 353 kcal (n = 8 women compared with n = 8 men), with power equal to 0.8 and alpha equal to 0.05.

Statistical analyses were performed using SPSS Statistics software (version 21, IBM, Armonk, NY, USA). Effects of gender, protein load and their interaction effect on energy intake and gastric emptying were determined using repeated measures ANOVA, with protein load as the *within-subject* factor, and gender as the *between-subject* factor. To adjust for baseline values at each visit as a covariate, a repeated measures mixed effect model, with protein load as the within-subject factor and gender as the between-subject factor was used to test for effects of gender and protein load and their interaction effect on perceptions of appetite and gastrointestinal symptoms, blood glucose and plasma hormone concentrations. Post-hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when there were significant main or interaction effects. *Within-subject* correlations were determined by using a general linear model with fixed slope and random intercept (277). Areas under the curve (AUC) were calculated using the trapezoidal rule. Assumptions of normality were verified for all outcomes before statistical analysis. Statistical significance was accepted at $P < 0.05$. All data are presented as mean values ± SEMs.

RESULTS

The study protocol was well tolerated by all subjects and there were no untoward effects.

Energy intake

Energy intake at the buffet meal was less in women than men (gender effect $P = 0.010$). On the control day energy intake was 34% lower in women than men (791 ± 87 kcal vs. 1205 ± 109 kcal, $P = 0.010$).

Energy intake at the buffet meal was suppressed by protein compared to control in men, but not women (protein-load effect $P = 0.008$, interaction-effect of gender by protein-load $P =$

0.046; **Figure 10.1**). The mean suppression of the 30 g and 70 g protein loads compared to control was 206 ± 39 kcal or 15 ± 2% for men, while it was 46 ± 54 kcal or 0 ± 26% for women (gender-effect $P = 0.032$, protein-load effect $P = 0.75$, interaction-effect $P = 0.19$). There was a protein-load effect ($P = 0.002$) on total energy intake (drink plus buffet meal), which was higher in women than men (gender-effect $P = 0.010$, interaction-effect $P = 0.046$). Compared to the total energy intake on the control day, total energy intake on the 30 g and 70 g protein days increased 22 ± 13% and 35 ± 15% respectively in women, and decreased 8 ± 3% and increase 10 ± 5%, respectively, in men. Total energy intake was higher after the 70 g compared to the 30 g protein load in men ($P = 0.021$), and after the 70 g protein drink compared to control in women ($P = 0.033$).

Macronutrient intakes at the buffet meal

At the buffet meal women consumed a higher percentage of their energy intake as protein than men (average of all three study days: women: 24 ± 1%, men: 20 ± 1%; gender-effect $P = 0.023$, protein-load effect $P = 0.31$, interaction-effect, $P = 0.60$) and fat (women: 36 ± 1%, men: 28 ± 1%; gender-effect $P = 0.006$, protein-load effect $P = 0.09$, interaction-effect $P = 0.85$), and less as carbohydrate (women: 41 ± 3%, men: 52 ± 2%; gender-effect $P = 0.001$, protein-load effect $P = 0.13$, interaction-effect $P = 0.98$).

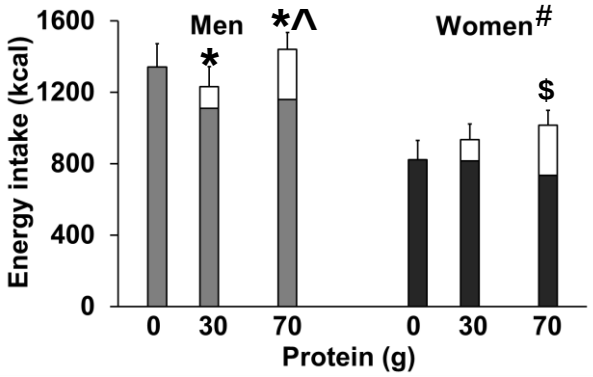

Figure 10.1: Mean (± SEM) energy intake at the buffet meal (kcal; energy intake in closed bars) in healthy young men (gray shading; n = 8) and women (black shading; n = 8) after intake of drinks (energy content in open bars) containing flavored water (control) and whey protein loads of 30 g (120 kcal) and 70 g (280 kcal). Effects of gender and protein-load and interaction effects were determined by using repeated-measures ANOVA. Interaction effect of gender by protein-load energy intake at the buffet meal $P = 0.046$ and total energy intake (drink + buffet meal) $P = 0.046$.
[#] Effect of gender: energy intake $P = 0.010$ and total energy intake $P = 0.010$ were higher in men than women.
Effect of protein load: energy intake $P = 0.008$ and total energy intake $P = 0.002$. * Post hoc effects: energy intake was lower after the 30 g ($P = 0.001$) and 70 g ($P = 0.049$) protein drink compared to control in men. [^] Post hoc effects: total energy intake was higher after the 70 g compared to the 30 g protein drink in men ($P = 0.021$); [$] Post hoc effects: total energy was higher after the 70 g protein drink compared to control in women ($P = 0.033$).

Perceptions of appetite and gastrointestinal symptoms

Baseline hunger, desire to eat, prospective food consumption, fullness, nausea and bloating were comparable in men and women (all $P > 0.05$). Protein drink ingestion was associated with a load-dependent decrease in perceptions (AUC and ratings immediately before the buffet meal at 180 min) of hunger ($P = 0.002$ and $P = 0.002$), desire to eat ($P = 0.001$ and $P < 0.001$) and prospective food consumption ($P = 0.001$ and $P = 0.005$).

Hunger ratings were lower in women than men during the control day, and decreased in men, but not women, after both 30 g ($P = 0.004$) and 70 g ($P < 0.001$) protein loads compared to control day values (gender-effect $P = 0.08$, interaction-effect of gender by protein-load $P = 0.014$; **Figure 10.2**).

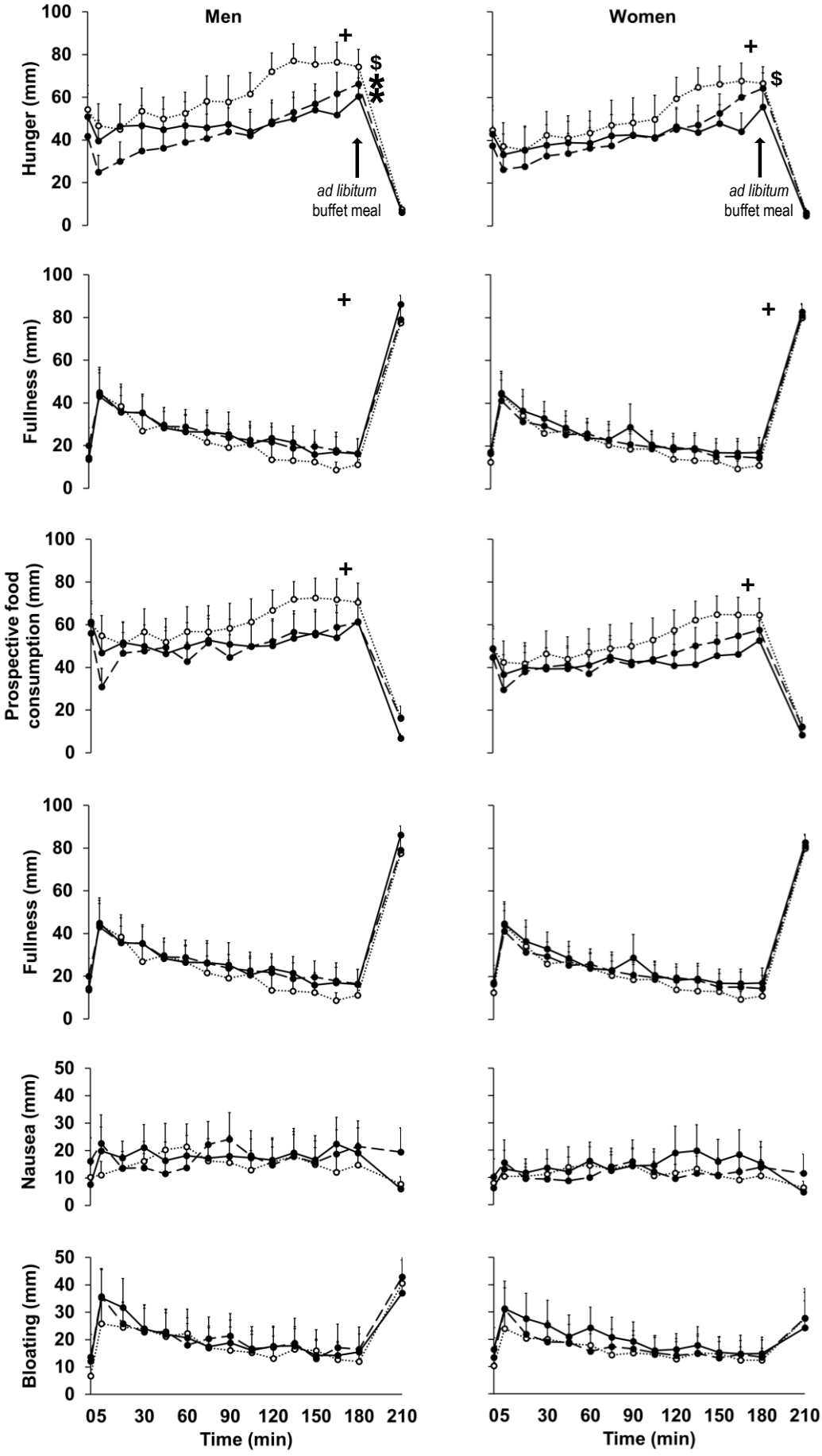
Men
Women
Hunger (mm)
Fullness (mm)
Prospective food consumption (mm)
Fullness (mm)
Nausea (mm)
Bloating (mm)
ad libitum buffet meal
ad libitum buffet meal
Time (min)
Time (min)
05 30 60 90 120 150 180 210

Figure 10.2: Mean (± SEM) Visual analogue score (VAS, mm) of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating in healthy young men (n = 8) and women (n = 8) after drinks containing flavoured water (control; dotted line with open circles) and whey protein loads of 30 g (dashed line with closed circles) or 70 g (solid line with closed circles). Effects of gender and protein-load and interaction effects were determined by using repeated-measures ANOVA including baseline values at each treatment visit as a covariate.
$^{+}$ $P < 0.005$ Effect of protein load: perceptions (area under the curve; AUC) of hunger ($P = 0.002$), desire to eat ($P = 0.001$) and prospective food consumption ($P = 0.001$) protein-load dependently increased after drink ingestion.
$^{\$}$ $P = 0.0016$ Interaction effect of gender by protein-load: perceptions of hunger were lower in women than men after the control drink.
* $P < 0.005$ Interaction effect of gender by protein-load: in men hunger was suppressed after both 30 g (Post-hoc $P = 0.004$) and 70 g (Post-hoc $P < 0.001$) protein loads compared to control.

Gastric emptying

Gastric emptying parameters are detailed in **Table 10.1**. Baseline gastric volumes were comparable in men (31 ± 6 mL) and women (34 ± 4 mL, $P = 0.69$) and between study days ($P = 0.41$). The control (water) and the 30 g protein drinks emptied in an overall non-linear pattern, whereas the pattern of the 70 g protein drink was linear (**Figure 10.3**). Gastric retention (AUC % decrease in stomach volume compared to directly after drink ingestion, $P < 0.001$), gastric emptying halftime (T50, $P < 0.001$), complete emptying time (T100, $P < 0.001$) and the rate of gastric emptying (kcal/min, $P < 0.001$) protein-load dependently increased after drink ingestion. The drinks emptied slower in women than men; gastric retentions were higher in women compared to men (gender-effect $P = 0.021$, interaction-effect of gender by protein-load $P = 0.34$).

Glucose and gut hormones

Baseline concentrations of blood glucose (5.4 ± 0.1 mmol/L) and plasma insulin (5.3 ± 0.6 mU/L), glucagon (68 ± 4 pg/mL), ghrelin (1507 ± 207 pg/mL), CCK (3.3 ± 0.4 pmol/L) and GIP (16 ± 2 pmol/L), and HOMA index (1.3 ± 0.1) were comparable in men and women (*P*

> 0.05) while plasma GLP-1 concentrations were marginally lower in women (16.5 ± 0.9 pmol/L) than men (20.6 ± 2.0 pmol/L, $P < 0.001$).

AUC blood glucose and plasma ghrelin decreased, and plasma insulin, glucagon, CCK, GIP, GLP-1 concentrations increased in a protein-load dependent fashion (all $P < 0.01$; **Figure 10.4**). 60 min and 180 min plasma ghrelin decreased and plasma insulin, glucagon, CCK, GIP and GLP-1 concentrations increased in a protein-load dependent fashion (all $P < 0.05$; **Table 10.2**). AUC blood glucose concentrations were lower after the 30 g protein drink compared to control. 60-min plasma ghrelin concentrations were lower after both protein drinks compared to control (all $P < 0.05$). AUC plasma ghrelin concentrations were lower after the 70 g protein drink compared to the 30 g protein and control drinks. 60-min and AUC plasma insulin, glucagon, CCK, GIP and GLP-1 concentrations were higher after both protein drinks compared to control, and 180-min and AUC concentrations after the 70 g compared to the 30 g protein drink (all $P < 0.05$). 60-min plasma GLP-1 concentrations were higher after 70 g compared to 30 g protein ($P = 0.036$).

Women compared to men had higher 60-min and AUC blood glucose concentrations ($P < 0.05$), and lower 60-min, 180-min and AUC plasma glucagon, CCK and GLP-1 concentrations (all $P < 0.05$). Women compared to men had higher 60-min and AUC plasma glucagon concentrations, lower 60-min and AUC plasma GLP-1 and 180-min and AUC GIP concentrations (gender by protein-load interactions all $P < 0.05$).

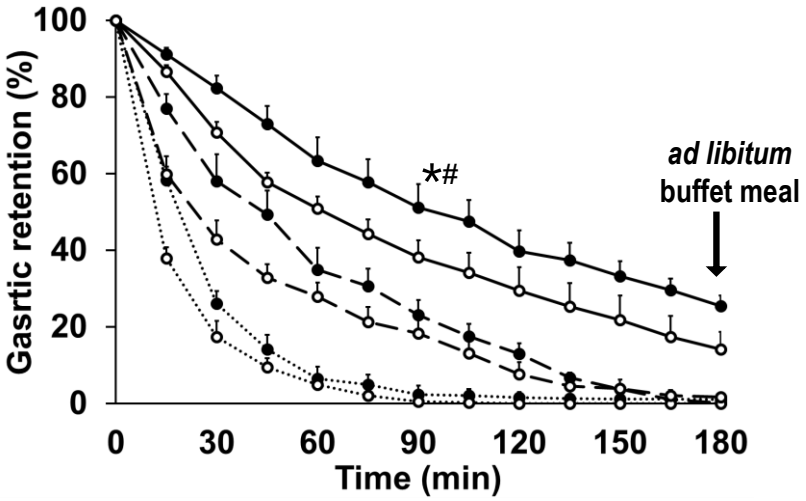

Figure 10.3: Mean (± SEM) gastric retention (%) in healthy young men (n = 8; closed circles) and women (n = 8; open circles) after drinks containing flavoured water (control; dotted line) and whey protein loads of 30 g (dashed line) or 70 g (solid line). Effects of gender and protein-load and interaction effects were determined by using repeated-measures ANOVA.

* $P < 0.05$ 50% gastric emptying time (T50): effect of gender and protein-load (interaction effect of gender by protein-load $P = 0.17$)

# $P < 0.05$ area under the curve (AUC): effect of gender and protein-load (interaction effect of gender by protein-load $P = 0.34$).

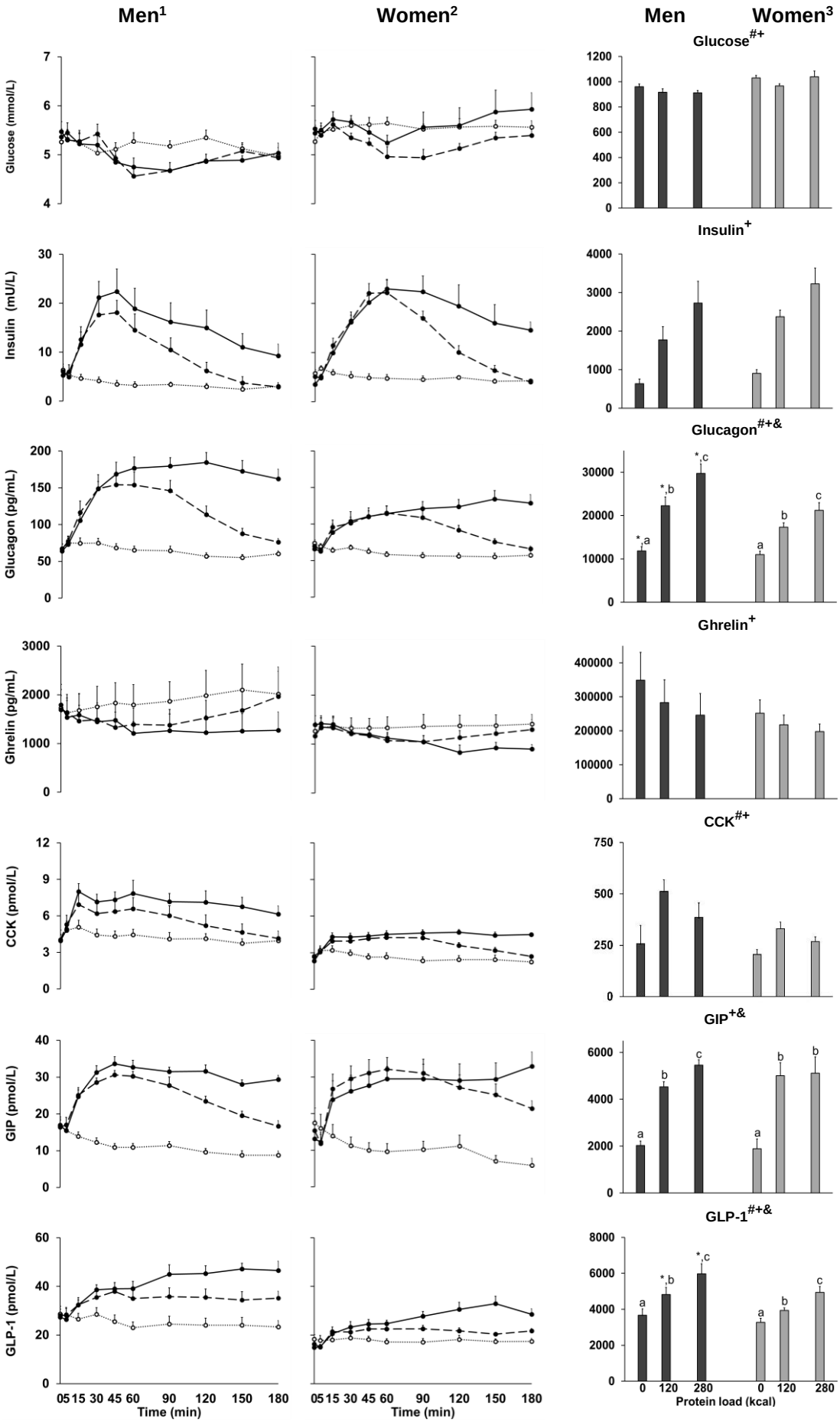
Men[1]
Women[2]
Men
Women[3]
Glucose (mmol/L)
Glucose[#+]
Insulin (mU/L)
Insulin[+]
Glucagon (pg/mL)
Glucagon[#+&]
Ghrelin (pg/mL)
Ghrelin[+]
CCK (pmol/L)
CCK[#+]
GIP (pmol/L)
GIP[+&]
GLP-1 (pmol/L)
GLP-1[#+&]
Time (min)
Time (min)
Protein load (kcal)

Figure 10.4: Mean (± SEM) blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP and GLP-1 concentrations in healthy young men (n = 8, n = 7 for GIP and GLP-1) and women (n = 8) after drinks containing flavoured water (control; dotted line with open circles) and whey protein loads of 30 g (dashed line with closed circles) or 70 g (solid line with closed circles). Effects of gender and protein-load and interaction effects were determined by using repeated-measures ANOVA including baseline values at each treatment visit as a covariate and post-hoc Bonferroni corrections.
$^{\#}$ $P < 0.05$ Effect of gender; $^{\$}$ $P < 0.05$ Post-hoc: plasma glucagon and GLP-1 concentrations were lower in women than men after all drinks
$^{+}$ $P < 0.001$ Effect of protein load; * $P < 0.05$ Post-hoc: plasma glucagon, GIP and GLP-1 concentrations were higher after both protein drinks compared control in men and women
$^{\&}$ $P < 0.05$ Interaction effect of gender by protein-load; ^ $P < 0.05$ Post-hoc: plasma glucagon and GLP-1 - and GIP only in men - concentrations were higher after the 70 g protein drink compared to the 30 g protein drink in men and women.

Relationships between energy intake, appetite, gastric emptying and gut hormones

Energy intake at the buffet meal was, *within subjects*, related to 180-min plasma insulin, (r = -0.37, $P = 0.032$), CCK (r = -0.36, $P = 0.041$), GIP (r = -0.37, $P = 0.033$) and GLP-1 (r = -0.37, $P = 0.001$) concentrations, and perceptions of hunger (r = 0.37, $P = 0.032$), desire to eat (r = -0.53, $P = 0.002$) and prospective food consumption (r = 0.40, $P = 0.022$). GIP was related to GLP-1 (r = 0.78 $P < 0.001$) while ghrelin was inversely related to insulin (r = -0.63 $P < 0.001$).

Table 10.1: Gastric emptying parameters of whey protein (30 g and 70 g) and control drinks in healthy young men and women

	Men (n = 8)			Women (n = 8)		
	0 g	30 g (120kcal)	70 g (280kcal)	0 g	30 g (120kcal)	70 g (280kcal)
50% emptying time (T50; min) [+]	12±1	25±4	72±13	19±1	39±5	98±14
100% emptying time (100, min) [+]	60±7	126±14	171±6	81±15	176±4	180±0
Rate of gastric emptying (kcal/min) [1, +]		1.0±0.1	1.5±0.1		0.9±0.0	1.2±0.0
Early phase rate of gastric emptying [1 +]		1.4±0.1	2.3±0.1		1.3±0.1	1.7±0.3
Late phase rate of gastric emptying [1 +]		0.3±0.1	1.0±0.2		0.5±0.1	1.0±0.1
Amount emptied at 60 min (%) [+]	95±1	72±4	49±3	93±3	65±6	37±6
Amount emptied at 180 min (%) [+, *]	100±0[a]	98±1[a]	86±5[b]	99±1[a]	100±0[a]	75±3[b]

All values are mean ± SEM. Effects of gender and protein-load and interaction effects were determined by repeated-measures ANOVA.
[1] Rate of gastric emptying was calculated as mean of rates of emptying during each 15-min interval respectively of the early phase (i.e. 0-60 min), the late phase (i.e. 60 min until 100% emptying time per individual) and total time period (i.e. 0 min until 100% emptying time per individual). [+] $P < 0.001$ Effect of protein load. $P \leq 0.1$. * $P < 0.005$ Interaction effect of gender by protein-load: amount emptied at 180 min
[a,b] $P < 0.05$, post hoc test: different letter indicates significant difference between drink-conditions.

Table 10.2: Concentrations of glucose, insulin, glucagon, ghrelin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1)

A	Gender effect			B	Protein-load effect			
	Men	Women	Average		Control	30g protein	70g protein	Average
60 min								
Glucose	4.9 ± 0.1	5.3 ± 0.1			5.5 ± 0.1[a]	4.8 ± 0.2[b]	5.0 ± 0.1[b]	
Insulin			14.4 ± 1.5		4.0 ± 0.5[a]	18.3 ± 2.3[b]	20.9 ± 2.3[b]	
Glucagon	132 ± 12	96 ± 6			62 ± 3[a]	134± 11[b]	145 ± 12[b]	
Ghrelin			1319 ± 180		1563 ± 236[a]	1230 ± 179[b]	1165 ± 130[b]	
CCK	6.3 ± 0.7	3.8 ± 0.3			3.5 ± 0.4[a]	5.4 ± 0.6[b]	6.2 ± 0.7[b]	
GIP			24.2 ± 1.3		10 ± 1[a]	31 ± 2[b]	31 ± 2[b]	
GLP-1	32.4 ± 3.1	21.4 ± 1.2			20 ± 2[a]	29 ± 3[b]	32 ± 2[c]	
180 min								
Glucose	5.0 ± 0.1	5.6 ± 0.2						5.3 ± 0.1
Insulin	5.1 ± 1.2	7.6 ± 0.7			3.6 ± 0.4[a]	3.5 ± 0.4[a]	11.9 ± 1.6[b]	
Glucagon	99 ± 6	84 ± 6			59 ± 3[a]	71 ± 7[b]	145 ±10[c]	
Ghrelin			1477 ± 253		1713 ± 294[a]	1633 ±281[a]	1085 ± 194[b]	
CCK	4.8 ± 0.5	3.1 ± 0.2			3.1 ± 0.3[a]	3.4 ± 0.4[a]	5.3 ± 0.4[b]	
GIP			19.2 ± 1.2		7 ± 1[a]	19 ± 1[b]	31 ± 2[c]	
GLP-1	35 ± 3	23 ± 1			20 ± 2[a]	28 ± 2[b]	38 ± 3[c]	

C	Interaction effect					
	Men			Women		
	Control	30 g protein	70 g protein	Control	30 g protein	70 g protein
60 min						
Glucagon	65±5*,[a]	154±18*,[b]	177±15*,[c]	58±4[a]	115±10[b]	114±11[b]
GLP-1	23.0±2.4[a]	35.0±4.2[b]	39.1±3.0*,[b]	17.2±1.3[a]	22.5±1.4[,b]	24.7±1.4[b]
180 min						

GIP	7.6±1.7*,a	19.6±1.8*,b	31.8±3.7c	5.9±1.9a	21.4±2.2b	32.9±3.9c

Main gender (A) and protein-load effects (B), and interaction effects (C) of gender by protein-load for mean (± SEM) 60-min and 180-min concentrations of blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL), ghrelin (pg/mL), cholecystokinin (CCK; pmol/L), gastric inhibitory polypeptide (GIP; pmol/L) and glucagon-like peptide-1 (GLP-1; pmol/L) in healthy young men (n = 8, n = 7 for GIP and GLP-1) and women (n = 8) after control (~2 kcal) and 30 g (120 kcal) or 70 g (280 kcal) protein loads. Results are presented separately for gender or protein-load if the main effect was significant, in case of non-significance the average was presented. Interaction effects of gender by protein-load are given if the effect was significant. Effects of gender and protein-load and interaction effects were determined by using repeated-measures ANOVA including baseline values at each treatment visit as a covariate and post hoc Bonferroni corrections. Post hoc effects: * $P < 0.05$: men vs. women; ^{a,b,c} $P < 0.05$: a different letter indicates a significant difference between drink-conditions within subject group.

DISCUSSION

This study examined the acute effects of oral whey protein ingestion on energy intake, perceptions of appetite and gastrointestinal symptoms, gastric emptying, blood glucose and plasma gut hormone concentrations in women and men. The current study is the first study we are aware of to compare the effect of gender on these parameters after pure protein intake. There was a load-dependent suppressive effect of protein on perceptions of hunger, desire to eat, prospective food consumption, and blood glucose and plasma ghrelin concentrations, slowing of gastric emptying, and increase of plasma insulin, glucagon, CCK, GIP and GLP-1 concentrations. Hunger and energy intake were less in women than men. The major finding was that hunger and *ad libitum* energy intake were suppressed by the whey protein ingestion in men, but not women. Men had a 15% reduction in food intake at the buffet meal after the protein drinks, whereas there was no suppression in women. The suppression in men represented almost 100% compensation for the energy content of the protein drinks (206kcal reduction vs 200kcal mean energy content of the two protein drinks), whereas there was no compensation in women. Consequently, compared to intake on the control day, total energy intake (protein drink *plus* buffet test meal) was increased by the protein drinks in women (~150 kcal [~30%]), with no effect on total intake in men. The drinks emptied from the stomach more slowly and plasma glucagon, CCK and GLP-1 concentrations were less in women than men.

There is evidence that protein has greater satiating effects than the other macronutrients (carbohydrate and fat (36-39)) and that enhanced protein diets can facilitate weight loss (40, 287); protein diets are widely used for this purpose by both men and women trying to lose weight. There is also evidence, however, that men lose weight more easily than women on energy-restricted diets (342), and that women, when compared to men compensate less for energy intake after mixed macronutrient drinks (322) and semi-liquid (yoghurt) preloads

(321). This may be due, at least in part, to the lower satiating effect of protein in young women than men we have demonstrated in the present study. The outcomes of this study may therefore have important implications for the types of dietary modifications recommended to promote weight loss in those trying to lose weight. Less emphasis on protein enrichment in women may be appropriate.

Appetite and energy intake are dependent on the precise co-ordination of interrelated 'gastric' and 'small intestinal' mechanisms triggered by the interaction with the nutrients ingested. Gastric emptying has an important role in mediating gut hormone release in response to protein, fat and carbohydrates (114, 273, 343, 344), and emptying of food content from the stomach itself is slowed by feedback mechanisms in the small intestine including the release of CCK and GLP-1 (345, 346). Gastric emptying was markedly and dose-dependently slowed by the whey protein in this study, with the 50% gastric emptying time more than doubling from control to the 30 g protein day, and doubling again from the 30 g to 70 g protein day. Gastric emptying was completed earlier after the 30 g than the 70 g whey protein load, which resulted in a time-dependent response (earlier return to baseline after 30 g vs. 70 g protein intake) in plasma concentrations of insulin, glucagon, ghrelin and CCK in men and women after 30 g vs. 70 g protein load. There was an immediate increase in plasma CCK and GIP concentrations, both mainly produced in the duodenum and proximal jejunum, reaching a plateau from 15-30 min onwards, while GLP-1, produced in the ileum more distally in the gut, showed a more constant increase. Rates of gastric emptying of the protein drinks were at the lower end of the normal range of gastric emptying i.e., 1-4 kcal/min (24, 41-43), with faster gastric emptying rates during the 70 g compared to the 30 g protein loads. Our findings that gastric emptying was slower in women compared to men were similar to the results of most (83, 330-332), but not all, previous studies (80, 332).

The findings of lower glucagon, CCK and GLP-1 concentrations after protein in women compared to men are consistent with previous reports that women have lower plasma

concentrations of glucagon and GLP-1 after a mixed-nutrient liquid meal (335), but not after glucose (347), and of CCK after mixed-nutrient drinks (336), but not after corn oil (348), than men. Both CCK and GLP-1 suppress appetite and food intake (288), so the reduced response of these hormones to protein in women than men provides one possible explanation for the observed, reduced satiating effect of whey protein in women than men. In the present study plasma insulin, ghrelin and GIP concentrations were comparable in men and women, consistent with most previous reports; for insulin after oral glucose (347, 349), insulin and ghrelin after mixed-nutrient ingestion (350), and insulin and GIP during intravenous glucose administration (349). Ghrelin concentrations have, however, also been reported to be higher in women than men after oral loads of glucose and lipids (351).

It has been suggested that sex hormones may affect food intake (352). Pre-menopausal women are reported to have slower gastric emptying and lower appetite, food intake and plasma GLP-1 concentrations during the follicular than luteal phase, without changes in CCK concentrations (338). The young adult, pre-menopausal women in the present study were investigated during the follicular phase of the menstrual cycle, so it is possible that some of the differences between women and men observed (e.g. slower gastric emptying, lower GLP-1 concentrations) would have been less marked or absent if the women were examined during the luteal phase of their cycles. Similarly, we do not know if the reduced suppression of appetite and food intake by protein observed in this study in women compared to men persists into the luteal phase.

Energy intake at the buffet meal was related to perceptions of appetite and plasma gut hormone concentrations immediately before the meal. Also energy intake at the buffet meal and perceptions of appetite were related to the rate of emptying of the whey protein drink from the stomach and plasma gut hormone responses, which were interrelated; the greater the increase in plasma insulin, glucagon, CCK, GIP and GLP-1 and decrease in ghrelin concentrations, the slower the drink emptied from the stomach within a subject – 70 g < 30

g < 0 g – the lower the perceptions of appetite, and the lower the subsequent energy intake at the buffet meal. GIP was related to GLP-1 (r = 0.78, $P < 0.001$) while ghrelin (AUC 0-180 min) was inversely related to insulin (r = -0.63, $P < 0.001$).

Although our study has several limitations, including the relatively small number of subjects, the results appear clear-cut. The protein preload drinks were selected to be iso-caloric for both men and women. Women are expected to have lower energy requirements when compared to men, and the drinks given to female group in this study could, therefore, be considered to be 'larger' than those given to the male group when considered in relation to energy requirements. While the drinks were matched for taste, we did not assess the subject's perceptions of taste, pleasantness and/or palatability of the drinks. Women were studied during the follicular phase of their menstrual cycle, and it is unsure whether the results can be translated to the luteal phase. Blood glucose was measured by a glucometer, which is less than optimal, however, the results appear to be clear-cut.

In summary, in young healthy women, when compared to men, whey protein drinks emptied slower from the stomach, and plasma glucagon, CCK and GLP-1 concentrations were lower associated with less suppression of energy intake and hunger by whey. These findings have potential implications for the efficacy of ingesting whey or other proteins to decrease overall food intake and achieve voluntary weight loss in women. Further studies are needed to determine how broadly these findings apply to other settings, including the use of other proteins, while longer-term studies will be needed to determine the effects of ingesting whey or other proteins on chronic changes in food intake, body weight and body composition.

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Table 10.3: *Within-subject* correlations between perceptions of appetite and gastrointestinal symptoms, gastric retention and blood glucose and plasma gut hormone concentrations after intake of whey protein (30 g and 70 g) and control drinks in healthy young men and women

	Hunger		Desire to eat		Prospective food consumption		Fullness		Nausea		Bloating	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Gastric retention	-0.39	0.024	-0.57	<0.001	-0.55	0.001	0.27	0.13	0.12	0.51	0.16	0.36
Glucose	-0.07	0.71	-0.17	0.35	-0.06	0.72	0.12	0.51	0.32	0.07	0.17	0.35
Insulin	-0.40	0.022	-0.51	0.003	-0.46	0.006	0.28	0.11	0.12	0.52	0.22	0.21
Glucagon	-0.41	0.017	-0.50	0.003	-0.49	0.003	0.30	0.08	0.12	0.49	0.16	0.39
Ghrelin	0.44	0.011	0.50	0.003	0.46	0.008	-0.15	0.39	-0.10	0.59	0.00	1.00
CCK	-0.35	0.047	-0.40	0.021	-0.47	0.006	0.23	0.20	0.14	0.43	0.29	0.12
GIP	-0.39	0.025	-0.47	0.006	-0.48	0.004	0.18	0.32	0.01	0.94	0.11	0.55
GLP-1	-0.51	0.002	-0.55	0.001	-0.51	0.002	0.32	0.07	0.24	0.18	0.23	0.19

r and *P* values of *within-subject* correlations, determined by using a general linear model with fixed slope and random intercept, between perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea, bloating [area under the curve (AUC 0-180 min) and blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL), ghrelin (pg/mL), CCK (pmol/L), GIP (pmol/L) and GLP-1 (pmol/L) concentrations (AUC 0-180 min) in healthy young men and women.

CHAPTER 11: EFFECT OF AGE ON BLOOD GLUCOSE AND PLASMA INSULIN, GLUCAGON, GHRELIN, CCK, GIP AND GLP-1 CONCENTRATIONS AFTER WHEY-PROTEIN INGESTION

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Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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ABSTRACT

Background: Protein-rich supplements are used widely to prevent and manage undernutrition in older people. We have previously shown that healthy older, compared to younger, adults have less suppression of energy intake by whey protein—although the effects of age on appetite-related gut hormones are largely unknown.

Objective: To determine and compare the acute effects of whey protein loads on blood glucose and plasma gut hormone concentrations in older and younger adults.

Design: Sixteen healthy older (eight men, eight women; mean ± SEM: age: 72 ± 1 years; body mass index: 25 ± 1 kg/m^2) and 16 younger (eight men, eight women; 24 ± 1 years; 23 ± 0.4 kg/m^2) adults were studied on three occasions in which they ingested 30 g (120 kcal) or 70 g (280 kcal) whey protein, or a flavoured-water control drink (~2 kcal). At regular intervals over 180 min, blood glucose and plasma insulin, glucagon, ghrelin, cholecystokinin (CCK), gastric inhibitory peptide (GIP), and glucagon-like peptide-1 (GLP-1) concentrations were measured.

Results: Plasma ghrelin was dose-dependently suppressed and insulin, glucagon, CCK, GIP, and GLP-1 concentrations were dose-dependently increased by the whey protein ingestion, while blood glucose concentrations were comparable during all study days. The stimulation of plasma CCK and GIP concentrations was greater in older than younger adults.

Conclusions: In conclusion, orally ingested whey protein resulted in load-dependent gut hormone responses, which were greater for plasma CCK and GIP in older compared to younger adults.

INTRODUCTION

Despite the well-recognised major adverse impact of nutritional impairment on the health of the elderly, including ageing-related muscle loss (190), and related increase in the use of high-energy drinks, usually rich in whey protein, few nutritional studies have involved older people. We have recently reported that healthy older, compared to younger, adults have less suppression of energy intake by whey protein, ether ingested orally (263) or infused directly into the proximal small intestine (194).

Appetite, energy intake, and blood glucose regulation are likely to be dependent on gastrointestinal mechanisms triggered by the interaction with the nutrients ingested. Mechanisms which reduce energy intake in younger adults include the stimulation of gut hormone secretion, e.g., cholecystokinin (CCK) and glucagon-like peptide (GLP-1), and the suppression of ghrelin. The incretin hormones gastric inhibitory polypeptide (GIP) and GLP-1 play major roles in the control of plasma insulin, glucagon, and blood glucose concentrations in response to nutrient ingestion (353). We, and others, have reported that age affects gut hormone responses; healthy older, compared to younger, adults had higher CCK concentrations after overnight fasting, after mixed nutrient intake (42, 48), and during intraduodenal glucose and lipid infusions (53), in addition to higher insulin in response to intraduodenal glucose infusion (44), higher GIP after glucose ingestion (132, 135), and higher GLP-1 after an overnight fast (89, 132, 231) as well as after glucose (132) and mixed macronutrient intakes (231), while the reported effects of age on fasting and postprandial ghrelin after mixed macronutrient intakes are inconsistent (48, 52, 88, 89, 91).

The aims of the study were to further determine the effects of oral whey protein loads on blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP, and GLP-1 concentrations in older as well as younger adults. We hypothesised that orally administered whey protein

would result in load-related responses of glucose and gut hormones, and that these responses to whey protein would be greater in older than younger subjects.

SUBJECTS AND METHODS

Subjects

Sixteen older adults (eight men and eight women, age: mean ± standard error of the mean (SEM): 72 ± 1 years; body weight: 70 ± 3 kg; height: 1.66 ± 0.02 m; body mass index (BMI): 25 ± 1 kg/m^2) and 16 younger adults (eight men and eight women, 24 ± 1 years; 68 ± 2 kg; 1.71 ± 0.02 m; 23 ± 0.4 kg/m^2) were included. The study protocol was approved by the Royal Adelaide Hospital Research Ethics Committee, and subjects provided written informed consent (clinical trial registration: ACTRN12612000941864).

Protocol

The protocol was identical to that of our previous studies comparing younger and older men (263), and older men and women (339) - results of blood glucose and plasma gut hormone concentrations in the healthy older women compared to men are published (339). The study had a randomised (using the method of randomly permuted blocks; www.randomization.com [16 subjects randomised in one block with random permutations)] double-blind cross-over design including three study days, separated by three to 14 days. Subjects consumed a standardised evening meal (beef lasagna (McCain Foods Pty Ltd., Wendouree, VIC, Australia), ~591 kcal) before the study days at ~19.00 h. They were instructed to fast overnight from solids and liquids thereafter and to refrain from strenuous physical activity. On the study day, subjects attended the laboratory at ~08.30 h and were seated in an upright position (263, 339).

Subjects ingested drinks containing 30 g (120 kcal) or 70 g (280 kcal) whey protein or a control drink (~2 kcal) (263, 339). The drinks were prepared by a research assistant who was not involved in the data analysis of the study results, flavoured with diet lime cordial (Bickford's Australia Pty Ltd., Salisbury South, SA, Australia), and served in a covered cup.

Measurements

Blood samples were collected, using an intravenous cannula, at 0, 5, 15, 30, 45, 60, 90, 120, 150, and 180 min, into ice-chilled ethylenediaminetetraacetic acid (EDTA) coated tubes. No inhibitors were added (264). Plasma was obtained by centrifugation for 15 min at 3200 rpm at 4 °C and samples were stored at −80 °C. Ad libitum energy intake (kcal) was determined from a buffet-style meal (180–210 min) (263). Gastric emptying was determined from total gastric volume measurements by three-dimensional (3D) ultrasonography (Logiq™ 9 ultrasound system, GE Healthcare Technologies, Sydney, NSW, Australia) (263).

Blood glucose (millimoles per liter) was determined immediately after collection by the glucose oxidase method using a portable glucometer (Optium Xceed, Abbott Laboratories, Doncaster, VIC Australia). Intra- and inter-assay coefficients of variation were 3.2 and 10.8%. Plasma total insulin [milliunits per liter (mU/L)] was measured by enzyme-linked immunosorbent *assay* (ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L. Intra- and inter-assay coefficients of variation were 3.0% and 8.7%. Plasma total glucagon [picogram per milliliter (pg/mL)], ghrelin (pg/mL), CCK-8 [picomoles per liter (pmol/L)], GIP (pmol/L), and GLP-1 (pmol/L) were measured by radioimmunoassay (RIA) (339). Minimum detectable limits were 20 pg/mL, 40 pg/mL, 1 pmol/L, 2 pmol/L, and 3 pmol/L. Intra- and inter-assay coefficients of variance were: insulin: 3.0% and 8.7%, glucagon: 4.3% and 7.1%, ghrelin: 6.7% and 12.1%, CCK: 5.4% and 13.9%, GIP: 3.9% and 9%, GLP-1: 6.3% and 10.3%.

Data and Statistical Analysis

Sixteen subjects per age group would allow detection of differences in the area under the curve (AUC) of the primary outcomes of 25,920 pg/mL.min ghrelin, 198 pmol/L.min CCK, and 1080 pmol/L.min GLP-1 between groups with power equal to 0.8 and alpha to 0.05. Statistical analyses were performed using SPSS software (version 22; IBM, Armonk, NY, USA). Effects of age and protein load and their interaction effect were determined using a repeated measures mixed-effect model, including baseline values as a covariate and Bonferroni's post hoc correction. AUC was calculated from baseline to 180 min using the trapezoidal rule and peak/nadir as the largest change from baseline. Statistical significance was accepted at $p < 0.05$. All data are presented as means ± SEMs.

RESULTS

Baseline concentrations after an overnight fast of blood glucose (mean ± SEM; older and younger: 5.4 ± 0.1 and 5.4 ± 0.1 mmol/L), plasma glucagon (64 ± 4 and 68 ± 4 pg/mL), ghrelin (1438 ± 156 and 1507 ± 207 pg/mL), and GIP (13 ± 2 vs. 16 ± 2 pmol/L) were comparable between age groups ($P > 0.05$), while insulin was lower (older vs. younger: 3.3 ± 0.4 vs. 5.3 ± 0.6 mU/L, $P = 0.006$) and CCK (4.8 ± 0.6 vs. 3.3 ± 0.4 pmol/L, $P = 0.033$) and GLP-1 (32 ± 4 vs. 22 ± 2 pmol/L, $P = 0.041$) were higher in healthy older compared to younger adults. AUC ghrelin dose-dependently decreased and insulin, glucagon, CCK, GIP, and GLP-1 dose-dependently increased (**Figure 11.1**, post hoc effects: 30 g and 70 g vs. control, 70 g vs. 30 g protein drink, all $P < 0.01$). Nadir glucose was lower ($P = 0.005$), and peak glucagon ($P = 0.001$) and GLP-1 ($P = 0.001$) were higher after 70 g compared to 30 g whey protein ingestion. Time to peak of glucagon was higher after 70 g compared to 30 g whey protein ingestion ($P < 0.001$). Plasma gut hormone concentrations were related (ghrelin positively, and insulin, glucagon, CCK, GIP, and GLP-1 negatively) to energy intake (energy intake after 0 g,

30 g, and 70 g whey protein intake: younger: 1082 ± 106 kcal, 963 ± 79 kcal, 948 ± 82 kcal; older: 843 ± 77 kcal, 803 ± 75 kcal, 793 ± 78 kcal) and gastric emptying (younger and older men (263) and older men and women (339) published previously; **Table 11.1**).

Older compared to younger adults had higher AUC and peak concentrations and time to peak of CCK (AUC: $P = 0.031$; older vs. younger: peak: 5.0 ± 0.7 pmol/L vs. 3.3 ± 0.3 pmol/L, $P = 0.007$; time to peak: 95 ± 12 min vs. 65 ± 8 min, $P = 0.046$) and GIP (AUC: $P = 0.036$; peak: 17.8 ± 2.7 pmol/L vs. 16.6 ± 1.4 pmol/L, $P = 0.028$; time to peak: 132 ± 11 min vs. 101 ± 9 min, $P = 0.037$). AUC interaction effects of age by protein load were not significant.

Table 11.1: Correlations between gut hormones, energy intake and gastric emptying

	Energy intake		**Gastric emptying (T50)**	
	r	***P***	**r**	***P***
Insulin	-0.41	0.001	0.80	<0.001
Glucagon	-0.34	0.005	0.81	<0.001
Ghrelin	0.36	0.003	-0.53	<0.001
CCK	-0.33	0.008	0.77	<0.001
GIP	-0.32	0.008	0.75	<0.001
GLP-1	-0.31	0.011	0.68	<0.001

r and *P* values of *within-subject* correlations between energy intake [control (~2kcal), 30 g (120 kcal) and 70 g (280 kcal) whey protein intake: younger n = 16: 1082 ± 106 kcal, 963 ± 79 kcal, 948 ± 82 kcal; older n = 16: 843 ± 77 kcal, 803 ± 75 kcal, 793 ± 78 kcal), gastric empting half time [T50; control, 30 and 70 g whey protein intake: younger n = 16: 16 ± 1 min, 32 ± 3 min, 85 ± 10 min; older n = 15: 23 ± 2 min, 65 ± 7 min, 130 ± 10 min] and concentrations (AUC 0-180 min) of blood glucose (mmol/L) and plasma insulin (mU/L), glucagon (pg/mL), ghrelin (pg/mL), CCK (pmol/L), GIP (pmol/L) and GLP-1 (pmol/L) in younger and older adults. *Within-subject* correlations were determined by using a general linear model with fixed slope and random intercept.

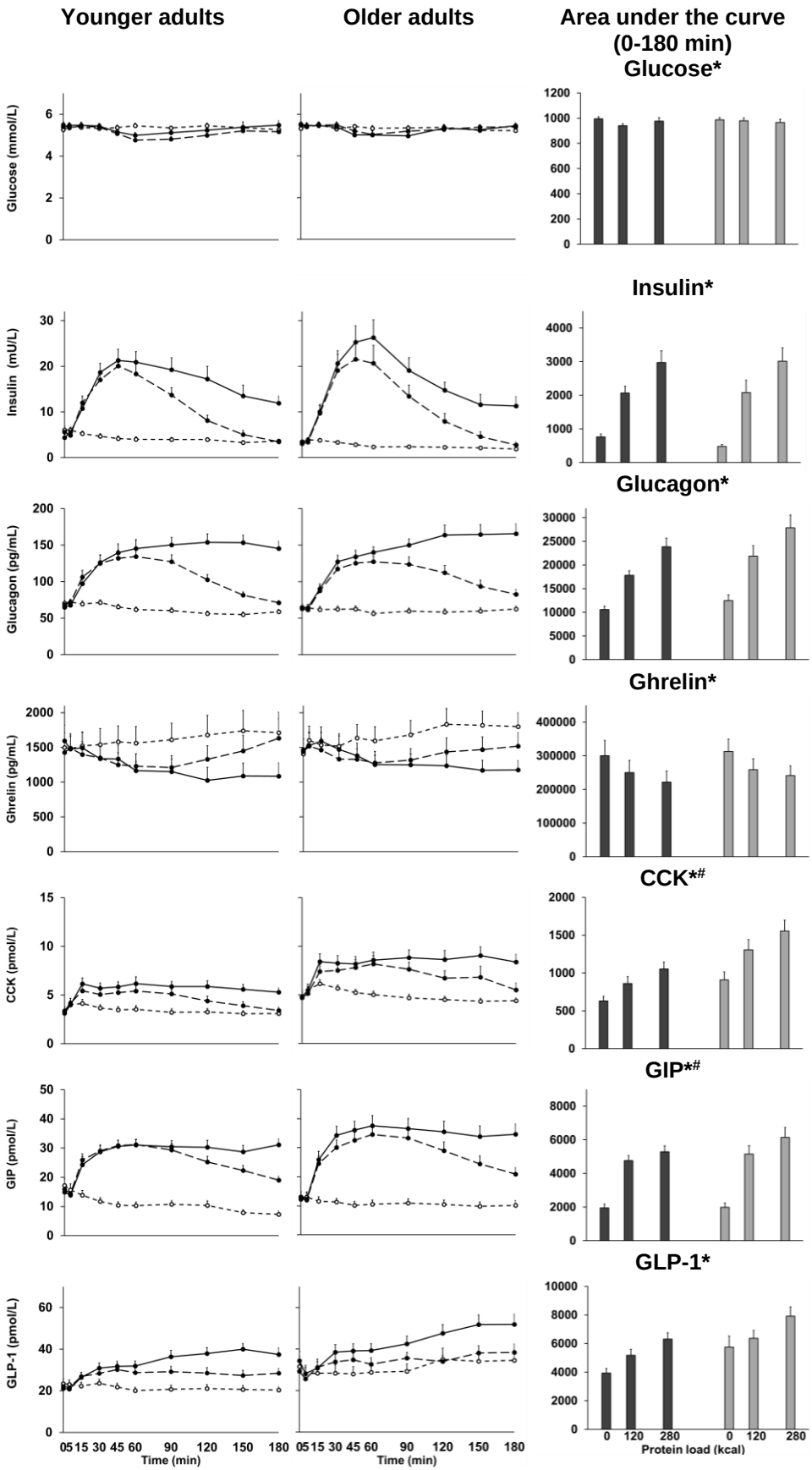

Figure 11.1: Mean and area under the curve (± SEM) values of blood glucose and plasma insulin, glucagon, ghrelin, CCK, GIP and GLP-1 concentrations in younger (n = 16) and older (n = 16) adults after 30 g (120 kcal; dashed line with closed circles) or 70 g (280 kcal; solid line with closed circles) whey-protein ingestion or control (~2 kcal; dashed line with

open circles). Main effects of age and protein load and the interaction effect of age by protein load were determined using a mixed-effect model with baseline concentrations as a covariate.
* $P \leq 0.005$, protein load effect: AUC blood glucose and plasma ghrelin dose-dependently decreased and plasma insulin, glucagon, CCK, GIP and GLP-1 dose-dependently increased after whey protein ingestion.
# $P < 0.05$, age effect: older compared to younger adults had higher AUC CCK and GIP concentrations.

DISCUSSION

This study examined the influence of age on the acute effects of orally ingested whey protein on blood glucose and plasma gut hormone concentrations in healthy adults. Plasma ghrelin was dose-dependently suppressed, while insulin, glucagon, CCK, GIP and GLP-1 concentrations were dose-dependently increased by the whey protein ingestion. Our observations extend the previously reported data of the acute effects of orally ingested whey protein on plasma insulin, glucagon, ghrelin, CCK, GIP, and GLP-1 concentrations in young adults (334, 354). The protein load effects were particularly evident after ~60 min, when the majority of the dose of 30 g whey protein had emptied from the stomach (339); plasma concentrations returned to baseline after 30 g, while they remained at their maximal increase/decrease after 70 g whey protein intake.

Our findings confirmed earlier reports that older, when compared to younger, adults have higher plasma CCK (42, 48, 53) and GLP-1 (132, 231) concentrations after an overnight fast, while fasting insulin concentrations were reduced in our study in healthy adults. Age also affected CCK and GIP, but not insulin, responses following whey protein ingestion; as previously reported after mixed macronutrient ingestion for CCK (42, 48) and oral, but not intraduodenally infused (133), glucose ingestion for GIP (132, 135), glucose, and insulin (44, 133), postprandial concentrations were greater. The higher plasma CCK and GIP concentrations in older rather than younger adults may be related to differences in the small intestinal transit of the whey protein, and clearance including GIP inactivation by dipeptidyl

peptidase IV (DPP-IV) and renal processes (132). The higher incretin hormone GIP response following whey protein ingestion in older compared to younger adults is likely to be beneficial for glycemic control in older people.

The causes of the age-related reduction in the suppression of energy intake by nutrients observed in this and other studies must include altered responses to the presence of nutrients in the small intestine, because the reduced suppression is observed after intraduodenal (194) as well as oral nutrient administration (46, 48, 263). CCK is a anorexigenic hormone and acts to suppress hunger and food intake (355). We have reported previously that older, when compared to younger, age in healthy subjects is associated with at least preserved, and possibly even increased, sensitivity to the satiating effects of exogenously administered CCK (43). Because plasma fasting and post-protein CCK concentrations were higher in older compared to young subjects in the present and previous studies, it is perhaps surprising that these higher concentrations were associated with reduced, not increased, protein-induced suppression of energy intake in the healthy older compared to young adult subjects (194, 263). It is possible that the test meal may have been given too late at 3 hours to assess the full effect of CCK changes, as plasma concentrations had returned to baseline by then after all but the highest whey protein load. Nevertheless, these findings are consistent with our previous finding that under-nourished older people have higher fasting and post-nutrient CCK concentrations in comparison to well-nourished older people, but reduced nutrient-induced suppression of food intake compared to well-nourished older people (48). Together, these findings suggest that age-related changes in CCK (circulating concentrations and/or action) are unlikely to contribute much, if anything, to the age-related reduction in food intake after the ingestion of protein and other nutrients.

The findings of this study do not exclude a role for GLP-1 or GIP in the lesser suppression of food intake by whey protein in healthy older subjects. Baseline circulating concentrations of the anorexigenic hormone GLP-1 were significantly higher in older compared to younger

subjects, with no difference between age groups in the subsequent whey protein-induced rise, consistent with responses during intraduodenal infusions of lipid and glucose (53). The higher baseline GLP-1 levels may have acted to further inhibit the suppression of appetite and thus food intake after whey protein ingestion. GLP-1 is mainly secreted more distally in the gastrointestinal tract (i.e., ileum and colon) than CCK and GIP (expressed mainly in the duodenum and jejunum), and the GLP-1 concentrations following whey protein ingestion increased more slowly than CCK and GIP concentrations. The emptying of food content from the stomach is slowed down by feedback mechanisms in the intestines including the release of CCK and GLP-1 (345, 346); indeed, gastric emptying of the whey protein was slower in the older compared to younger adults (263). Although the effect of GIP on human appetite and food intake, if any, is not clear, there is limited animal evidence to suggest it may act to stimulate food intake; GIP receptor-deficient mice are resistant to diet-induced obesity (356). The greater increase in circulating GIP concentrations after whey protein in healthy older compared to younger subjects might therefore act to reduce the protein-induced suppression of food intake. More studies will be required to investigate the role of these hormones in age-related feeding changes. Also, psychological factors, including increased dietary restraint, particularly in women (357), may affect the short-term energy intake regulation of older adults.

Healthy older and younger adults had comparable plasma ghrelin concentrations following whey protein ingestion, consistent with responses to mixed-nutrient intake in some (88, 89) but not all previous studies (48, 52, 91). It has been suggested that aging-related changes in body composition (i.e., a decrease in lean mass and increase in fat mass) may act to decrease fasting (92) and postprandial (48) ghrelin concentrations, as body fat is negatively correlated to ghrelin concentrations (295) and tends to increase with older age. Other studies, however, have found higher postprandial and fasting ghrelin concentrations in older adults than those in

younger adults and impaired suppression of ghrelin after the consumption of a mixed-nutrient meal in older compared to younger subjects (52, 91).

This study has several limitations, including the relatively small subject numbers. Total ghrelin instead of active ghrelin was measured, which could be considered to be less than optimal; however, the results appeared to be clear-cut, with significant dose-dependent suppressive effects of the protein loads on ghrelin in the direction expected.

The finding that plasma gut hormone responses to whey protein are not blunted in healthy older compared to younger men is likely to have implications to the composition of dietary supplements for older people, and warrants further research to their relation to food intake and glycemic control in older people.

CHAPTER 12: EFFECTS OF SUBSTITUTION, AND ADDITION, OF CARBOHYDRATES AND FAT TO WHEY-PROTEIN ON ENERGY INTAKE, APPETITE, GASTRIC EMPTYING, GLUCOSE, INSULIN, GHRELIN, CCK AND GLP-1 IN HEALTHY OLDER MEN

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Contribution	Conception and design of the study, coordination, participant recruitment, data collection and interpretation, statistical analysis and drafting of the manuscript.		
Overall percentage	75%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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

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ABSTRACT

Background Protein-rich supplements are used widely for the management of malnutrition in the elderly. We reported previously that the suppression of energy intake by whey protein is less in older than younger adults.

Objective: To determine the effects of substitution, and adding of carbohydrate and fat to whey protein, on ad libitum energy intake from a buffet meal (180–210 min), gastric emptying (3D-ultrasonography), plasma gut hormone concentrations (0–180 min) and appetite (visual analogue scales), in healthy older men.

Design In a randomised, double-blind order, 13 older men (75 ± 2 years) ingested drinks (~450 mL) containing: (i) 70 g whey protein (280 kcal; 'P_{280}'); (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'); or (iv) control (~2 kcal).

Results: The caloric drinks, compared to a control, did not suppress appetite or energy intake; there was an increase in total energy intake (drink + meal, $p < 0.05$), which was increased most by the M_{504}-drink. P_{280}- and M_{504}-drink ingestion were associated with slower a gastric-emptying time ($n = 9$), lower ghrelin, and higher cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) than M_{280} ($P < 0.05$). Glucose and insulin were increased most by the mixed-macronutrient drinks ($P < 0.05$).

Conclusions: In conclusion, energy intake was not suppressed, compared to a control, and particularly whey protein, affected gastric emptying and gut hormone responses.

INTRODUCTION

Over recent decades, the prevalence of malnutrition, both under-nutrition and obesity, has increased in older men and women in Western societies (285, 319). A growing awareness of the prevalence and adverse effects of the major muscle loss that occurs during aging, irrespective of body mass index (BMI, kg/m^2) - e.g., reduced functional capacity and decreased quality of life (190, 285, 358)- has led to the development of nutritional strategies designed specifically to preserve and/or restore skeletal muscle mass and function. A 'common' strategy is the use of supplements, which are usually high-energy drinks rich in whey protein (26, 27, 35, 286, 320).

Despite this increasing use of protein-rich drinks, information about their effects on energy intake and underlying gastrointestinal mechanisms in older people is limited. In younger adults, preloads high in protein suppress appetite and energy intake (36, 359-363) more than iso-caloric preloads high in fat or carbohydrate. In young adults, variations in gut hormone secretion and/or action [e.g., ghrelin, cholecystokinin (CCK) and glucagon-like polypeptide-1 (GLP-1)], as well as gastric emptying, are likely to regulate energy intake (70, 112-114, 234, 237, 288).

Compared to younger adults, healthy older people exhibit decreased taste and food palatability, are less hungry and fuller during the fasting and postprandial states, and consume less food and energy (315). This has been termed the 'anorexia of aging' (190, 358). Healthy aging is also associated with reduced responsiveness to the suppressive effects of nutrients on appetite and energy intake (46, 63, 194, 263). We have recently demonstrated that acute administration of 30 g (120 kcal), and 70 g (280 kcal) whey protein drinks, 180 min before a meal, suppressed subsequent energy intake by 12–17% in young, but without suppression in healthy older, men (263) so that in older men protein ingestion increased total energy intake (drink plus energy intake) compared to a control (~0 kcal) to a greater extent

than in the young men. Gastric emptying of the whey protein drink was shown to be slower in older than younger men (263). In young adults, gastric emptying of 500 mL of protein (375 kcal) has been reported to be comparable to carbohydrate (400 kcal) or fat (375 kcal), when expressed as the rate of emptying in mL/min (364), as well as slower when ingested as a mixed macronutrient dairy breakfast (high-protein compared to high-carbohydrate; ~400 kcal) (365). The high-protein dairy breakfast had higher plasma CCK and GLP-1 responses - gut hormones known to slow gastric emptying (365).

The aim of this study was to determine the effects of substitution and addition of carbohydrate and fat to whey protein on ad libitum energy intake at a buffet meal, gastric emptying, gut hormones and perceptions of appetite and gastrointestinal symptoms, in healthy older men. We hypothesised that the replacement of protein by carbohydrate and fat would result in less suppression of subsequent energy intake, more rapid gastric emptying, more pronounced changes in plasma gut hormone concentrations (insulin, ghrelin, CCK, GLP-1), and that the addition of carbohydrate and fat would result in greater suppression of subsequent energy intake, slower gastric emptying, more pronounced changes in plasma gut hormone concentrations and decreased perceptions of appetite, compared to a 'pure' whey—protein drink.

SUBJECTS AND METHODS

Subjects

Thirteen older healthy men, 65 years or older [mean ± standard error of the mean (SEM); age: 75 ± 2 years; body weight: 79 ± 2 kg; height: 1.75 ± 0.01 m; BMI: 26 ± 1 kg/m^2], were recruited by advertisement. Subjects were excluded if they failed to comprehend the study protocol, had donated blood in the 12 weeks prior to the study days, had known lactose intolerance or food allergies, or were undernourished [score < 24 on the Mini Nutritional

Assessment (276)]. Further exclusion criteria were low plasma ferritin levels, diabetes, gallbladder or pancreatic disease, significant gastrointestinal symptoms (abdominal pain, gastro-esophageal reflux, diarrhea, or constipation) or surgery, depression [score ≥ 11 on the Geriatric Depression Questionnaire (275)], alcohol abuse, smoking, use of illicit substances or medications known to potentially affect energy intake, or had impaired cognitive function [score < 25 on Mini Mental State (274)]. The Royal Adelaide Hospital Human Research Ethics Committee approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki. The study is a sub-analysis of a larger study, which is registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au; ACTRN12614000846628). All subjects provided written informed consent prior to their inclusion.

Protocol

Subjects were studied on 4 occasions, separated by 3–14 days, to determine the effects of drinks (~450 mL) containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'); (ii) 14 g whey protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'); or (iv) an iso-palatable control drink (~2 kcal; 'control') on energy intake, gastric emptying, gut hormones, and perceptions of appetite and gastrointestinal symptoms, in a randomised (using the method of randomly permuted blocks; www.randomization.com), double-blind, cross-over design.

Drinks were prepared on the morning of the study day, by homogenizing olive oil (Bertolli Australia Pty Ltd., Unilever Australasia, Sydney, NSW, Australia) and dissolving whey protein isolate (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) and dextrose, in varying volumes of demineralised water and diet lime cordial (Bickford's Australia Pty Ltd., Salisbury South, SA, Australia), to achieve the desired composition, by a

research officer (SH) who was not involved in the data analysis. Both the investigator and the subject were blinded to the treatment. The drinks were matched for taste and served in a covered cup.

Before each study day, subjects consumed a standardised meal [beef lasagne (McCain Foods Pty Ltd., Wendouree, VIC, Australia), containing ~591 kcal], at ~19:00 p.m. Thereafter, subjects fasted overnight from solids and liquids until they attended the laboratory at ~08:30 a.m. Subjects refrained from strenuous physical activity for 24 h before the study day.

Subjects removed all metal objects and were seated in an upright position on a wooden chair. An intravenous cannula was inserted for blood sampling and subsequent measurement of glucose and gut hormones. In each subject, blood samples and ultrasound measurements of gastric volume, and perceptions of appetite and gastrointestinal symptoms were performed before (during fasting) and after ingestion of the drink, until 180 min. Subjects were instructed to consume the drink within 2 min. At 180 min, each subject was presented with a standard, cold, buffet-style meal, in excess of what they were expected to consume (total energy content of 2.457 kcal; 19% protein, 50% carbohydrates, 31% fat), in a room by themselves to limit external distractions, and were allowed to eat for 30 min (180–210 min) until comfortably full.

Measurements

Energy intake

The buffet-style meal consisted of bread, chicken, ham, cheese, margarine, mayonnaise, yoghurt, custard, fruit, fruit salad, orange juice, iced coffee and water (263). The amount eaten at the meal (g) was quantified by weighing the food before and after consumption. Energy intake (kcal), as intake at the buffet meal, and as the cumulative energy intake, defined as the sum of energy intake at the buffet meal and the energy content of the preload

drink, proportions of protein, carbohydrate and fat (Foodworks version 8; Xyris Software Pty Ltd., Spring Hill, QLD, Australia), and change in energy intake at the buffet meal (expressed as % of energy intake of the control day) by a given protein load, compared to the control, were calculated.

Gastric emptying

Total gastric volume was measured by a Logiq™ 9 ultrasound system (GE Healthcare Technologies, Sydney, NSW, Australia) with TruScan Architecture [built-in magnetically-sensored 3D positioning and orientation measurement (POM)], including a 3D sensor, attached to a 3.5C broad spectrum 2.5–4 MHz convex transducer, and a transmitter, placed at the level of the stomach, immediately behind the subject, at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 min (264). The stomach was scanned along its longitudinal axis, whilst the subject was holding their breath and sitting still. Stomach volumes were calculated using EchoPAC-3Dsoftware (GE Vingmed Sound, Horten, Norway). Intragastric retentions were calculated as total gastric volume minus fasting gastric volume (baseline) at each time point, and expressed as percentage of the maximal gastric volume (100%). Data were imput by linear interpolation when ultrasound images lacked sufficient clarity (263). The rate of gastric emptying was calculated during each 15-min interval in the early phase (i.e., 0–60 min), and the late phase (i.e., 60 min until 100% emptying time per individual). Fifty percent of the gastric emptying time (T50; min) and 'complete' (residual volume of the drink in the stomach was ≤5%) gastric emptying time (100% gastric emptying time; T100; min) were calculated (263).

Blood Glucose and Plasma Insulin, Ghrelin,* Cholecystokinin *(CCK) and Glucagon-Like Peptide-1 (GLP-1) Concentrations

Blood samples were collected, at 0, 5, 15, 30, 45, 60, 90, 120, 150, 180 min, into ice-chilled, EDTA-coated tubes. No inhibitors were added (264). Plasma was obtained by centrifugation for 15 min, at 3200 rpm, at 4 °C, and samples were stored at −80 °C for further analysis of hormone concentrations.

Blood glucose (millimoles per liter) was determined immediately after collection, by the glucose oxidase method, using a portable glucometer (Optium Xceed, Abbott Laboratories, Sydney, NSW, Australia). Intra- and inter-assay coefficients of variation were 2.6% and 15.2%.

Total plasma insulin (milliunits per liter) was measured by enzyme-linked immunosorbent *assay (*ELISA) immunoassay (10-1113; Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L. Intra- and inter-assay coefficients of variation were 3.0% and 6.8%.

Total plasma ghrelin (picograms per milliliter) was measured using a radioimmunoassay (RIA) with some modifications to a published method (323). The radiolabel was supplied by Perkin Elmer (NEX388, Boston, MA, USA). The standard and samples were incubated with the antibody and radiolabel for 3–4 days, at 4 °C. The detection limit was 40 pg/mL. Intra- and inter-assay coefficients of variation were 5.1% and 10.1%.

Plasma CCK-8 (picomoles per liter) was measured by RIA, using an adaption of a previous method (324). Samples were extracted in 66% ethanol; extracts were dried down and re-suspended in assay buffer (50 mM phosphate, 10 mM EDTA, 2 g/L gelatin, pH = 7.4). Standards were prepared using synthetic sulphated CCK-8 (Sigma Chemical, St Louis, MO, USA), antibody (C2581, Lot 105H4852, Sigma Chemical) was added at a working dilution of 1/17,500 and sulphated CCK-8 ^{125}I-labeled with Bolton and Hunter reagent (Perkin Elmer, Boston, MA, USA) was used as tracer. Incubation was for 7 days at 4 °C. The

antibody bound fraction was separated by the addition of dextran-coated charcoal containing gelatin (0.015 g gelatin, 0.09 g dextran, 0.15 g charcoal in 30 mL assay buffer), and the radioactivity was determined in the supernatants following centrifugation. The detection limit was 1 pmol/L. The intra- and inter-assay coefficients of variation were 8.1% and 11.5%.

Total plasma GLP-1 (picomoles per liter) was measured by RIA (GLPIT-36HK; Millipore, Billerica, MA, USA), with a detection limit of 3 pmol/L. Intra - and inter -assay coefficients of variation were 2.7% and 7.1%.

Peak/nadir and time to peak/nadir concentrations for glucose, insulin, ghrelin, CCK and GLP-1 were calculated for the caloric drink conditions.

Perceptions of appetite and gastrointestinal symptoms

Perceptions of hunger, desire to eat, prospective consumption, fullness, nausea and bloating were rated using a visual analogue scale (VAS) questionnaire at 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210 min (47). The questionnaire consisted of 100-mm horizontal lines, where 0 represented that the sensation was 'not felt at all' and 100 represented that the sensation was 'felt the greatest'. Subjects placed a vertical mark on each horizontal line to indicate the strength of each sensation at the specified time points.

Data analysis

On the basis of our previous work, with an observed within-subject standard deviation (SD) of 267 kcal for suppression of energy intake by whey protein, and 31 min for gastric emptying half time (263), we calculated that 13 subjects would allow detection of a within-group difference between treatments for suppression in energy intake of 272 kcal and T50 of 35 min, with power equal to 0.8 and alpha equal to 0.05.

Statistical analyses were performed using SPSS software (version 22; IBM, Armonk, NY, USA). Differences between study conditions for energy intake, gastric emptying, perceptions of appetite and gastrointestinal symptoms (visual analogue scores) and glucose and hormone concentrations were determined using one-way repeated-measures ANOVA, with the treatment as the within-subject factor. Post hoc comparisons were adjusted with the Bonferroni method. Interaction effects of time by treatment, for concentrations of blood glucose and plasma insulin, ghrelin, CCK and GLP-1, and perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating, were determined using a two-way repeated measures ANOVA, with treatment and time as the within-subject factors. Within-subject correlations were determined using a general linear model with fixed slope and random intercept (277). Areas under the curve (AUC) for gastric emptying, perceptions of appetite and gastrointestinal symptoms, and concentrations of glucose, insulin, ghrelin, CCK and GLP-1, were calculated from baseline to 60 min (i.e., 'early' phase of gastric emptying) and 60 to 180 min (i.e., 'late' phase of gastric emptying), using the trapezoidal rule. Peak/nadir and time to peak/nadir perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating were calculated for the all conditions. Assumptions of normality were verified for all outcomes before the statistical analysis. Statistical significance was accepted at $p < 0.05$. All data are presented as mean values ± SEMs.

RESULTS

The study protocol was well-tolerated by all subjects. Baseline gastric volumes (mean ± SEM of four study days: 33 ± 4 mL), blood glucose (5.7 ± 0.1 mmol/L), plasma insulin (5.1 ± 1.8 mU/L), ghrelin (1659 ± 165 pg/mL), CCK (2.0 ± 0.2 pmol/L) and GLP-1 concentrations (15 ± 1 pmol/L), and perceptions of hunger (31 ± 13 mm), desire to eat (30 ± 12 mm), prospective

food consumption (46 ± 14 mm), fullness (2 ± 1 mm), nausea (3 ± 1 mm) and bloating (3 ± 1 mm), were not different between study days.

Energy intake

Ad libitum energy intake at the buffet meal (**Figure 12.1**) and energy percentages of protein, carbohydrate and fat were not different between study days (mean of four study days: energy intake: 994 ± 76 kcal, $P = 0.53$; protein: 20 ± 0.4%, $P = 0.60$; carbohydrate: 52 ± 1%, $P = 0.25$; fat: 30 ± 1% $P = 0.83$). There was no suppression of energy intake by the caloric drinks, compared to the control ($P > 0.05$). Total energy intake (drink plus meal) was higher after all caloric drinks, compared to the control ($P < 0.001$; post hoc tests versus control (972 ± 87 kcal): P_{280} (1300 ± 95kcal) $P < 0.001$; M_{280} (1306 ± 76 kcal) $P = 0.003$; M_{504} (1461 ± 76 kcal) $P < 0.001$).

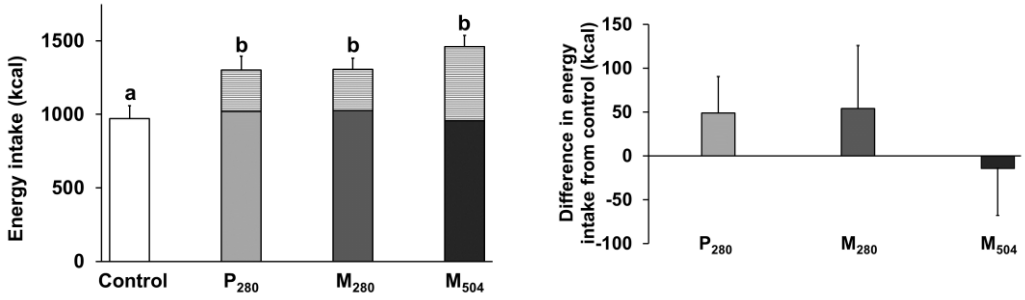

Figure 12.1: Left: mean [± standard error of the mean (SEM)] energy intake at the buffet meal (kcal) in healthy older men (n = 13) after drinks (~450 mL; energy content of the drink as the striped part of each bar) containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'); (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'); or (iv) an iso-palatable control drink (~2 kcal; 'control'). Right: mean (±SEM) suppression of energy intake after caloric drinks (P_{280}, M_{280} and M_{504}) compared to control. a,b $p < 0.05$ Total energy intakes (meal plus drink) for P_{280}, M_{280} and M_{504} (b) were higher compared to the control (a).

Gastric emptying

In four subjects, the quality of ultrasound stomach images was insufficient to determine gastric emptying in one or more conditions, and all data related to gastric emptying in these

subjects were, therefore, excluded from the analysis. The control and the M_{280} drink emptied in an overall non-linear pattern, whereas the pattern of emptying of P_{280} and M_{504} was linear (**Figure 12.2**). Gastric emptying of P_{280} and M_{504} was slower ($P < 0.001$), and gastric retention greater ($P < 0.001$), than M_{280}, with 50% gastric emptying times being ~three-fold higher ($P < 0.001$, **Table 12.1**).

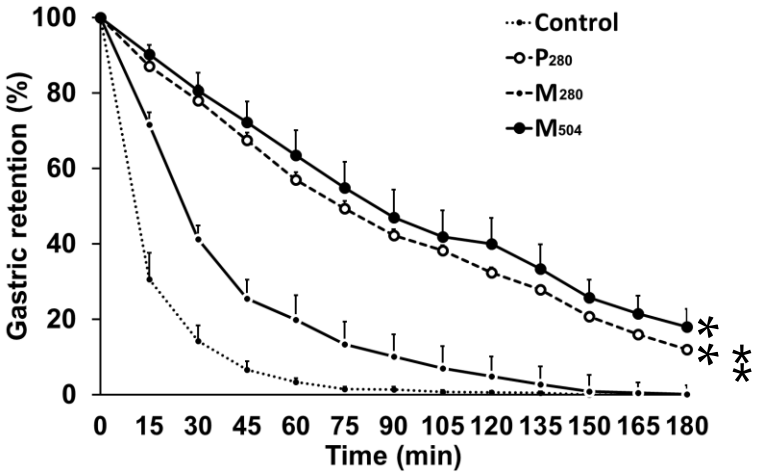

Figure 12.2: Mean (± SEM) gastric retention (%) in healthy older men (n = 9), after drinks containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'; dashed line with open circles); (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'; solid line with open circles); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'; solid line with closed circles); or (iv) an iso-palatable control drink (~2 kcal; 'control'; dotted line). Gastric emptying half time (T50) was higher after P_{280} and M_{504}, compared to M_{280} and control (*$P < 0.05$).

Table 12.1: Gastric emptying parameters after drink ingestion in healthy older men

Gastric emptying parameters	Control	P_{280}	M_{280}	M_{504}
50% emptying time (T50; min)	12 ± 2 [a]	78 ± 11 [b]	26 ± 2 [a]	93 ± 13 [b]
100% emptying time (T100; min)	58 ± 7 [a]	180 ± 0 [b]	120 ± 8 [c]	170 ± 7 [b]
Gastric retention (%)				
$AUC_{0\text{-}60min}$	1546 ± 200 [a]	4666 ± 222 [b]	2979 ± 83 [c]	4871 ± 240 [b]
$AUC_{60\text{-}180min}$	94 ± 42 [a]	3907 ± 559 [b]	552 ± 122 [c]	4331 ± 771 [b]
Rate of gastric emptying (kcal/min) [1]				
Early phase		2.0 ± 0.3 [a]	3.7 ± 0.1 [b]	3.1 ± 0.6 [b]
Late phase		1.1 ± 0.1[a]	1.3 ± 0.3 [a]	2.2 ± 0.3 [b]
Amount emptied (%)				
at 60 min	98 ± 1 [a]	61 ± 11 [b]	86 ± 4 [a]	56 ± 12 [b]
at 180 min	100 ± 0 [a]	89 ± 3 [b]	100 ± 0 [a]	85 ± 5 [b]

Mean (± SEM) 50% and 100% emptying time (min), gastric retention (%), rate of gastric emptying (kcal/min) and amount emptied (%) at 60 and 180 min in healthy older men (n = 9), after drinks containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'); (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'); or (iv) an iso-palatable control drink (~2 kcal; 'control'). [1] The rate of gastric emptying was calculated as the mean of rates of emptying during each 15-min interval, respectively, in the early phase (i.e., 0–60 min) and late phase (i.e., 60 min until 100% emptying time per individual). Different letters indicate a significant difference (P < 0.05) between drink conditions; gastric emptying time and retention and amount emptied were higher after P_{280} and M_{504} (^{b}) than M_{280} (^{a,c}) and control (^{a}), rate of gastric emptying was higher after M_{504} (^{b}) and M_{280} (^{a,b}) than P_{280} (^{a}).

Glucose

Blood glucose concentrations increased after M_{280} and M_{504}, returned to baseline ~60 min, and stayed below baseline until the buffet meal (interaction effect of time by drink condition: P < 0.001). Peak glucose concentrations were higher after M_{280} and M_{504}, compared to P_{280} (P = 0.001, **Table 12.2**). Early phase $AUC_{0–60\ min}$ glucose concentrations were higher after M_{280} and M_{504}, compared to the control, and after M_{280}, compared to P_{280} (P < 0.001, **Figure 12.3**). Late phase $AUC_{60–180\ min}$ glucose concentrations were lower after M_{280} than the control (drink condition effect: P < 0.001). Glucose concentrations at 180 min were lower after M_{280} compared to control and M_{504} (P = 0.003).

Insulin

Plasma insulin concentrations increased after all caloric drinks (P_{280}, M_{280} and M_{504}; interaction effect of time by drink condition: P = 0.038). The mixed-macronutrient drinks evoked a rapid increase in insulin, insulin peak concentrations were higher after M_{280} and M_{504} compared to P_{280} (P < 0.001, **Table 12.2**), and the drinks containing 70 g of whey protein (P_{280} and M_{504}) remained elevated (**Figure 12.3**). Early phase $AUC_{0–60\ min}$ insulin concentrations were higher after M_{504}, compared to P_{280} (P = 0.008).

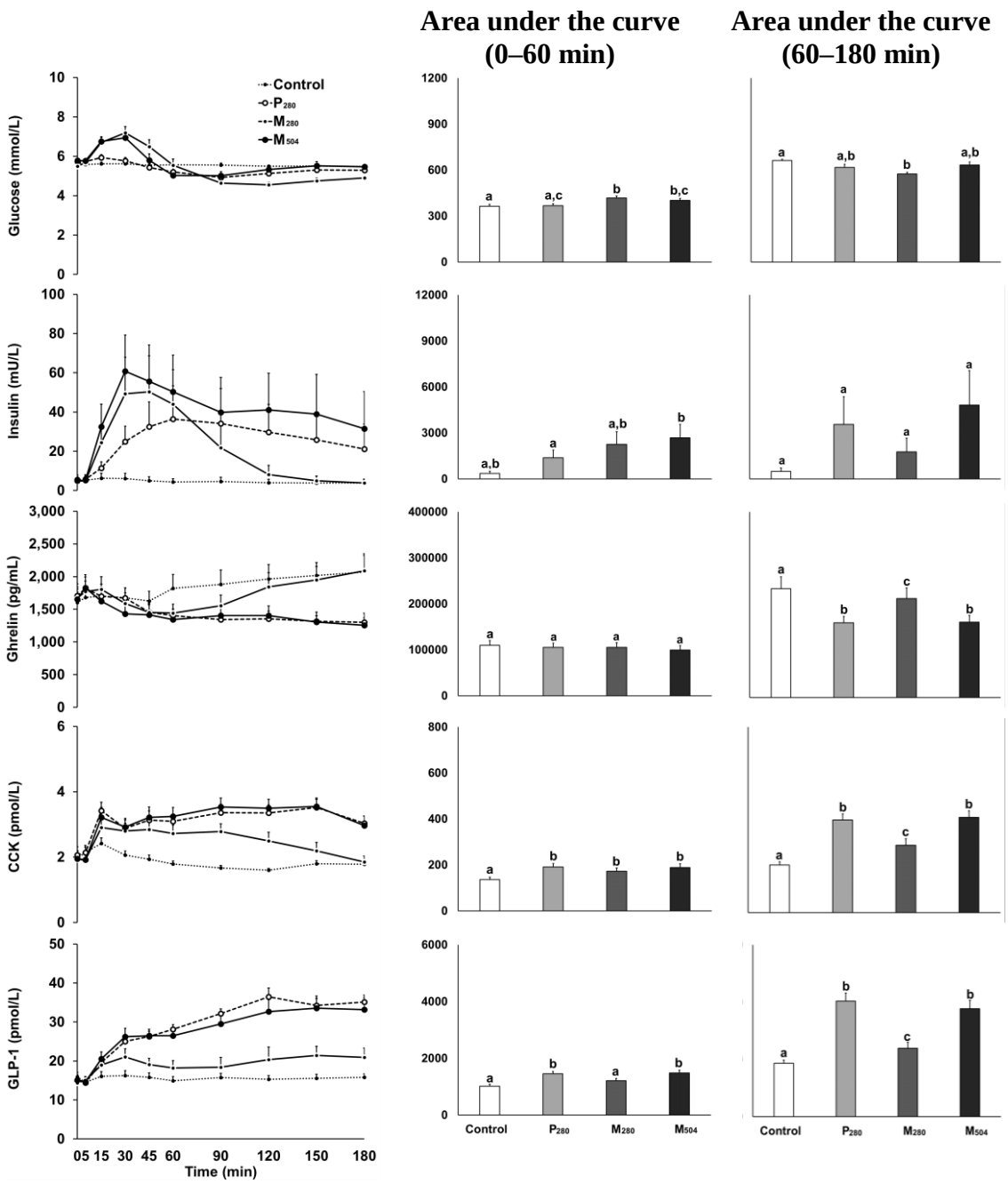

Figure 12.3: Mean (± SEM) concentrations of blood glucose and plasma insulin, ghrelin, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) in healthy older men (n = 13), after drinks containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'; dashed line with open circles); (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'; solid line with open circles); (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'; solid line with closed circles); or (iv) an iso-palatable control drink (~2 kcal; 'control'; dotted line). There was an interaction effect of time by drink condition for concentrations of blood glucose ($P < 0.001$), insulin ($P = 0.038$), ghrelin ($P < 0.001$), CCK ($P < 0.001$) and GLP-1 ($P < 0.001$). Different letters indicates significant difference ($P < 0.05$) in area under the curves (0–60 or 60–180 min) between drink-conditions: control vs. P_{280} vs. M_{280} vs. M_{504}.

Ghrelin

Plasma ghrelin concentrations decreased after all caloric drinks (P_{280}, M_{280} and M_{504}; interaction effect of time by drink condition: $P < 0.001$). Nadir ghrelin concentrations were lower after P_{280} and M_{504}, compared to M_{280}, ($P = 0.001$, **Table 12.2**), and remained suppressed after P_{280} and M_{504} (**Figure 12.3**). Late phase $AUC_{60–180\ min}$ ghrelin concentrations were lower after all caloric drinks, compared to the control, and after P_{280} and M_{504}, compared to M_{280} ($P < 0.001$). Ghrelin concentrations at 180 min were lower after M_{504} and P_{280}, compared to the control and M_{280} ($P < 0.001$).

CCK

Plasma CCK concentrations increased after all caloric drinks (P_{280}, M_{280} and M_{504}; interaction effect of time by drink condition: $P < 0.001$). Peak CCK concentrations were higher after P_{280} and M_{504}, compared to M_{280} ($P < 0.001$, **Table 12.2**), and remained elevated after P_{280} and M_{504} (**Figure 12.3**). Early phase $AUC_{0–60\ min}$ CCK concentrations were higher after the caloric drinks, compared to the control ($P < 0.001$). Late phase $AUC_{60–180\ min}$ CCK concentrations were higher after the caloric drinks, compared to the control, and after P_{280} and M_{504}, compared to M_{280} ($P < 0.001$). CCK concentrations at 180 min were higher after P_{280} and M_{504}, compared to the control and M_{280} ($P < 0.001$).

GLP-1

Plasma GLP-1 concentrations increased after all caloric drinks (P_{280}, M_{280} and M_{504}; interaction effect of time by drink condition: $P < 0.001$, **Figure 12.3**). Early phase $AUC_{0–60\ min}$ GLP-1 concentrations were higher after P_{280} and M_{504}, compared to the control and M_{280} ($P < 0.001$). Late phase $AUC_{60–180\ min}$ GLP-1 concentrations were higher after all caloric drinks, compared to the control, and after P_{280} and M_{504}, compared to M_{280} ($P < 0.001$). GLP-

1 concentrations at 180 min were higher after the caloric drinks, compared to the control, and after P_{280} and M_{504}, compared to M_{280} ($P < 0.001$).

Perceptions of appetite and gastrointestinal symptoms

Early phase $AUC_{0–60\ min}$ and late phase $AUC_{60–180\ min}$ perceptions of hunger, desire to eat, prospective food consumption, fullness, nausea, and bloating were not different between study days ($P > 0.05$, **Figure 12.4**). Hunger (mean decrease over four study visits: 9 ± 2 mm, time to nadir: 29 ± 7 min, time effect: $P < 0.001$), desire to eat (9 ± 1 mm, 21 ± 5 min, $P < 0.001$) and prospective food consumption (11 ± 2 mm, 35 ± 7 min, $P < 0.001$) initially decreased after drink ingestion and increased thereafter to ratings higher than baseline, immediately before the buffet meal (180 min). Fullness (mean increase over four study days: 16 ± 5 mm, time to peak: 38 ± 8 min, $P = 0.001$) increased after the drink to return to baseline thereafter. Nausea and bloating did not change over time (nausea: $P = 0.51$, bloating: $P = 0.10$).

Correlations between gastric retention and hormones

Plasma insulin (AUC 0–180 min; $r = 0.41$ $P = 0.029$) ghrelin ($r = −0.70$ $P < 0.001$), CCK ($r = 0.76$ $P < 0.001$) and GLP-1 ($r = 0.78$ $P < 0.001$) concentrations were, within subjects, related to gastric emptying (AUC 0–180 min).

Table 12.2: Glucose, insulin, ghrelin, CCK and GLP-1 after drink ingestion in healthy older men

	Control	$Protein_{280kcal}$	$Mixed_{280kcal}$	$Mixed_{504kcal}$
Peak/nadir concentration				
Glucose	5.9 ± 0.1^{a}	$6.1\pm0.1^{a,b}$	7.4 ± 0.3^{b}	7.2 ± 0.3^{b}
Insulin	7 ± 3^{a}	$39\pm17^{a,b}$	$56\pm18^{a,b}$	67 ± 18^{b}
Ghrelin	2171 ± 240^{a}	1855 ± 183^{a}	2194 ± 251^{b}	1862 ± 198^{b}
CCK	2.7 ± 0.2^{a}	3.9 ± 0.3^{b}	$3.1\pm0.3^{a,b}$	3.9 ± 0.3^{b}
GLP-1	19 ± 1^{a}	39 ± 3^{b}	25 ± 2^{a}	36 ± 3^{b}
Time to peak/nadir				
Glucose	42±13	20±5	29±3	22±2
Insulin	15 ± 3^{a}	53 ± 7^{b}	39 ± 3^{b}	44 ± 7^{b}
Ghrelin	44 ± 13^{a}	126 ± 14^{b}	58 ± 7^{a}	114 ± 18^{b}
CCK	39 ± 15^{a}	$87\pm16^{a,b}$	$45\pm12^{a,b}$	99 ± 16^{b}
GLP-1	87 ± 22^{a}	$138\pm13^{a,b}$	$98\pm18^{a,b}$	155 ± 7^{b}
180-min concentration				
Glucose	5.5 ± 0.1^{a}	5.3 ± 0.4^{a}	4.9 ± 0.4^{b}	5.5 ± 0.4^{a}
Insulin	3.9±1.9	21±13	3.8±1.6	32±9
Ghrelin	2069 ± 257^{a}	$1302\pm137^{a,b}$	2091 ± 259^{a}	1254 ± 117^{b}
CCK	1.8 ± 0.1^{a}	3.0 ± 0.2^{b}	1.9 ± 0.2^{a}	3.0 ± 0.3^{b}
GLP-1	16 ± 1^{a}	35 ± 2^{b}	21 ± 2^{a}	33 ± 3^{b}

Mean (± SEM) peak/nadir concentrations, time to peak/nadir (min) and 180-min concentrations of blood glucose and plasma insulin, ghrelin, CCK and GLP-1 in healthy older men (n = 13), after drinks containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'), (ii) 14 g protein, 28g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'), (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'), or (iv) an iso-palatable control drink (~2 kcal; 'control'). ^{a,b,c,d} $P < 0.05$, post hoc test: different letter indicates significant difference between drink-conditions: control vs. P_{280} vs. M_{280} vs. M_{504}.

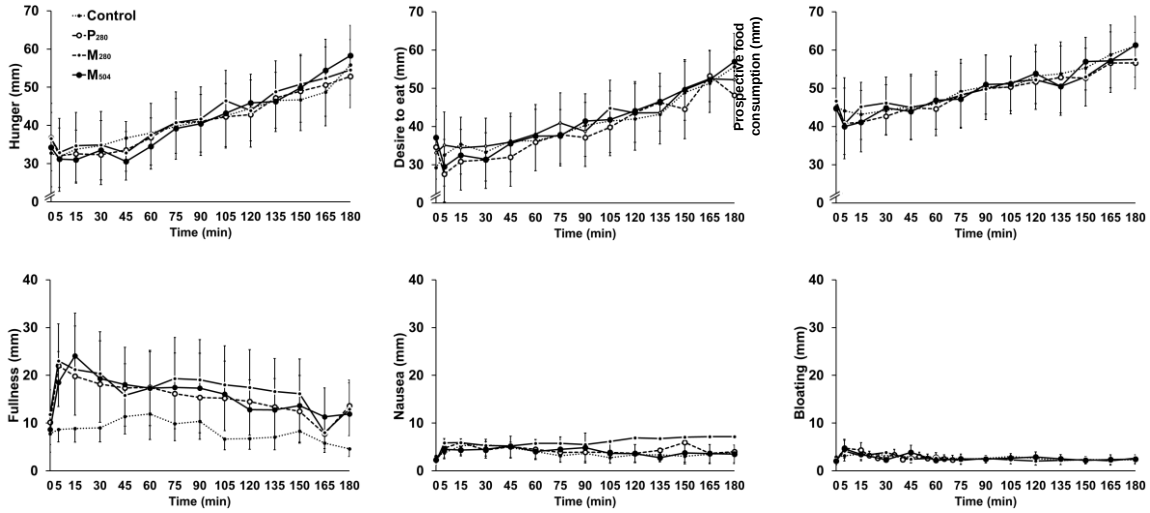

Figure 12.4: Mean (± SEM) visual analogue score (VAS, mm) of hunger, desire to eat, prospective food consumption, fullness, nausea and bloating in healthy older men (n = 13), after drinks containing either: (i) 70 g whey protein (280 kcal; 'P_{280}'; dashed line with open circles), (ii) 14 g protein, 28 g carbohydrate, 12.4 g fat (280 kcal; 'M_{280}'; solid line with open circles), (iii) 70 g protein, 28 g carbohydrate, 12.4 g fat (504 kcal; 'M_{504}'; solid line with closed circles), or (iv) an iso-palatable control drink (~2 kcal; 'control'; dotted line). Effects of time were significant for hunger ($P < 0.001$), desire to eat ($P < 0.001$), prospective food consumption ($P < 0.001$) and fullness ($P = 0.001$).

DISCUSSION

This study examined the effects of substituting fat and protein for, or adding them to, whey protein, on energy intake, gastric emptying, blood glucose and plasma gut hormone concentrations, perceptions of appetite and gastrointestinal symptoms in healthy older men. The major novel observation is that ingestion of a whey protein drink of 280 kcal (P_{280}: 70 g protein) or a mixed macronutrient drink of 504 kcal (M_{504}: 70 g protein, 28 g carbohydrate, 12.4 g fat,) was associated with slower gastric emptying, lower ghrelin, and higher CCK and GLP-1 concentrations than an iso-caloric mixed-macronutrient drink (M_{280}: 14 g protein, 28 g carbohydrate, 12.4 g fat: 280 kcal). There was no suppression of energy intake or appetite by the caloric drinks, compared to the control.

The use of high protein supplements by older people is widespread, and increasing, in response to greater awareness of the prevalence of undernutrition and sarcopenia in older people [38] and evidence that protein supplementation may increase muscle mass and

function (20, 271). If timing and preparation are optimised, it may be possible to give sufficient protein [probably at least 35 g (23)] to older people, to preserve, or increase muscle mass and function, without suppressing energy intake. Indeed, our observations suggest that optimal protein administration may increase overall energy intake in older people. None of the caloric drinks suppressed subsequent ad libitum energy intake at a buffet meal, compared to a non-caloric control, and consequently, there was an increase in total energy intake. This observation is consistent with our recent finding that the suppression of subsequent energy intake by oral ingestion and intraduodenal infusions of whey protein is less in healthy older men (~1%) than in young controls (~15–19%) (194, 263). Total energy intake (drink plus meal) was predictably increased most by the drink with the highest energy content (504 kcal) - a substantial increase of ~50% or 490 kcal, compared with an increase of ~34% or ~330 kcal after both 280 kcal drinks. Comparable amounts of protein could reasonably be given as protein supplements several times during the day. We have reported that variation in the timing of protein ingestion does not affect energy intake at a subsequent meal in healthy older people, and that total energy intake is higher on the protein days compared to a control (366). These findings raise the intriguing possibility that appropriately designed protein supplements, administered in divided doses, might increase energy intake in undernourished people by meaningful amounts (>300–500 kcal/day), without the need to encourage and supervise additional energy intake.

We, and others, have shown that healthy aging is associated with modest slowing of gastric emptying of both solids and liquids, although the rate of emptying generally remains within the relatively wide normal range for young subjects (i.e., ~1–4 kcal/min) (50, 74, 82, 263). In healthy older men, the addition of 28 g carbohydrate and 12.4 g fat to the 70 g (280 kcal) whey protein did not affect gastric emptying time; both P_{280} and M_{504} had comparable T50 and T100 and therefore, the rate of gastric emptying was higher for M_{504} than P_{280} (e.g., initial rates of gastric emptying of ~3 and 2 kcal/min, respectively). Iso-caloric substitution

of 56 g (224 kcal) protein with carbohydrate and fat resulted in faster gastric emptying; M_{280} compared to P_{280} had lower T50 and T100 and thus a faster rate of gastric emptying (~4 vs. 2 kcal/min).

The M_{280} drink had emptied completely, and P_{280} and M_{504} were ~90% emptied immediately before the meal. It should be appreciated that, as the subjects were seated, it is possible that, despite being mixed for ~45 min prior to, until immediately before, consumption, the fat (olive oil) separated from the protein/carbohydrate solution, and emptied from the stomach slower than the aqueous phase, by 'layering' on the denser aqueous components (367).

The hormones, insulin, ghrelin, GLP-1 and PYY, are secreted by the gastrointestinal tract, in response to the ingested nutrients. Plasma gut hormone concentrations were, within subjects, related to gastric retention; lower ghrelin, and higher insulin, CCK and GLP-1 concentrations correlated with slower gastric emptying. Both drinks containing 70 g protein (P_{280} and M_{504}) had comparable gut hormone responses, which were greater than the responses evoked by the M_{280} drink. These observations suggest that in healthy older men, gastric emptying and plasma gut hormone concentrations were more likely dependent on the amount of protein, rather than the energy content of the drink.

In young subjects, the addition of protein to a glucose meal increases the insulin response (368-370). It has been reported that whey protein, which has a high content of insulinotropic amino acids (371), resulted, when compared to casein protein, in a higher increase of plasma insulin concentrations (372). In our study, both mixed-macronutrient drinks evoked a rapid increase in plasma insulin concentrations, while insulin remained elevated for longer in the drinks containing 70 g of whey protein. Glucose concentrations immediately before the meal were lower after M_{280} than M_{504}, which is likely related to the M_{504} drink still being emptied from the stomach.

In young people, it has been reported previously that effects were larger and more sustained after high compared to low protein on ghrelin concentrations (39, 365, 373-375), protein

compared to glucose (39, 365), but not fat (376), on CCK concentrations, and protein compared to carbohydrate (235, 365, 377, 378) or fat (377-379) - in several studies but not all studies (380-382) - on GLP-1 concentrations. In our study, the drinks containing 70 g of protein resulted in a comparably more sustained lower decrease in ghrelin concentrations and higher increases in CCK and GLP-1 than M_{280}, which is likely to be related to the potency of the higher content of whey protein, and for the M_{504} drink, an additional caloric content.

Our study has several limitations. The subject numbers were relatively small. This applies particularly to the gastric emptying measurements (n = 9). Nevertheless, the findings were clear-cut. We studied only men, as they appear to have the greatest ability to regulate energy intake in response to energy manipulation (46) and in women, particularly the menstrual cycle may have a confounding effect on appetite and energy intake. We have also recently reported that there is no effect of gender on gastric emptying, concentrations of glucose or gut hormones, perceptions of appetite and gastrointestinal symptoms in older people (339). Energy intake at the buffet meal was assessed three hours after drink ingestion, to allow for complete emptying of the drinks from the stomach, and not during the remainder of the day—accordingly, potential compensatory changes in energy intake after lunch were not evaluated. While the drinks were palatable and matched for taste, we did not assess the subjects' perceptions of taste and/or pleasantness of the drinks. Blood glucose was measured by a glucometer, which is less than optimal; however, the results appeared clear-cut.

A drink containing 70 g whey-protein (280 kcal), and a mixed-macronutrient drink containing 70 g protein, 28 g carbohydrate and 12.4 g fat (504 kcal), were associated with slower gastric emptying time, lower ghrelin, and higher CCK and GLP-1 concentrations than a mixed-macronutrient drink containing 14 g protein, 28 g carbohydrate and 12.4 g fat (280 kcal). The caloric drinks did not suppress energy intake, compared to the non-caloric control and, consequently, there was an increase in total energy intake, particularly with the mixed-

macronutrient drink with the highest caloric content. Our findings are likely to have implications for the composition of protein-rich supplements, for both undernourished and obese older people as well as for targeting gastric emptying and gut hormone responses by preload intakes, in relation to, for example, glycemic control in older people.

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CHAPTER 13: CONCLUSIONS OF THE THESIS

The studies included in this thesis provide novel insights into the acute effects of dietary whey protein ingestion on energy intake, perceptions of appetite and gastrointestinal symptoms, gastric emptying, blood glucose and plasma gut hormone concentrations (i.e. insulin, glucagon, ghrelin, CCK, GIP, GLP-1 and PYY) in healthy older and younger men and women.

Collectively, the studies described in this thesis have produced clear-cut and exciting results – whey protein ingestion is less suppressive of feeding behaviour in healthy older than younger adults, and may even increase overall energy intake in the elderly. These findings will aid the development of ways to effectively increase energy and protein intake in older adults at risk of undernutrition.

Suppression of energy intake by whey protein, compared to control, was shown to be less in healthy older than younger men after 60-min intraduodenal infusions (bypassing 'orosensory' and 'intragastric' factors) of partly-hydrolysed whey protein at loads lower than (0.5 kcal/min, 30 kcal, 8 g), similar to (1.5 kcal/min, 90 kcal, 24 g) and at the upper end (3 kcal/min, 180 kcal, 48 g) of normal gastric emptying rates (i.e. 1-4 kcal/min; **Chapter 5**), and 3 hours after oral isolate whey protein ingestion [30 g (120 kcal) and 70 g (280 kcal), **Chapters 7, 9 and 10**]. Suppression of hunger during the intraduodenal protein infusions and of desire to eat and prospective food consumption after oral protein ingestion was less in the healthy older, compared to younger, men. Whey protein did not induce adverse gastrointestinal symptoms - nausea and bloating were not different after protein ingestion/infusion compared to control in older adults.

The timing of protein ingestion did not affect energy intake in healthy older men; *ad libitum* energy intake at the buffet meal did not differ from control when 70 g whey protein was

ingested orally either 3, 2 or 1 hour(s), or just before the meal (**Chapter 8**). Furthermore, substitution carbohydrates and fat for whey-protein or adding them to whey protein did not affect energy intake in older men; there was no suppression of energy intake by the caloric drinks compared to control (**Chapter 12).**

Energy intake was dose-responsively suppressed by whey protein infusions (30, 90, 180 kcal) compared to saline control infusion in younger men (7%, 17% and 33% suppression), whereas in healthy older men suppression was observed only with the highest protein-load infusion (7% and 6% increase and 17% suppression **Chapter 5**). Suppression of energy intake by 30 g and 70 g oral whey protein ingestion compared to a non-caloric control drink was 17% and 12% in younger men, whereas there was no suppression in older men and younger and older women (**Chapters 7, 9 and 10**). The lack of suppression of energy intake in healthy older adults is concordant with the concept of reduced responsiveness to the suppressive effects of nutrients on appetite and energy intake.

Total energy intake (infusion/drink plus meal) was suppressed by protein compared to control in the younger men, while, in contrast, the older men and women exhibited dose-dependent increases in total energy intakes (~10% and ~30% after 30 g and 70 g protein compared to control **Chapters 7, 9 and 10**). The protein doses used in these studies are likely to have the capacity to increase body weight, particularly when ingested several times per day.

Gastrointestinal mechanisms are likely to play a role in the regulation of energy intake, particularly in the short-term after nutrient ingestion in younger, but not older, adults. Isolated pyloric, antral and duodenal pressure waves (**Chapter 5**), gastric emptying (**Chapters 7, 9 and 10**) and gut hormones (**Chapters 6, 9-12**) were dependent on the protein

load in younger and older men. Older men (**Chapter 7**) and younger women (**Chapter 10**) had slightly slower gastric emptying than younger men, while gastric emptying was comparable in older men and women (**Chapter 9)**. Plasma GIP concentrations were higher in healthy older compared to younger men after the intraduodenal protein infusions, while blood glucose, plasma ghrelin, CCK, GLP-1 and PYY concentrations were comparable between the age groups and the release of plasma insulin, glucagon and amino acid concentrations were delayed in the older compared to younger men (**Chapter 6**). After oral protein administration, plasma CCK and GIP were higher in older compared to young adults (**Chapter 11**). Older men and women had comparable plasma gut hormone concentrations after oral whey protein ingestion (**Chapter 9**), while younger women had higher blood glucose and lower plasma glucagon, CCK and GLP-1 concentrations than younger men (**Chapter 10**). The drink containing 70 g whey-protein was associated with lower ghrelin, and higher CCK and GLP-1 concentrations than an iso-caloric (280 kcal) mixed-macronutrient drink containing 14 g protein, 28 g carbohydrate, 14 g fat, but comparable gut hormone responses compared to a drink containing 70 g protein, 28 g carbohydrate, 14 g fat (504 kcal; **Chapter 12**).

From the results of the studies in the thesis, we can conclude that although older people are less hungry and eat less than younger adults (**Chapter 3**), they appear to be less susceptible to further suppression of appetite and eating behaviour by ingestion of energy and nutrients, including whey protein. Protein is the most satiating macronutrient in younger people, and high-protein diets promote and facilitate deliberate weight loss during energy restricting diets in younger adults. The demonstrated lack of suppression of energy intake and appetite by whey protein ingestion (3 hours to immediately before the meal) in older adults is an important finding, given that it alleviates concerns that administration of protein-enriched nutritional supplements could be counter-productive in older people, particularly those with,

or at risk of under-nutrition, by suppressing subsequent food intake. Accordingly, administration of whey protein in doses sufficient to stimulate muscle protein synthesis (≥ ~30 g), may be beneficial to preserve or increase muscle mass and function without decreasing appetite or energy intake. The finding that plasma gut hormone responses to whey protein are not blunted during healthy ageing is likely to have implications to the composition of dietary supplements for older people, and could potentially play an important role in the glycaemic control of older people. The observation of comparable gastrointestinal hormone response to protein in older people without suppression of energy intake, suggests that older people may experience less sensitivity to the anorexigenic effects of these hormones.

The studies presented in this thesis have several strengths and limitations. The subject numbers were relatively small, and most studies included only men, however, we did not find any effects of gender on suppression of energy intake by whey protein in healthy older people (**Chapter 9**). We determined the effects of whey protein ingestion a protein source which is frequently used in high energy supplements due to its high leucine content and 'rapid' digestion, when compared to other proteins such as casein. It has to be determined whether our observations apply to other proteins. It is also unknown whether suppression of protein ingestion occurs beyond 3 hours after intake, e.g., during the remainder of the day or during the next day, in healthy older adults. The main strength of the studies was their randomised cross over design in which the participants acted as their own control.

The effect of ageing on the acute effect of suppression of energy intake by whey protein is a novel, and important, contribution to the current knowledge of energy intake regulation in older people. The outcomes from the studies dictate the need for future research, including determining the effects of long-term intake of whey protein supplements on 24-hour energy

intake in older people. Also, although the results in this thesis support the use of protein supplements in older people at risk of undernutrition, the lack of suppression of energy intake suggests that the use of such supplements as a strategy to decrease energy intake in older obese individuals may not be effective; this warrants further investigation. Older people may be less sensitive to the orexigenic effects of ghrelin, and anorexigenic effects of insulin CCK, GLP-1 and PYY, however, except for CCK, there are no studies which have assessed the effects of ageing on the sensitivity to these hormones. There is evidence that diet induced thermogenesis is higher after high-protein compared to low-protein intake in younger adults; effects of protein intake on postprandial energy expenditure in older adults are poorly characterised, and this information is of major relevance to the understanding of postprandial protein metabolism in the ageing population.

In conclusion, the main finding of this thesis was that the suppression of energy intake by whey protein was less in healthy older than younger adults, so that in the older adults compared with the younger controls whey protein ingestion increased overall energy intake more which support the use of 'pure' whey protein supplements in older people at risk of undernutrition.

GLOSSARY

AgPR; Agouti-related peptide

ANOVA; Analysis of variance

AUC; Area under the curve

BBP; Basal pyloric pressures

BMI; Body mass index

CCK; Cholecystokinin

DPP-IV; dipeptyl peptidase-4

EDTA; ethylenediaminetetraacetic acid

ELISA, enzyme-linked immunosorbent assay

GHS; Growth hormone secretagogue

GIP; gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide

GLP-1; Glucagon-like peptide-1

HOMA-IR; Homeostatic model assessment of insulin resistance

ID; Intraduodenal

IPPW; Isolated pyloric pressure wave

MMC; migrating motor complex

NPY; neuropeptide Y

PRISMA; Preferred reporting items for systematic reviews and meta-analysis

PYY; Peptide tyrosine tyrosine

RIA, radioimmunoassay

SD; Standard deviation

SEM; Standard error of the mean

SMD; Standard mean differences

T50, 50% gastric emptying time

TMPD; Transmucosal potential difference

VAS, visual analogue scale

WMD; weighted mean difference

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APPENDIX 1

Table 3.1: Studies included in the meta-analysis.

Study (No in References)	N Young/Older	Age (Years) Young/Older	Mean Body Mass (kg) Young/Older	Mean BMI (kg/m^2) Young/Older	Outcomes Used for Meta-Analysis
Alam *et al.* 2012	131/526	34 ± 9/69 ± 6	62.4 ± 13.5/63.5 ± 10.2 †	23.2 ± 2.2/22.3 ± 1.7 †	Energy intake of 24-h food intake recalls
Apolzan *et al.* 2009	24/32	25 ± 5/71 ± 6	75.5 ± 21.1/74.1 ± 18.7 †	25.2 ± 3.9/26.0 ± 5.1 †	Energy intake of 24-h food intake recalls
Arciero *et al.* 2009	0 M; 10 F/0 M; 10 F	19 ± 2/55 ± 5	62.5 ± 7.3/72.1 ± 9.4 *		Energy intake of 3-day weighed food records
Bell *et al.* 2003	7 M; 5 F/12 M; 9 F	23 ± 3/68 ± 5	70.4 ± 11.8/77.2 ± 13.7 †	23.7 ± 2.4/26.6 ± 3.7 *	Energy intake of 4-day weighed food records
Cheng *et al.* 1978	8 M; 0 F/7 M; 0 F	26 ± 3/67 ± 5	66.5 ± 7.2/61.6 ± 11.3 †		Energy intake of weighed food records
Church *et al.* 1984	7 M; 8 F/6 M; 8 F	20-35/36-53	45.0-95.3/52.6-85.4		Energy intake of weighed food records
Clarkston *et al.* 1997]	10 M; 9 F/5 M; 9 F	30 ± 35/76 ± 19		25.3 ± 3.4/25.2 ± 1.7 †	Hunger/fullness during fasting and postprandial (456 kcal oral mixed nutrient preload) conditions
Cook *et al.* 1997, MacIntosh *et al.* 1999 #	7 M; 0 F/8 M; 0 F	27 (20–34)/70 (65–75)		26.8 (24.4–31.8)/25.8 (18.2–30) †	- Energy intake of 5-day weighed food records - Energy intake during postprandial conditions ‡ (348 kcal intraduodenal lipid infusion) - Hunger/fullness during fasting conditions

Davy *et al.* 2001	6 M; 0 F/5 M; 0 F	25 ± 2/63 ± 7	79.0 ± 7.3/82.0 ± 8.9 †		Energy intake of 4-day weighed food records
Di Francesco *et al.* 2010	6 M; 6 F/5 M; 7 F	28 ± 2/75 ± 6		18.9–26.5/21.1–28.3 †	Hunger during fasting and postprandial (800 kcal oral mixed nutrient preload) conditions ^
Di Francesco *et al.* 2006	4 M; 4 F/4 M; 4 F	30 ± 3/78 ± 3		22.7–25.7/22.1–29.4 †	Hunger/fullness during fasting and postprandial (800 kcal oral mixed nutrient preload) conditions
Di Francesco *et al.* 2005	5 M; 4 F/5 M; 5 F	32 ± 8/77 ± 3		22.7–28.1/23.5–29.3	Hunger/fullness during fasting and postprandial ‡ (800 kcal oral mixed nutrient preload) conditions
Drewnowski *et al.* 1996	12 M; 12 F/12 M; 12 F	23 ± 1/67 ± 2		22.7 ± 1.0/24.5 ± 1.2	Energy intake of 14-day weighed food records
Flint *et al.* 2008	16 M; 14 F/16 M; 14 F	25 ± 4/68 ± 5	71.0 ± 10.4/73.8 ± 17.0 †	24.6 ± 2.2/24.7 ± 2.2 †	Energy intake of 4-day weighed food records
Fukagawa *et al.* 1990	6 M; 0 F/6 M; 0 F	21 ± 2/72 ± 7			Energy intake of 14-day dietary recalls
Giada *et al.* 1995	24 M; 0 F/24 M; 0 F	24 ± 4/57 ± 6		23.7 ± 2.4/26.8 ± 2.5 †	Energy intake of 7-day weighed food records
Howarth *et al.* 2007	1021 M; 771 F/491 M; 402 F	39 ± 17/71 ± 12		25.2 ± 4.2/25.4 ± 6.0 †	Energy intake of 24-h food intake recalls
Ishikawa *et al.* 1999	53 M; 16 F/50 M; 32 F	30-49/50-69	69.2 ± 10.4/62.9 ± 8.6	25.2 ± 3.0/25.0 ± 2.7	Energy intake of 2-day weighed food records
Keene *et al.* 1998	7 M; 5 F/4 M; 6 F	25/75			Energy intake during postprandial conditions ‡ (447 kcal oral mixed nutrient preload)
Kos *et al.* 1996	0 M; 38 F/0 M; 17 F	29 ± 3/59 ± 4	61.6 ± 9.7/57.4 ± 8.3 †	21.7 ± 3.1/21.8 ± 2.8 †	Energy intake of 4-day weighed food records
Lieberman *et al.* 1989	21 M; 20 F/21 M; 24 F	26 (20–35)/73 (65–95)			Energy intake of 4-day weighed food records

Macintosh *et al.* 2001	5 M; 7 F/5 M; 7 F	23 (20-26)/72 (65-84)		24.7 ± 2.4/25.0 ± 1.7 †	- Energy intake during fasting conditions ‡ - Energy intake of 3-day weighed food records - Hunger/fullness during fasting conditions
Macintosh *et al.* 2001	6 M; 6 F/6 M; 6 F	23 ± 4/71 ± 5		23.5 ± 2.8/24.1 ± 2.4 †	- Energy intake during fasting conditions ‡ - Energy intake of 3-day weighed food records - Hunger during fasting conditions
Macintosh *et al.* 2001	13 M; 0 F/13 M; 0 F	24 ± 5/72 ± 6		23.9 ± 2.2/23.5 ± 3.6 †	- Energy intake during postprandial conditions ‡ (347 kcal intraduodenal lipid infusion) - Energy intake of 3-day weighed food records - Hunger/fullness during postprandial conditions ‡ (347 kcal intraduodenal lipid preload)
McGandy *et al.* 1966	13 M; 0 F/37 M; 0 F	20-34/75-99	74.5 ± 1.2/70.9 ± 1.0		Energy intake of 7-day weighed food records
Morais *et al.* 2000	4 M; 3 F/3 M; 5 F	28 ± 5/72 ± 3	63.5 ± 10.6/64.2 ± 10.2 †	21.4 ± 2.1/24.8 ± 3.1 *	Energy intake of 6-day weighed food records
Morais *et al.* 1997	8 M; 7 F/8 M; 8 F	28 ± 5/73 ± 5	62.6 ± 7.4/64.1 ± 8.7 †	21.2 ± 1.8/23.8 ± 3.2 *	Energy intake of 6-day weighed food records
Moriguti *et al.* 2000	5 M; 6 F/9 M; 9 F	26 ± 3/68 ± 3	65.6 ± 9.6/80.0 ± 14.9 *	23.2 ± 1.6/27.5 ± 3.4 *	Energy intake of provided food items (7 days)
Nagengast *et al.* 1988	5 M; 6 F/6 M; 5 F	22 ± 6/67 ± 5	67.6 ± 5.0/69.1 ± 12.3 †		Food intake recalls
Poehlman *et al.* 1990	42 M; 0 F/26 M; 0 F	25 ± 5/67 ± 5	75.5 ± 10.7/78.4 ± 7.6 †		Energy intake of 3-day weighed food records

Polito *et al.* 2005	48 M; 47 F/103 M; 96 F	61 ± 4/74 ± 4	71.5 ± 8.1/67.9 ± 9.0	26.1 ± 2.4/25.3 ± 2.7	Energy intake of 4-day weighed food records
Rayner *et al.* 2000	5 M; 0 F/5 M; 0 F	23 (22–27)/71 (68–73)		24.4 (20.7–31.2)/25.6 (22.4–30.7)	- Energy intake during fasting conditions ‡ - Hunger/fullness during fasting conditions ^
Roberts *et al.* 1996	7 M; 0 F/9 M; 0 F	24 ± 1/70 ± 7	76.2 ± 12.4/72.9 ± 9.3 †	23.9 ± 3.4/23.4 ± 3.3 †	Energy intake of provided food items (10 days)
Roberts *et al.* 1994; 1995 #	17 M; 0 F/18 M; 0 F	23 ± 2/68 ± 6	71.6 ± 11.1/78.8 ± 12.6	23.4 ± 2.6/25.2 ± 3.6	Energy intake of provided food items (7 days)
Rolls *et al.* 1995	16 M; 0 F/16 M; 0 F	24 ± 5/69 ± 6	74.0 ± 7.2/84.3 ± 12.8 *	22.7 ± 2.0/26.2 ± 3.6 *	- Energy intake during fasting and postprandial (510 kcal oral mixed nutrient preload) conditions - Hunger/fullness during fasting and postprandial ‡ (510 kcal oral mixed nutrient preload) conditions
Rolls *et al.* 1991	12 M; 12 F/12 M; 12 F	26 ± 4/75 ± 5	68.9 ± 3.0/66.0 ± 3.2	23.5 ± 3.0/24.1 ± 2.8	Hunger/fullness during fasting conditions
Sawaya *et al.* 2001	9 M; 0 F/10 M; 0 F	23 ± 1/69 ± 1	72.9 ± 2.7/74.7 ± 3.4 †	22.7 ± 0.5/24.4 ± 0.9 †	Hunger during fasting conditions
Sawaya *et al.* 1996	0 M; 10 F/0 M; 10 F	25 ± 4/74 ± 4	54.8 ± 4.1/58.7 ± 9.8 †	20.9 ± 1.9/24.1 ± 2.8 *	Energy intake of 7-day weighed food records
Schneider *et al.* 2008	5 M; 5 F/3 M; 6 F	34 ± 8/76 ± 9		22.5 ± 2.9/23.6 ± 1.8 †	Hunger/fullness during fasting conditions ‡
Serra-Prat *et al.* 2013	7 M; 12 F/13 M;7 F	38 ± 11/81 ± 8	67.3 ± 9.0/72.6 ± 16.2	23.7 ± 2.8/27.9 ± 4.9 †	- Hunger during fasting and postprandial ‡ (400 kcal oral mixed nutrient preload) conditions
Serra-Prat *et al.* 2009 [66]	7 M; 10 F/6 M; 4 F	40 ± 10/80 ± 8		25.2 ± 3.3/26.7 ± 3.0 †	- Hunger during fasting and postprandial ‡ (380 kcal oral mixed nutrient preload) conditions

Soenen *et al.* 2014	10 M; 0 F/10 M; 0 F	23 ± 4/74 ± 4	73 ± 7/79 ± 7 †	22 ± 2/26 ± 2 *	- Energy intake during fasting and postprandial (180 kcal intraduodenal protein infusion) - Hunger/fullness during fasting and postprandial (180 kcal intraduodenal protein infusion) conditions
Stafleu *et al.* 1994	0 M; 97 F/0 M; 97 F	25 ± 3/76 ± 6	64.2 ± 10.6/70.5 ± 10.7	22.5 ± 3.5/26.8 ± 4.1	Energy intake of food frequency questionnaires
Sturm *et al.* 2004	6 M; 6 F/6 M; 6 F	24 ± 1/74 ± 1		23.2 ± 2.1/24.1 ± 3.5 †	- Energy intake during fasting and postprandial ‡ (750 kcal oral mixed nutrient preload) conditions - Energy intake of 3-day weighed food records - Hunger/fullness during fasting and postprandial ‡ (750 kcal oral mixed nutrient preload) conditions
Sturm *et al.* 2003	0 M; 8 F/0 M; 8 F	22 ± 4/77 ± 3	57.5 ± 5.4/58.0 ± 5.9 †	20.5 ± 1.1/23.7 ± 2.3 *	- Energy intake during fasting and postprandial (280 kcal oral mixed nutrient preload) conditions - Energy intake of 3-day weighed food records - Hunger/fullness during fasting and postprandial (280 kcal oral mixed nutrient preload) conditions ‡
Surrao *et al.* 1998	0 M; 10 F/0 M; 10 F	25 ± 4/74 ± 4	54.8 ± 4.1/58.7 ± 9.8 †	20.9 ± 1.9/24.1 ± 2.5 *	Energy intake of a 7-day weighed food record
Temme *et al.* 2010	413 M; 460 F/389 M; 355 F				Energy intakes of 24-h food intake recalls and food frequency questionnaires
Toth *et al.* 1996	18 M; 0 F/30 M; 0 F	23 ± 4/69 ± 5	79 ± 8/75 ± 5 †		Energy intake of 3-day weighed food records

Van Pelt *et al.* 2001	71 M; 0 F/66 M; 0 F	27 ± 8/62 ± 8	75.1 ± 16.0/77.4 ± 16.2	23.4 ± 4.7/25.1 ± 4.4	Energy intake of 4-day weighed food records
Van Walleghen *et al.* 2007	14 M; 15 F/11 M; 10 F	25 ± 5/69 ± 9	67.9 ± 1.7/70.8 ± 2.9	23.3 ± 3.7/24.7 ± 3.2	- Energy intake during fasting conditions ‡ - Energy intake of 4-day weighed food records - Hunger/fullness during fasting conditions
Van Walleghen *et al.* 2007	14 M; 15 F/13 M; 12 F	24 ± 5/68 ± 10	67.6 ± 15.5/71.1 ± 16.5	23.3 ± 4.3/24.6 ± 3.8	- Energy intake during fasting and postprandial (476 kcal for males and 360 kcal for females oral mixed nutrient preloads) conditions - Energy intake of 4-day weighed food records - Hunger/fullness during fasting and postprandial (476 kcal for males and 360 kcal for females oral mixed nutrient preload) conditions ‡ - Hunger/fullness during fasting conditions
Vaughan *et al.* 1991	33 M; 31 F/17 M; 21 F	24 ± 4/71 ± 6	84.5 ± 23.1/71.2 ± 13.5 *		Energy intake of provided food items (1 day)
Winkels *et al.* 2010	15 M; 0 F/17 M; 0 F	24 (20–34)/68(64–85)	75.8 ± 11.3/75.8 ± 7.6	23.0 ± 2.3/24.5 ± 1.9	Energy intake of provided food items (14 days)
Wolk *et al.* 2004	72 M; 0 F/94 M; 0 F	42–54/65–76		25.6 ± 2.7/26.5 ± 3.7	Energy intake of 24-h food intake recalls
Wright *et al.* 1995	41 M; 42 F/28 M; 43 F	20–64/74–90	70.1 ± 10.4/64.8 ± 10.1		Energy intake of 7-day weighed food records
Wurtman *et al.* 1988	21 M; 20 F/21 M; 24 F	26 (19–35)/72 (65–94)			Energy intake of provided food items (5 days)

Yukawa *et al.* 2006	8 M; 13 F/7 M; 11 F	25 ± 5/75 ± 4	72.9 ± 12.4/73.6 ± 12.7 †	24.7 ± 3.0/26.9 ± 3.0 *	Energy intake of provided food items (14 days)
Zandstra *et al.* 2000	5 M; 28 F/6 M; 18 F	22 ± 2/76 ± 5	71.0 ± 9.6/72.4 ± 8.9	23.3 ± 2.3/26.6 ± 3.5	Energy intake during fasting and postprandial (502 kcal for young subjects) or 430 kcal for older subjects oral mixed nutrient preload) conditions ‡
Zhou *et al.* 2013	49 M; 10 F/15 M; 21 F	20–29/50–59	59.0 ± 10.8/69.0 ± 12.3	21.7 ± 3.0/24.6 ± 3.1	Fullness during fasting and postprandial (896 kcal oral mixed nutrient preload) conditions