Aspects of Obesity: From aetiology to weight loss and maintenance

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DECLARATION OF ORIGINALITY

I declare that the work presented in this dissertation is, to the best of my knowledge, original and my own work, except as acknowledged in the text, and the material has not been submitted, either in whole or in part, for the award of a degree at this or any other institution.

Namson Shon-Chung Lau

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ABSTRACT

Background

Overweight and obesity are serious conditions that place a significant health and financial burden on individuals and the wider community. While the knowledge of the issues producing obesity are well known, the prevalence of the condition continues to rise. Despite advances in treatments, the cornerstone of management remains a combined lifestyle intervention.

Recently, insights from novel research have shed light on different aspects of obesity: from aetiology to weight loss and obesity remission.

The experiments

In the first experiment, the effect of covertly and randomly varying the protein content of the diet was tested in lean and healthy adults (n=22). Following 4 days of an *ad libitum* diet and a standardised in energy intake breakfast, for the lowest protein diet, fasting ghrelin was highest while post-prandial cholecystokinin lowest. The direction of these changes favoured increased appetite and decreased satiety.

In the second experiment, isocaloric low and high protein diets were tested in obese and lean mice (n=20 per group). The daily food intake varied and 12 - 15% more consumed with the low protein compared to the high protein chow.

These novel results demonstrate the intake for dietary protein is prioritised, even at the expense of an increased total energy intake and it occurs in the obese state.

In the third experiment, the effects of a combined lifestyle intervention on the secretion of key gut hormones and adipokines that are involved in the regulation of appetite and body weight was tested. Following a 12 weeks of the weight loss intervention, obese subjects (n=60) lost a mean 5.6% off baseline weight and this was associated with changes in the fasting levels of ghrelin and glucagon-like peptide-1. The direction of the changes would prevent further weight loss whilst favouring weight regain.

Discussion

The results from these experiments challenge conventional thinking about overweight and obesity. The secular trends of reduced protein consumption may be a powerful driver for the increased rate of overweight and obesity. Changes arise in the peripheral mediators of appetite and body weight after successful conventional weight loss therapies, which militate against further weight loss while promoting weight regain. These changes help explain the high rates of recidivism seen following weight loss by lifestyle interventions.

Further research into these different aspects of obesity may provide new therapeutic opportunities in the management of overweight and obesity: to reduce its incidence; to help facilitate weight loss; and to increase the rates of obesity remission.

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List of abbreviations

ADP	Adiponectin
AUC	Area Under the Curve
BMI	Body Mass Index
C57BL6	C57 Black 6 (mouse)
CCK	Cholecystokinin
CD	Conventional diet
CHO	(dietary) carbohydrates
DIO	diet induced obesity
EI	Energy Intake
F	(dietary) fat
FFAs	free fatty acids
GLP-1	Glucagon-Like Peptide-1
HFC	high fat chow
*hs*CRP	*high sensitivity* C reactive-protein
KD	Korean diet
LEP	Leptin
P	(dietary) protein
PLH	Protein Leverage Hypothesis
QCs	Quality Control
RCTs	randomised controlled trials
RIA	radioimmunoassay
SC	standard chow
T2DM	Type 2 Diabetes Mellitus
TC	total cholesterol
VAS	Visual Analogue Score

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The effects of weight loss arising from a diet and exercise program on fasting serum levels of Adiponectin, Total Ghrelin, Glucagon-Like-Peptide 1, Insulin and Leptin in overweight and obese subjects

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The effects following a 3-month combined lifestyle and exercise based weight loss program in overweight and obese subjects on fasting Total Ghrelin, Glucagon-Like-Peptide 1, Leptin, Adiponectin and other measures. Lau NS, Fuller NR, Conigrave AD, Caterson ID.

Percent dietary protein and Cholecystokinin (CCK), total Ghrelin, Glucagon-Like Peptide 1 (GLP1) and Insulin levels in lean humans after an isocaloric meal and 4 days of ad libitum feeding. Lau NS, Gosby AK, Conigrave AD, Simpson SJ, Caterson ID.

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Dietary Protein and food intake in lean and obese C57BL6 mice. Lau NS, Conigrave AD, Caterson ID. *Proceedings from the 20th Annual European Congress on Obesity;* Obesity Reviews Suppl *accepted Jan 2013*

Percent dietary protein and Cholecystokinin (CCK), total Ghrelin, Glucagon-Like Peptide 1 (GLP1) and Insulin levels in lean humans after 4 days of ad libitum feeding and an isocaloric meal. Lau NS, Gosby AK, *et al. Proceedings from the 19th Annual European Congress on Obesity 2012;* Obesity Facts. 2012; Vol 5 Suppl 1

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A 12-month, randomised, controlled trial to examine the efficacy of the Korean Diet in an Australian overweight and obese population - a follow up analysis. Fuller NR, Lau NS et al. Obesity Research & Clinical Practice; Oct 2012

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CHAPTER 1

Aspects of Obesity: the challenges of aetiology, weight loss and remission

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1.1 INTRODUCTION

1.1.1 Definitions, epidemiology, global trends, burden of illness

Definitions, epidemiology and global trends

An individual who continually accumulates excess body fat will become overweight and/or obese. These conditions are defined by the Body Mass Index (BMI), a calculated ratio of weight to height and a simple method of quantifying excess adiposity. The BMI is calculated by dividing body weight (in kilograms [kg]) by height (in meters [m]) squared (BMI = kg/m^2).

Adults with a BMI ≥ 25 kg/m^2 are defined as overweight while those with a BMI ≥ 30 kg/m^2 are obese. Obese range BMIs are graded into three degrees of severity (Grade I: 30 – 34.9 kg/m^2, Grade II: 35 – 39.9 kg/m^2, and Grade III: ≥40 kg/m^2)[1] with severe obesity defined as a BMI ≥40 kg/m^2 (or ≥ 35 kg/m^2 in the presence of multiple co-morbidities).

National and international studies of overweight and obesity have shown a high prevalence of overweight and obesity in children, adolescents and in adults. The Australian Bureau of Statistics has estimated that in 2011- 2012[2], 63.4% of adult Australians were overweight or obese, with the prevalence increasing compared to previous. This trend has also been noted in other high-income nations and in these countries for the last three decades the prevalence of overweight and obesity has increased rapidly (though the most recent five year Australian and U.S. health surveys showed that the rate of increase had slowed)[1-6].

Finally it is worth noting that these conditions are no longer a problem of high-income nations alone but also a problem for countries in transition. The World Health Organisation has estimated that in 2008 1.4 billion adults were overweight and of these over 200 million men and nearly 300 million women were obese. For the same period more global deaths were linked to overweight and obesity than to underweight[1].

The health burden from overweight and obesity

Obesity is a major risk factor for conditions such as cardiovascular disease, Type 2 diabetes mellitus, osteoarthritis, chronic kidney disease and cancers including those of the breast, prostate, oesophagus, kidney and colon[7-10]. Elevated BMI has long been correlated with an increased mortality risk[10] with the results of a meta-analysis of close to 100 studies and 2.9 million subjects confirming the strength of the relationship between mortality and severe obesity[11], the category of elevated BMI of which the prevalence has continued to increase[5,6].

The economic burden from overweight and obesity

The increasing global prevalence and the impact on health from overweight and obesity is well recognised and this has led to a focus on assessing the burden that it places not just on individuals, but on health care services and the wider community.

It has been estimated that overweight and obesity accounts for between 0.7 – 2.8% of a nation's total healthcare expenditure and that the obese have 30% greater health care costs than their lean counterparts[12]. In Australia, the annual direct health care cost from overweight and obesity has been calculated to range from $8.3 to 10.7 billion. When indirect costs such as lost productivity, cost of carers and government welfare are considered, the annual figure rises to an astonishing $45 to 58 billion[13,14].

With costs rising by 3 – 10% per annum, there are strong incentives to develop and implement effective strategies to reduce the burden of the condition, especially as noted earlier, the rates of Australian overweight and obesity have continued to rise.

1. 1.2 Challenges in managing overweight and obesity

The recommended clinical management of overweight and obesity is to assist patients to implement a combined lifestyle intervention with the goals of decreasing energy intake (by reducing food consumption) and increasing energy expenditure (through regular physical activity). This should also include promoting positive behavioural changes to alter habits that lead to increased energy intake and providing psychological methods and support to achieve such changes.

The antecedents of this current approach go as far back as Hippocrates[15] and Galen[16], physicians of classical antiquity, who perceptively recognised that obesity was a disease of imbalance (though of the *humours* rather than of hormones). Their treatment strategies involved recommending that their corpulent patients re-balance their *humours* by increasing their levels of physical activity and by decreasing their indulgence of fine foods.

Since these times, while our knowledge of obesity's underlying pathophysiology has significantly improved, the guidelines for the prevention and clinical management of overweight and obesity, as put forward by the national bodies of Australia (National Health and Medical Research Council[17]), the United Kingdom (National Institute for Health and Clinical Excellence[18]) and the United States of America (National Heart Lung and Blood Institute[19]) all still recommend the same type of combined lifestyle intervention as the cornerstone of management. The now three decade old increases in rates of overweight and obesity places the effectiveness of these strategies into question.

Weight loss or weight regain?

Meta-analyses of combined lifestyle-based weight loss studies have shown that 5 years after any lifestyle intervention only 17 – 30% of subjects maintained weight loss while an even smaller 2 - 7% of subjects continued to lose weight[20]. This suggests that on following a lifestyle intervention, the norm is not long-term weight loss but rather recidivism or weight regain.

Modern advances: pharmacotherapy and bariatric surgery

Modern advances in the management of obesity include several pharmacological agents including *Phentermine*, *Sibutramine*, *Rimonabant* and *Orlistat*, which target central appetite centres or influence the intestinal absorption of macronutrients. While these agents are modestly effective at facilitating weight loss[21,22] studies into their clinical use have been marked by high rates of subject drop-out, which have also been a feature of their clinical use in the *"real world"*.

Furthermore all of these agents have their own individual and problematic side-effect profiles and over the last 5 years *Sibutramine* and *Rinomabant* have had to be withdrawn from national pharmaceutical registries across several jurisdictions (including Australia) due to an increased rate of significant adverse events (cardiovascular for *Sibutramine* and psychiatric for *Rinomabant*)[23].

The most effective advance in obesity management has been with modern bariatric surgery, which functionally either restricts the capacity of the stomach (e.g.

laparoscopic adjustable gastric band or sleeve gastrectomy), leads to nutritional malabsorption (switch and bypass procedures) or combines both effects in a single procedure (Roux-en-Y gastric bypass). The effectiveness of these procedures compared to conventional lifestyle interventions in producing greater and more sustained weight-loss and in inducing the remission of obesity related complications has been firmly established[24].

Interestingly, while originally it was thought that the reason for the success of bariatric surgery was due to the procedures themselves, more recently focus has shifted to the favourable effects on the peripherally secreted hormonal regulators of appetite and body weight that are seen following bariatric surgery[25,26].

The superior weight loss, weight maintenance and remission of obesity complications associated with bariatric surgery has led to an increase in its popularity. However there remain problems when considering bariatric surgery as first line in the management of obesity. In particular, surgery itself carries a (small) risk of morbidity and mortality[27,28], and a considerable financial cost with access to surgery not distributed equitably across national health care sectors[29,30].

In summary, despite their limitations, conventional weight loss strategies remain the cornerstone of the management of overweight and obesity. Insights gained from reviewing the aetiology of obesity may shed light and provide opportunities to address these limitations.

1.1.3 Multi-factorial aetiology of obesity

Most individuals gain weight due to a complex and dynamic interaction between evolutionary, genetic, prenatal, environmental, psychological and behavioural factors that contribute to a long-term misalignment between their diet and energy intake and their levels of physical activity and energy expenditure. The resulting excessive energy intake leads to fat deposition and weight gain[9,31].

Genetic, pre-natal and social factors

Only a minority of people excessive weight gain is due to a specific genetic defect which comprises a clinical syndrome (the best known of these being Prader-Willi and Bardet-Biedl syndromes). However research into these and other genetic conditions has advanced the knowledge of common overweight and obesity[32]. More broadly, studies of adoptees, families and twins suggest that important metabolic traits, including the regulation of energy intake, energy expenditure and fat storage, have a genetic component and are in part heritable[33-35], although attenuating these factors remains beyond the scope of current therapies.

In contrast, the prenatal environment has long been recognised as both contributing to offspring overweight and obesity while providing an opportunity to implement obesity prevention strategies. Maternal factors such as smoking history[36], nutrition and weight change[37] and diabetes status[38,39] are all independently associated with an increased risk for obesity in the woman's offspring. More recently novel factors such as the weight profile of an individual's social network, incorporating family members,

spouses and friends, has been independently associated with a risk of obesity[40], raising this as another area of possible intervention.

Diet and lifestyle

Internationally there has been a several decades long increase in the global food supply, which has been driven by innovation in agricultural technology that has become coupled with changes in food manufacture favouring the increased use of refined carbohydrates (CHO) and fats (F) at the expense of protein (P)[41].

While research into the aetiology of overweight and obesity has traditionally focused on the role of CHO and F, there has recently been a focus on the role dietary P, which has declined in a smaller magnitude than the increases in the consumption of CHO and F. Whether the modest reduction in the consumption of dietary P has contributed to the increased prevalence of overweight and obesity is an open and relevant question[42].

Across high-income nations on a population level, the well-recognised decline in the levels of physical activity across all age groups has been associated with an increased risk of obesity[43-46]. Some of this decline can be attributed to a shift in mass entertainment to types which are less participatory and more sedentary[47], while factors such as the increase in vehicular transportation, the increase use of technology and the design of work spaces and urban living areas all contribute to this problem[41].

Taking diet and lifestyle together, it is unsurprisingly that the availability of cheap, energy dense foods and a decline in physical activity both play highly significant roles in the increased rates of overweight and obesity[41].

Evolutionary and environmental factors

Evolutionary theory has lessons for the aetiology of overweight and obesity. As modern man (*homo sapiens*) spent much of his existence living in a small hunter-gatherer bands and following a subsistence lifestyle, the theory predicts that *homo sapiens* adapted to the reality of unpredictable food supplies by evolving to be able to readily accumulate fat stores whenever food sources became available[48].

The complex homeostatic system of appetite and body weight regulation that evolved during *homo sapiens'* long existence living in subsistence nutrient environments is much more sensitive to states of energy deficit than to states of energy excess. The secretion of multiple peripheral hormones is quickly altered by any sustained decrease in food intake, which triggers a central response that involves increases to hunger and to food seeking behaviour. In comparison the physiological response to chronically increased food intake is blunted and easily down-regulated[48,49].

While this system was advantageous for the hundreds of thousands of years, when *homo sapiens* lived in small, nomadic band societies, the rapid transit from pre-agricultural societies to the modern *post-industrial* age has seen technological advances in agricultural productivity and the creation of labour saving devices that have become coupled with physically less demanding employment, more sedentary

lifestyles and greater urban living. In a little over 6,000 years, this has resulted in *homo sapiens* facing a completely different living environment than was encountered for much of human history[50]. The modern lifestyle is one where energy dense foods are readily available and where little physical effort is required to obtain and consume this food. This results in modern living conflicting with our evolutionary heritage, creating a situation where living in the here and now is to live in an *"obesogenic"* world[51]. Furthermore, the maladaptive interplay between the *obesogenic* world and our evolutionary heritage is highlighted when considering the neuro-endocrine homeostatic regulation of appetite and body weight. This results in an insidious conflict between human physiology, that always seeks to retain body weight, and the modern living environment, where excess energy intake is the norm. It is not surprising that the physiological response to this world is one of overweight and obesity[50].

1.2 AIMS OF THE THESIS

Overweight and obesity are complex clinical conditions the prevalence of which has increased globally. This due to multiple factors including an evolutionary susceptibility, technological and socio-economic shifts, changes to agricultural and food manufacture practices in addition to individual lifestyle choices. It is a significant health and economic issue both for individuals and their wider society. Despite pharmacological and surgical advances, the cornerstone of clinical management is the combined lifestyle intervention, which is marked by (at best) modest long-term outcomes.

Recent studies focusing on the role of dietary protein in the regulation of food intake and the impact of weight loss on peripheral regulators of appetite and body weight have challenged conventional theories as to the aetiology of obesity and to the limitations of combined lifestyle therapies. Further research in these areas will provide novel insights and approaches for the complex condition.

The aims of this thesis are:

1. To review the role of dietary protein in the homeostatic control of body weight and the role of declining dietary protein intake in the causation of overweight and obesity. Whether short and medium term changes in the intake of dietary protein are associated with changes in total energy intake, hunger and satiety will be tested and changes in the peripheral mediators of appetite and body weight regulation with differences in protein intake will be tested. This will be examined in detail in

Chapter 2: Testing the *Protein Leverage Hypothesis* in lean humans: The Fixed Energy Meal Experiment.

2. To examine whether the intake of dietary protein is prioritised by obese phenotypes, as it is in their lean counterparts and to examine whether changes in dietary protein influence the secretion of peripheral mediators of the appetite and body weight regulation and markers of obesity related sub-clinical inflammation. This will be examined in detail in **Chapter 3**: Testing the *Protein Leverage Hypothesis* in lean and obese mice.

3. To review the effects of weight change, produced by a combined lifestyle intervention, on the homeostatic regulation of body weight with a particular focus on the impact on the secretion of key peripheral mediators of appetite and body weight regulation. This will be examined in detail in **Chapter 4**: Lessons from South Korea and its diet: Lifestyle-induced weight loss and the secretion of appetite and body weight influencing hormones.

Finally the results of the three experiments will be reviewed in **Chapter 5** with focus on the insights that the experiments bring to different aspects of the challenging conundrum of overweight and obesity.

CHAPTER 2

Testing the *Protein Leverage Hypothesis* in lean humans: The Fixed Energy Meal Experiment

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2.1 INTRODUCTION

2.1.1 Dietary Protein

It has been well established that when dietary protein (P) is compared isocalorically to the other macronutrients (carbohydrates [CHO] and fat [F]), P is the most satiating. Additionally, following the consumption of a standard unit of each macronutrient P will provide the lowest net energy output because of its greater thermogenic properties. When these two facts are taken together, it suggests that modestly increasing the consumption of P at the expense of CHO and F will promote satiety and facilitate weight loss through two means; a decrease in total energy consumption and an increase in diet induced thermogenesis[52-55].

2.1.1.1 High Protein Diets

There have been four recent reviews[52-55] that have examined the evidence from randomised controlled trials (RCTs) on the relationship between high P diets, increased satiety and decreased total energy intake critically. As a summary of their

findings and while acknowledging the differing methodologies of the individual studies, it is apparent when higher P meals (20-80% of Energy Intake as P - hence abbreviated as 20-80% P) were compared to isocaloric meals of lower protein content (5- 18% P or 100% CHO or 100% F) higher % P meals were associated with greater post-meal satiety for up to 24 hours and a decrease in *ad libitum* food intake.

The typical design of those RCTs that included healthy lean to overweight subjects used a single meal test study protocol with diets randomly crossed over, following an overnight fast. The test meals were presented as either "*real-world*" foods (such as sandwiches or yogurt) or liquid meals made from protein powder in combination with milk or water. The types of dietary P varied with animal (meat, egg, whole milk, whey or casein) and plant (soy) sources being used. Following the consumption of the experimental meal, satiety was measured subjectively in the post-prandial period by the use of a Visual Analogue Score[56]. The RCTs that included an assessment of protein and its effects on subsequent caloric intake compared isocaloric liquid or solid snacks of differing %P (randomly allocated and crossed over) followed by a main standardised meal. However the reviews all noted that to date, large-scale and conventional meta-analyses techniques were not feasible because of the large methodological variance in the RCTs.

One of the limitations of these reviews is that only *Halton et al*[52] commented in detail on studies with negative findings. Regarding dietary P, satiety and energy intake in adults they found six of nine studies reported positive findings[57-62] but the three that reported negative findings[63-65] were marked by unique methodological traits such as the use of nose clips, subjects being placed in whole body calorimeter chambers rather

than free living arrangements and the use of foods of significantly different palatability and caloric content. These non-standard design traits made it more difficult to generalise from the negative studies and *Halton et al* concluded that the weight of evidence supported the notion that higher P diets are associated with increased satiety and reduced energy intake, especially as the six positive studies used design methodologies closer to "real life *(living)* situations".

2.1.1.2 Dietary Protein, Satiety and Appetite Hormones

While there is strong evidence to support a relationship that diets higher in P increase satiety and decrease energy intake, the mechanisms remain unresolved.

The first explanation put forward to explain the link between higher P diets and increased satiety was by *Mellinkoff*[66] in 1956. He suggested that plasma levels of amino acids act directly on then unidentified centres in the brain to regulate and control appetite and satiety. In a recent review by *Westerterp-Plantenga et al*[55] the authors re-examined the still fragmentary evidence to support Mellinkoff's hypothesis, highlighting the evidence from neurophysiology, clinical trials and appetite studies that show that elevated levels of the amino acids of tryptophan, tyrosine and histidine, known precursors for the neuro-transmitters of serotonin, dopamine, noradrenaline and histamine respectively appear to influence central satiety centres and are slightly elevated in high P diets. However the authors of the review readily acknowledged that there still remains insufficient evidence to support or refute Mellinkoff's hypothesis.

A second hypothesis put forward to explain the relationship between P and satiety is based on the observation that the consumption of diets higher in P are associated with post-prandial changes in secretion of appetite and satiety hormones: increased secretion of anorexigenic hormones and significantly decreased secretion of the orexigenic hormone ghrelin[54].

Bowen et al[67] demonstrated in 72 lean and overweight men fed pre-meal soups (high protein [70% P] vs. low protein [87% CHO, 1.5% P]) in a randomised, cross-over fashion that during the post-prandial period up to 180 minutes, there was a prolonged suppression of ghrelin secretion and an elevation in Glucagon-like peptide-1 (GLP-1) secretion. The authors also reported that while no differences on subjective assessments of hunger and satiety were found between the high and low P soups, the higher P soups were associated with a reduction in *ad libitum* food intake from a buffet lunch offered afterwards.

Raben et al[68] studied 19 healthy, lean men and women offered *ad libitum* breakfasts differing in ratios of %P | %CHO | %F (High P: 32% | 3% | 31%; High CHO: 11% | 65% | 24% and High F: 11% | 24% | 65%) in an randomised, crossover fashion. The authors reported the high P breakfasts were associated with post-prandial increased diet-induced thermogenesis and greater secretion of GLP-1 to 300 minutes but failed to find any relationship between the high P breakfasts and increased subjective measures of satiety.

Lejeune et al[69] and *Westererp-Plantenga et al*[70] demonstrated in lean women and men respectively fed a high (30% P) or low (10% P) P diet for 24 hours in a randomised, cross-over fashion, that the high P diet was associated with increased meal-related and 24-hour levels of satiety, increased diet induced thermogenesis and increased GLP-1 secretion to 12 hours but there were no differences in the 24-hour levels of ghrelin.

In contrast, *Veldhorst et al*[71] studied 25 healthy lean people offered an *ad libitum* standardised breakfast varying in % P but fixed in % CHO (10% or 25% P) in a randomised, cross over fashion. These authors found that the higher P breakfast was associated with increased satiety and decreased hunger but not with any changes to the post-prandial secretion of ghrelin and GLP-1 up to 180 minutes.

However, not all have reported a link between higher P diets and increased levels of satiety hormones. *Smeets et al*[72] studied 30 lean to overweight but healthy men and women fed a fixed energy breakfast followed four hours later by a fixed energy lunch both fixed in %F (30%) but varying in % P and % CHO (High P: 25% P |45% CHO; Low P: 10% P | 60% CHO) in a randomised, cross-over fashion and reported that the high P meal was associated with increased diet-induced thermogenesis and post-prandial satiety but also with a reduced peak in the secretion of GLP-1 and with no changes in the secretion of ghrelin for up to 180 minutes after lunch.

Similarly *Weigle et al*[73] assessed 19 healthy, lean to overweight men and women provided with 2 and then 12 weeks of diets fixed in % CHO (50%) but varied in % P and % F (% P | % F: 15% | 35% vs. 30% | 20%) and reported that the high P diet was associated with increased satiety and decreased energy intake but, in contrast to the

studies above, the high P diet was also associated with increased (and not decreased) 24-hour levels of ghrelin when assessed at the end of each diet treatment.

On balance the evidence from these studies suggest that following a high P meal, there is an increased secretion of GLP-1 and a reduced secretion of ghrelin and that these changes are not always correlated with differences to subjective satiety, however the presence of negative studies suggests that the strength of the relationships remains unresolved.

Compared to the studies above, examining the relationships between ghrelin, GLP-1 and high P diets, the relationship between cholecystokinin (CCK) and dietary P is even more under-explored with only three studies found. Two studies that measured CCK relative to the intake of dietary P have been identified with both studies being by the same team of authors[67,74]. In both studies, isocaloric liquid meals of 70% P or 100% CHO were provided to healthy, lean to obese men in a randomised, cross-over fashion and the authors reported that the high P meals were associated with the following changes to CCK: prolonged post-prandial elevation (120 minutes compared to 30 minutes) and increased total secretion to 180 minutes.

In the third study[75] liquid drinks made from 100 % P from different sources of P (mixed whey and casein vs. whey vs. casein vs. pea protein) were compared. The authors reported that after the consumption of a milk drink made up of a mix of dietary P (whey and casein proteins), an increase in the post-prandial secretion of CCK (up to 120 minutes) was demonstrated compared to the other drinks. This suggests

that CCK secretion is influenced by very high dietary P meals and that it may be more responsive to meals composed of mixed rather than single source dietary P.

The third theory explaining the finding of increased post-prandial satiety after high P diets links satiety with increased diet-induced thermogenesis (DIT) with high P diets and assumes that it is DIT itself which contributes to increased satiety. This association was initially reported in 1999 by *Westerterp-Plantenga et al*[76] in healthy, lean subjects who had resided in a respiratory chamber for 36 hours and were fed isocaloric, matched for palatability high and low P diets (9% P vs. 29% P). Compared to the low P diet, measures of DIT and of short- and long- term satiety were significantly greater on the high P diet and increased satiety significantly correlated with DIT. However due to the practical difficulties of measuring DIT this has remained relatively unexplored with the exception being *Smeets et al*[72] who used indirect-calorimetry to assess DIT. In their study of thirty healthy, lean to overweight men and women, they found no correlation between DIT and increased satiety.

2.1.1.3 High Protein Diets and Weight

High P diets are also associated with the metabolically favourable changes of increased total weight loss, increased % fat loss and following weight loss, decreased weight regain. *Paddon-Jones et al*[53] and *Westerterp- Plantenga et al*[55] summarised the evidence from individual studies that explored these relationships and noted that the evidence supporting these findings comes from studies with small numbers and short follow-up (between 6 - 12 months).

More contemporary studies have re-examined these issues but the results from the two largest studies are mixed. *Sacks et al*[77] examined 811 overweight and obese adults for two years who were randomised to one of four low energy diets varying in CHO, P and F (% CHO | % P | % F: 65 | 15 | 20 or 55 | 25 | 20 or 45 | 15 | 40 or 35 | 25 | 40). The authors found between the four interventions neither significant differences for weight loss at 6 or 24 months nor differences in satiety or to compliance with the diets. However one significant criticism of the study was that the between the diet treatments, the measured differences in % P were small (2.5 – 3.4%) and lower than that reported in other studies of dietary P.

The DIOGENES group[78] studied 1209 overweight and obese adults for 6 months and compared *ad libitum* diets varying in glycaemic index (high vs. low) and dietary protein (%P: 13 vs. 25). The diet treatments were prescribed for weight maintenance after at least 8% weight loss was achieved by 8 weeks of a liquid meal replacement / very low calorie diet. The authors reported that the high P and low glycaemic index diets were associated with less weight regain and lower study drop-out rates. While these results are generally positive for the role of dietary P, a subset of the participants had active support in the form of financial payments, more regular contact and the direct provision of high P food and these protocol differences may have affected compliance and the study outcomes.

2.1.1.4 Secular Trends in Dietary Protein

Epidemiological evidence from large health surveys from the United States[79,80], Australia[81,82] and Spain[83] demonstrate that in these Western countries there is a

secular pattern of a reduction in the % energy intake derived from P compared to the CHO and F.

Data from 1970 to 2000 from the United States National Health and Nutrition Examination Surveys ([NHANES] an annual survey of 5000 nationally representative Americans) demonstrates that among adult Americans, over the last 30 years the total daily EI has increased by 5%[80], made up of an increase from CHO (+7%) and a decrease from P (-1.4%). Among American men dietary P has fallen by -1.1% to 15.4% of daily EI and among American women it has fallen by -1.8% to 15.1% of daily EI. Futhermore an updated analysis[79] incorporating results from NHANES surveys to 2008 has confirmed that over the last 10 years, these trends have remained constant.

Analysis of the two Australian nutrition surveys, the 1983 National Dietary Survey of Adults and the National Nutrition Survey 1995[81,45,82] (surveys of 6254 and 2367 capital city based Australian adults aged 25 – 64 years) demonstrated that between 1983 - 1995 while the daily consumption of P remained relatively constant (+0.9% to 112 gm/day in men; -1.2% to 76 gm/day in women) over the same period total EI rose by on average +350 kj/day (+3.5% in men and +4.5% in women). This increase was predominately made up by an increased intake of CHO (+17% in men and +16% in women) and so when dietary P is calculated as a % of total EI over the period 1983 to 1995 the contribution of % total EI from P actually fell (-0.3% in men and -1% in women). By the end of 2013, the 2011/2013 Australian Health Survey, which incorporates a detailed assessment of dietary and physical activity habits, will be completed and the release of an updated national nutritional data and analysis is expected afterwards.

The most applicable European study, which was of 8470 randomly selected adults in regional Spain[83] and surveyed in 2000 and in 2005, did not demonstrate a similar significant increase in total EI but did show a significant drop in % EI obtained from dietary P (-0.3%) and an increased intake of dietary F, unlike the US and Australian data.

In summary, diets higher in P are associated with increased satiety (though the mechanisms for this remains unclear), increased thermogenesis and in the overweight and obese favourable changes to body weight. However the broader historical trends in macronutrient consumption have been towards an overall increased total EI but with a slight but significant reduction in EI derived from dietary P.

2.1.2 The Protein Leverage Hypothesis

In 2005 Simpson and Raubenheimer published the *Protein Leverage Hypothesis*[42], which holds that the driver of food consumption is the prioritisation of the absolute intake of dietary P at the expense of regulating the intake of CHO and F. It follows that the effort to maintain the intake of P constant in a food environment that has experienced even a small decline in the availability of P will lead to increased total food consumption and greater energy intake. Unless this is matched by a commensurate increase in energy expenditure, it will lead to weight gain. This is the process that is hypothesised to be a key and previously under recognised contributor to the increased rates of overweight and obesity.

2.1.2.1 The Geometric Framework

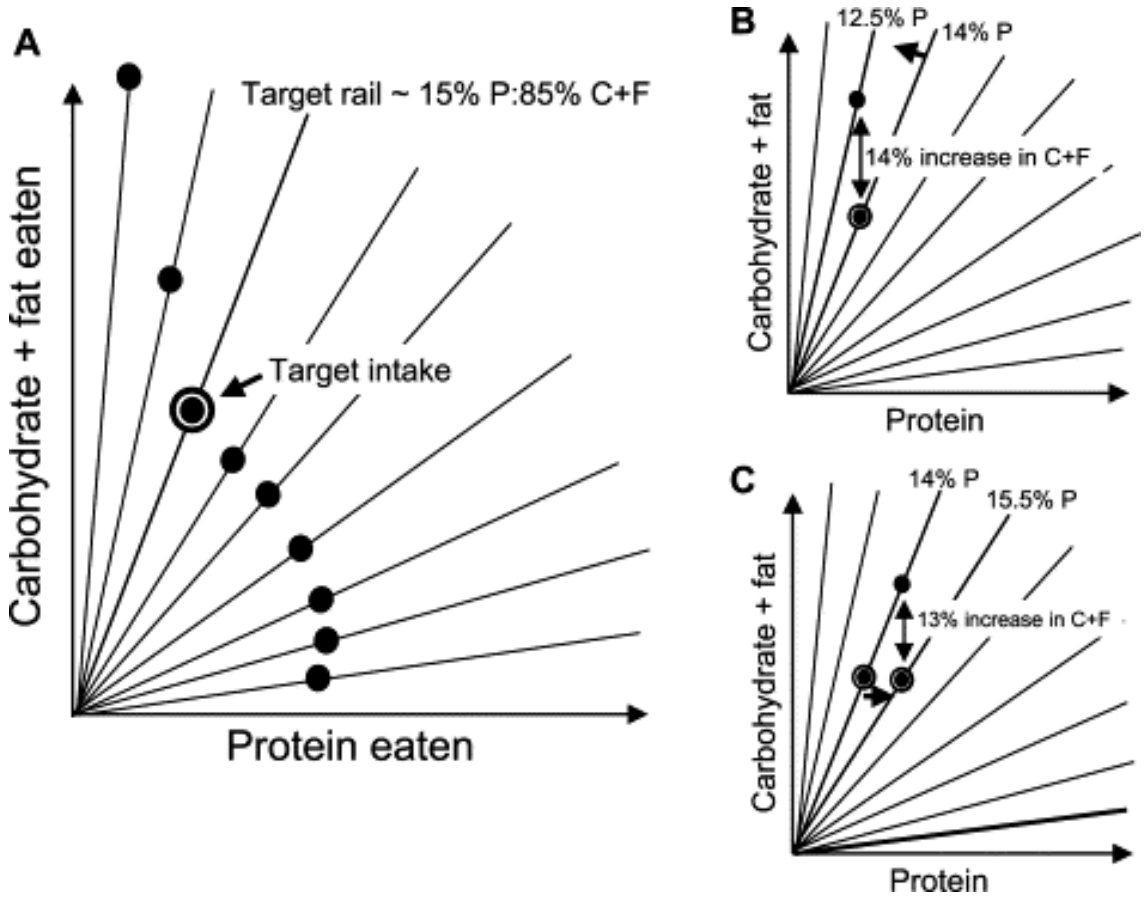
The precursor to the *Protein Leverage Hypothesis (*or *PLH)* was published in 2003 by Simpson *et al*[84]. The interaction between the *Geometric Frameworks* and the *Protein Leverage Hypothesis* are illustrated in ***Figure 2.1.***

In the article, the authors described the *Geometric Framework* model that envisaged a state-space model which framed the animal's nutritional environment as an interaction between the macronutrients that make up the diet and the metabolic responses of the animal when feeding upon diets of differing macronutrient ratios.
The animal's nutritional intake targets are visualised in the model and represent the optimal points that balance the nutritional and physiological needs of the animal. These are represented as target points within the model. Whether an animal can meet its intake target is determined by the availability of food in the dietary environment and these are illustrated as linear lines or "nutrient rails" which reflect the macronutrient balance of a particular diet (see ***Figure 2.1 (a)*** below for the nutrient target intake and nutrient target rails)

The *Geometric Framework* model demonstrates that when an animal is in a food environment that is unbalanced in macronutrients, it can still achieve its macronutrient intake targets by over-consuming some macronutrients whilst under-consuming others.

Figure 2.1 The prioritisation of dietary P in humans: Balancing dietary P versus CHO + F

From Simpson et al[84] with the kind permission of the author



A Different nutrient rails reflecting diets of differing P to CHO + F ratios. The optimal intake target presented here is 15% P: 85% CHO + F.

B The effects of shifting from a diet of 14% P to 12.5% P: To maintain the same absolute amount of dietary P, there needs to be an associated 14% increase in the consumption of CHO + F

C The effects from an increase in P requirements by 1.5% such as might occur due to enhanced needs secondary to obesity and Type 2 Diabetes Mellitus; if diet composition is maintained, a 13% increase in CHO + F is required to meet the relatively small increased dietary P need.

Key: Figures not to scale, the X axis represents the % of daily EI as Protein, the Y axis represents the % of daily EI as CHO + F

2.1.2.2 *Evidence to support the Geometric Framework and Protein Leverage Hypothesis*

Evidence supporting the *Geometric Framework* model and *PLH* has been found in several animal studies including locusts[85,86] (which showed that over differing food pairings varying in distance and frequency protein intake was maintained despite dilution of food with indigestible bulk); rats[87] (where with over 8 different complementary food pairings varying in macronutrient ratios, the absolute amount of protein consumed by the rats remained relatively constant); and mice and pigs[88] (which demonstrated that over numerous diets varying in macronutrient ratios, P intake was prioritised above CHO and F). The evidence from these studies supports the notion that animals of many species regulate their nutrient intake to prioritise the intake of dietary P above that of CHO and F.

The evidence for the *PLH* in humans to date has come from a pilot study[84] published in 2003 with ten healthy volunteers demonstrating that diets with low %P then CHO + F were over-consumed to keep the absolute intake of P relatively constant. Conversely, when diets with a high %P were offered, CHO + F were proportionately more under-consumed than P intake over-consumed.

While the strength of protein regulation appeared to vary between species, what was striking was that in the many species examined, when %P was lowered, total energy intake increased in an effort to maintain constant the absolute intake of protein.

The *PLH* developed from the above animal studies, the evidence of a secular decline in the intake of %P and the *Geometric Framework* model. It is proposed that humans also balance their intake of dietary P against that of CHO and F. If this theory is proven correct, it will have important implications and help explain the recent trends in human overweight and obesity.

The nutritional survey data demonstrates a trend for the dilution of P in the diet, which is linked with or possibly caused by an economically driven increase in the use of cheaper sources of fat and sugar by food manufacturers. If this is coupled with the evolutionary tendency for humans to find fat and sugar highly palatable then the food environment becomes nutritionally unbalanced. Human physiology is being forced to trade-off the intake of protein against the intake carbohydrate and fat. In the words of the proposers of the *PLH*[42] "*... it is unsurprising that the regulatory systems controlling (energy) intake by modern humans are prone to becoming unstable, driving a catastrophic cycle of over-consumption and obesity*".

The *PLH* in humans had yet to be assessed and confirmed under controlled experimental conditions and what else remained unclear was the relative power of protein leveraging in humans. A further area of uncertainly was the physiological mechanisms that underlie protein leveraging and whether the hormones that influenced satiety and appetite have any role to play.

2.1.3 The regulation of appetite and satiety: Key gut hormones and adipokines

2.1.3.1 Cholecystokinin

Cholecystokinin (CCK) is secreted by the proximal small intestine in response to the detection of the nutrients that arise from the consumption and digestion of food. Its secretion is followed by local intestinal effects that include delayed gastric emptying, altered intestinal motility and the regulation of the secretions from the gallbladder and pancreas. Distal CCK effects include the inhibition of food consumption via action on the local and central centres that regulate appetite and satiety.

CCK was first isolated by *Mutt* and *Jorpes*[89,90] in work published in the late 1960's and early 1970's. The CCK polypeptide is initially expressed as a 115-residue pre-prohormone precursor which is processed to carboxyl-terminal amino-polypeptides of various lengths including 8 and 33 unit length residues. The CCK polypeptides are widely distributed throughout the gastrointestinal tract (GIT) and the central nervous system (CNS) with CCK-8 the most abundant form in the CNS while CCK-8, -33 and -58 all present in GIT in significant amounts[91-93].

Thus far two CCK receptors have been identified[94] and were initially named according to their locations (CCK Receptor A for "alimentary" and CCK Receptor B for "brain"). As it has since been established that there is significant overlap in their distribution, the receptors have been renamed CCK_1 and CCK_2[95].

Following the ingestion of food and processing in the stomach, the digested food moves into the small intestine where the presence of intestinal nutrients of fatty acids, amino acids and glucose (the digestive products that arise from dietary F, P and CHO) trigger the secretion of CCK, which is otherwise secreted at a basal rate. It is known that the products arising from the breakdown of fat and protein are more potent stimulators of the secretion of CCK than those from carbohydrates. Post-prandial secretion is rapid and within 30 minutes from the ingestion of food, concentrations increase over five-fold (to 5-8 pMol/L) before gradually falling back to basal levels (normally~1 pMol/L) over a period of three to five hours[96].

CCK has local effects on the gut and is the main regulator of gall bladder function and through action on CCK1 receptors modulates post-prandial biliary contraction and the release of bile into the duodenum. Through action on the vagus nerve CCK also stimulates pancreatic enzyme release[96,97]. CCK has inhibitory properties that include inhibiting gastric emptying through altering gut motility of the antrum and pylorus of the stomach and the duodenum[98,99]. These effects are seen in studies of exogenously administered CCK that demonstrate that levels of CCK are associated with delayed gastric emptying[99] and in studies using CCK1 receptor antagonists that demonstrate accelerated gastric emptying especially after the ingestion of fat- and protein- rich meals[100].

CCK is also known to have actions on central centres that regulate appetite and satiety. This was first demonstrated by *Gibbs et al*[101] in a landmark experiment published in 1973. The authors showed that following the administration of exogenous CCK-8 to rats, the intake of both solid and liquid foods was suppressed in a dose related fashion

for up to three hours. The administration of CCK-8 was also associated with reduced meal sizes and a reduction in the time the rats spent consuming food. Finally the authors noted that the rats demonstrated behaviours that were suggestive of within-meal fullness (or satiation) and postulated that this was due to action on CCK receptors located in the brain. These findings have been replicated in numerous species including in humans. In studies of healthy volunteers (lean and overweight) administered physiological amounts of exogenous CCK-8[102-104], a reduction in meal size and subjective levels of hunger as assessed by Visual Analogue Scores proportional to the rate of infusion of CCK has repeatedly been demonstrated.

2.1.3.2 Ghrelin

Ghrelin is a 28 amino-acid peptide first isolated from the stomachs of rats in 1999. Ghrelin is primarily released from specialised endocrine cells found in the gastric mucosa but is also expressed by cells in the small intestine and CNS[105]. To become physiologically active, ghrelin undergoes a post-translational modification to its third amino acid –serine- residue[105] and it was as recent as in 2008 that the enzyme responsible for the acylation process was first reported[106]. Ghrelin's physiological effects on the central nervous[107] and gastrointestinal systems[105] are predominately mediated by its acylated form. Studies suggest its physiological effects are correlated when measured in either acylated or total form[108]. To date, ghrelin is still the only orexigenic gut hormone identified.

Ghrelin levels rise during the fasting period before rapidly falling after meal initiation and food consumption. The pre-prandial rise of ghrelin is thought to serve as a

physiological signal to initiate meal consumption and the subsequent fall proportional to the caloric and macronutrient content of the meal[109,110].

The acute and chronic administration of ghrelin to rats was found to increase their intake of food and weight[111,112] and similar effects have been reported in healthy human volunteers who received intravenous ghrelin and who then increased their food intake by +25% (kcals) when compared to a sham infusion[113].

Physiologically, ghrelin is thought to increase food intake by actions in the CNS; by stimulating the arcuate nucleus region of the hypothalamus and increasing the production of neuropeptide Y and agouti-related peptide[114]. It has other CNS mediated properties that include acting on the same hypothalamic receptors to reduce the utilisation of stored body fat[111] and on pituitary receptors to stimulate growth hormone production[105].

In studies of obese humans compared to normal weight controls, the obese display suppressed levels of fasting ghrelin[115,116] and an attenuated 24 hour ghrelin secretion profile[116]. In contrast fasting ghrelin and 24hr ghrelin profiles are increased in those with anorexia nervosa[116,117] compared to normal weight individuals.

In states of negative energy balance, long term prandial changes to ghrelin secretion have been reported with ghrelin secretion up-regulated. The opposite occurs in states of positive energy balance with post-prandial ghrelin secretion down-regulated[115]. These findings suggest that ghrelin has both a short and long term role in the

regulation of food consumption and in the physiological processes involved in body homeostasis and the maintenance of body weight.

The relationship between meals of varying macronutrient content and ghrelin secretion has been studied in rodents and in humans[118-121]. The studies report that high F meals suppress ghrelin secretion less than meals high in CHO or P.

Comparing CHO and P, studies in humans using a single meal test diet protocol have found that CHO is the most powerful macronutrient suppressing post-prandial ghrelin secretion[122-124]. However experiments using liquid drinks differing in their ratio of CHO to P to F have demonstrated only weakly that P suppressed post-prandial ghrelin secretion more than either CHO or F[67,74,125]. Finally other studies using liquid and solid meal protocols have failed to demonstrate differences between the macronutrients for post-prandial ghrelin secretion[126,122,123,127]. With this conflicting evidence it has recently been suggested that the signal that influences post-prandial ghrelin secretion does not arise directly from the macronutrient content of meals but rather is due to shifts in intestinal osmolarity arising from the passage of amino acids, glucose and fatty acids from intestine into the circulation of the gut[120].

It has also been suggested that the post-prandial secretion of insulin effects the post-prandial secretion of ghrelin[109] but the evidence to support this hypothesis remains limited to few studies reporting conflicting results[121,128].

2.1.3.3 Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is a 30 amino-acid peptide that is released from the L-cells of the distal gut in response to sensed nutrients (in particular CHO[129]) in mixed solid / liquid meals[130]. GLP-1 is synthesised by the L-cells in two forms that undergo post-synthesis cleavage at their N-terminals to produce two active forms: GLP-1 $_{7\text{-}37\ \text{amide}}$ and GLP-1 $_{7\text{-}36\ \text{amide}}$ with the -$_{7\text{-}36\ \text{amide}}$ the most biologically active isoform[131]. Within 30 minutes of the ingestion of a meal, GLP-1 levels peak remaining elevated for over 120 minutes[130]. GLP-1 exhibits physiological effects on satiety, meal termination and post-prandial insulin secretion.

GLP-1 exerts this activity through the GLP-1 receptor, which is expressed throughout the CNS, gut and other peripheral tissues[132]. The central GLP-1 receptors located in the hypothalamus and brainstem play a role in inhibiting food intake while receptors located in other tissues are involved in meal termination, satiety and gastric emptying[133]. The latter effects have been demonstrated in rodents and humans from studies that exogenously administered GLP-1 and examined food consumption[134,135] with the studies demonstrating a dose dependent relationship between the rate of the GLP-1 infusion and food intake.

GLP-1 is also a powerful stimulator of pancreatic β cell mediated post-prandial insulin release[129] and it is due to this property that GLP-1 was historically classified as belonging to a group of gut-derived hormones known as *incretins*. These *incretin* effects are demonstrated by the efficacy of the novel Type 2 Diabetes mellitus (T2DM) medications: *Exenatide* and *Liraglutide;* both belonging to the same pharmacological

class and whose main action is to activate the GLP-1 receptor[136]. These agents have been widely studied in randomised, placebo controlled clinical trials in T2DM prior to their global release and not only did the agents demonstrate improved glycaemic control but their use was also associated with increased satiety, reduced food intake and clinically significant weight loss[136].

The effects of dietary P, either compared to meals made entirely from the other macronutrients or consumed as differing %P ratios, on post-prandial GLP-1 secretion remains unclear with the few studies reporting conflicting findings.

In one of the earliest studies on GLP-1, *Elliot et al*[137] examined the effects of consuming isocaloric meals consisting entirely of CHO, F or P on post-prandial GLP-1 secretion and reported that there were no significant differences for post-prandial GLP-1 secretion between the meals. More recently in 2009, *Veldhorst et al*[71] compared breakfasts differing in % P (CHO | P | F: 55| 10| 25 vs. 55| 25| 35) on post-prandial GLP-1 secretion. As in the earlier study, the authors reported that varying dietary %P had no significant effects on post-prandial GLP-1 secretion. In a third study, *Diepvens et al*[75] compared different types of dietary P (100% plant based vs. mixed plant and animal-based) that were consumed as pre-meal liquid drinks on post-prandial GLP-1 secretion and subjective assessments of satiety. They also failed to demonstrate any link between the different P pre-loads and post-prandial GLP-1 secretion or to satiety.

In contrast *Bowen et al*[67] examined the effects of consuming pre-meal liquid snacks made entirely of the different macro-nutrients on post-prandial GLP-1 secretion, the *ad libitum* consumption of a standardised meal and subjective satiety. The authors

demonstrated that compared to the 100% CHO pre-load; the 100% P pre-loads, regardless of whether they were animal, plant or mixed, all prolonged post-prandial GLP-1 secretion and reduced the caloric intake from the subsequent meal.

2.1.3.4 Insulin

Insulin is a well-studied 51 amino-acid peptide synthesised, packaged and secreted by pancreatic β cells primarily in response to the concentration of glucose in blood. Its major action is to regulate the concentration of glucose in serum through action on almost all the tissues of the body, mediated by binding to the insulin receptor on the surface of cells.

It is well known that insulin affects the concentration of serum glucose directly by inhibiting glycogenolysis and gluconeogenesis, by increasing transport of glucose into fat and muscle cells and by stimulating glycogen synthesis.

Insulin also moderates the body's use of the alternative fuel substrates of fat (by promoting the storage of triglyceride into fat cells and decreasing lipolysis) and protein (by facilitating intra-cellular transportation of amino acids and inhibiting protein breakdown to support protein synthesis).

While virtually no insulin is produced in the brain, it is present in the cerebral circulation after transport through the blood brain barrier via a saturable, receptor mediated process[138]. Once present in the intra-cerebral circulation, insulin exerts an anorexigenic effect by acting on insulin receptors predominately distributed in the

hippocampus and hypothalamus[139]. This has been shown by studies that have demonstrated that the sustained intra-cerebral administration of insulin in rodents[140,141] and primates[142] leads to a dose-dependent reduction in food consumption and to body weight.

Circulating levels of insulin are predominately regulated by, and are highly sensitive to, changes in the concentration of glucose in the serum. However, basal insulin secretion is also influenced by body fat[143] and the strong relationship between body weight, abdominal adiposity, increased fasting serum glucose concentrations and elevated levels of fasting insulin, highlights the link between adiposity (in particular visceral fat) and insulin sensitivity[144].

2.1.4 Rationale for the experiment

The Human PLH trial was designed to the test the validity of the *Protein Leverage Hypothesis* in a healthy population. In its overall design, the Human PLH trial allowed for the testing of three associated hypotheses: that the key gut and fat hormones impacting on appetite and satiety are in turn influenced by the macronutrient composition of the diet; that diets higher in protein (P) are associated with higher levels of the hormones that increase satiety and lower levels of the hormones that reduce satiety when compared to lower P diets; and that diets higher in P are associated with higher levels of subjective satiety. The author was an associate investigator for the Human PLH trial and had direct responsibility for the design and implementation of the experiment that tested the associated hypotheses.

The National Health and Research Council provided funds for this clinical trial: Research Project Grant no. 457522 *"Testing the Protein Leverage Hypothesis in Humans or, can humans lose weight by eating a diet with a higher percentage of protein"*.

2.2 MATERIALS AND METHODS

2.2.1 Study participants

Participants were recruited through advertising placed on the casual employment websites of five Sydney based tertiary academic institutions: Macquarie University, the University of New South Wales, the University of Sydney, the University of Technology Sydney and the University of Western Sydney. While advertising was placed in other forms of media, it did not result in any successful participant recruitment.

To be eligible for the study participants had to be healthy, not be taking regular prescribed medications, have a normal body weight (defined as BMI= 18.5 – 24.9 kg/m^2) and have had at least three months of a stable body weight and be on no diet for weight loss. Participants had to be willing to follow the Human PLH trial study protocols.

Volunteers who were pregnant or had any form of Diabetes Mellitus were automatically excluded. Specific exclusion criteria included these chronic medical conditions: inflammatory bowel disease or other gastro-intestinal disorders, hypertension, dyslipidaemia, thyroid disease, asthma and chronic kidney or hepatic disease.

Vegans and vegetarians and those with a strong dislike of any of the study foods or food allergies were also automatically excluded. Potential participants then completed

the Eating Attitudes Test-26 questionnaire[145] and excluded if they revealed a previous history of an eating disorder, displayed current disordered eating habits or scored higher than 20 (which correlates with increased risk of disordered eating) on the questionnaire.

All participants were given detailed verbal and written information regarding the design and purpose of the study and its protocols. All participants provided their written, informed and personally signed and dated consent prior to commencing any study related matters. *"Testing the Protein Leverage hypothesis in humans"* was approved by the human ethics committees of the University of Sydney: Protocol ID No. 10153 and the Sydney South West Area Health Service: Royal Prince Alfred Hospital: Protocol ID No. X07-0044.

Eighty-one people (53 females and 28 males) responded to the advertising and attended screening interviews. From these eighty-one, thirty (20 female and 10 male) signed informed consent forms and were placed onto the waiting list for the investigators to organise a start time for their involvement in study.

From these thirty, three females withdrew before they commenced the study due to logistical difficulties, extreme phlebotomy-related phobia and inter-current illnesses. Three males and two females who commenced their first study diet treatment were subsequently withdrawn; one male was newly diagnosed with overt hyperthyroidism, another male expressed a previous unrevealed extreme dislike of the trial foods and the remainder were due to logistical difficulties. The results from these five individuals have not been included in any of the following analyses.

Here we report on the analysed results from twenty-two participants (15 female and 7 male) who enrolled in the Human PLH trial and completed all of the study protocols. The twenty-two participants were aged 24.7 ± 1.4 years (mean ± SEM; range 18-51 years) and had a mean BMI of 21.8 ± 0.4 (17.7 – 25.2) kg/m^2

2.2.2 Study design and experimental protocols

The primary outcome was change in total energy intake after 4 days of *ad libitum* diet. Participants attended for three 4 day trial periods (diet treatments) that consisted of in-house covert dietary manipulation Monday to Friday culminating in the Fixed Energy Meal Experiment on the Friday morning. Each diet treatment was separated by at least one 1 week washout period where participants were instructed to return to their regular pre-study eating habits.

Participants were placed into single-sex groupings of two to four participants with each study group remaining together throughout the whole experiment. Groups of participants resided in the long-stay facilities of the Woolcock Medical Research Institute (Glebe, NSW).

Prior to commencing their first residential diet treatment period, participants attended the Endocrine and Metabolism Unit, Royal Prince Alfred Hospital for the measurement of their resting metabolic rate and for the collection of baseline blood and urine samples.

The resting metabolic rate was determined by assessing each participant's oxygen and carbon dioxide exchange while they rested (awake) for 60 minutes underneath a plastic canopy attached to the indirect calorimeter (Parvo Medics True One 2400-Metabolic Analyser System; software version OUSW 4.3 (20080219), USA). This allowed the investigators to calculate the energy requirements of each participant accurately.

During each diet treatment, participants consumed *ad libitum* diets matched for palatability and variety but differing in % energy intake by P (% Energy Intake (EI) as P: 10% P, 15% P or 25% P) in a random order. For example during the first diet treatment one group consumed food contained 10% P; for the next diet treatment, they consumed food that contained 25% P and for the last diet treatment, all of the consumed food contained 15% P. The order of three diet treatments was randomly determined by computer program prior to the group commencing their first treatment.

Food was prepared in advance of each diet treatment by Human PLH trial staff working in the kitchens located in the Old Teacher's College, University of Sydney. Palatability was assessed on day 4 of each diet treatment to ensure that this was kept consistent across each study period and throughout the trial. Food was kept refrigerated or frozen until required.

The participants' daily *ad libitum* food menu was made up of three main meals and free access to sweet and savoury snacks throughout the day. The diet treatments followed the same menu of 28 food items, which included 12 sweet and 16 savoury foods as has been reported earlier[146]. During the diet treatments, participants were

instructed to only consume foods provided by the study investigators and their food intake was measured by recording the weight of main meals before and after food service, by returning the snack wrappings and by the return of any uneaten food. Participants were instructed to consume 150 millilitres of skim milk per day and were provided with free access to chilled and boiled water, tea and decaffeinated coffee.

The energy values for the food was provided by reference to manufacturers' food labelling or by reference to the Nutrient Tables used in Australia (NUTTAB 2006, Food Standards Australia New Zealand[147]). In these tables the assigned energy value of the macronutrients was: protein 17 kJ/g, simple carbohydrates 16 kJ/g, complex carbohydrates 17 kJ/g and fat 37 kJ/g.

The % Energy from F was fixed at 30% for all three diet treatments. The % Energy from CHO and P was manipulated by the addition of maltodextrin (Polyjule, Nutricia Australia Pty Ltd) and/or 1:1:1 mix of whey protein, calcium caseinate and egg white powder (Whey, casein and egg white protein powders, Mycopure, Sydney, Australia). Food contained all essential amino acids at levels exceeding WHO requirements. **Table 2.1** describes the macronutrient content and standardised menu for the diet treatments.

Further and more detailed descriptions of the design and testing of the study menu and the process of dietary manipulation were reported in 2010[146] and in 2011[148].

Table 2.1 (a) % Energy Intake by macronutrient for each diet treatment

% Protein	% Carbohydrates	% Fat
10	60	30
15	55	30
25	45	30

Table 2.1 (b) Weekly & standardised menu for the diet treatments

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
BREAKFAST FASTING	**BREAKFAST** Apricot Muesli Pear, Raspberry and Coconut Bread Savoury Breakfast Muffins	**BREAKFAST** Raspberry Muesli Banana Bread Savoury Breakfast Muffins	**BREAKFAST** Apricot Muesli Pear, Raspberry and Coconut Bread Savoury Breakfast Muffins	**BREAKFAST** Raspberry Yoghurt
LUNCH Tuna Bake Beef and Vege pastry Green Salad mix fruit salad yoghurt	**LUNCH** Savoury Mince and rice wrap Teriyaki Chicken Sushi Green salad mix apple crumble muffins	**LUNCH** Tandoori Chicken Wrap Beef and Vege Pastry Green Salad mix fruit salad yoghurt	**LUNCH** Pasta Salad Sweet Potato and Ricotta Wrap apple crumble muffins	**LUNCH** END OF DIET TREATMENT
DINNER Goulash Cheese Scones Green salad mix Orange and Poppyseed cake Custard	**DINNER** Chow Mein Mince Mushroom Pasta Choc, Apple & Ricotta Cake Custard	**DINNER** Pasta Bolognaise Cheese Scones Green salad mix Orange and Poppyseed cake Custard	**DINNER** Hokkien Noodles Beef Massaman Curry Choc, Apple & Ricotta Cake Custard	**DINNER**
SNACKS carrot cake savoury scones	**SNACKS** raspberry yoghurt cheese scones	**SNACKS** apricot muffins savoury scones	**SNACKS** raspberry yoghurt cheese scones	**SNACKS** NOT AVAILABLE

2.2.3 The Protocol for the Fixed Energy Meal Experiment

The Fixed Energy Meal Experiment was conducted on the Friday morning at the end of the week of each diet treatment and involved study subjects consuming a standardised breakfast and for the next 180 minutes having their blood collected whilst completing a standardised appetite questionnaire.

2.2.3.1 Breakfast

The subjects were asked to fast from Friday midnight. Prior to their waking, the study investigators prepared a standard breakfast with a fixed Energy content (0.3 X Resting Metabolic Rate) for each participant. The macronutrient composition (by % Energy) of the breakfasts differed according to the current experimental week diet treatment of the subjects. Between 0800 - 0845 the subjects were instructed to consume all of their breakfast within 15 minutes and afterwards, not to eat or drink anything further for the remainder of the Fixed Energy Meal Experiment.

Breakfast itself consisted of a fruit and yoghurt mix made up of packaged raspberries (Coles Brand, Coles Pty Ltd, Australia), vanilla yoghurt (Yoplait Creamy Lite, Lion Dairy & Drinks Pty Ltd, Australia). The %P was varied by the addition of different quantities of the 1:1:1 whey: casein: egg white protein powder mix (Mycopure, Sydney, Australia). Breakfast was accompanied by a glass of water. Photographs illustrating the Fixed Energy Meal breakfasts are show in ***Figure 2.2*** below.

Figure 2.2 Breakfast as part of the Fixed Energy Meal Experiment

2.2.3.2 The Appetite Visual Analogue Scale

Visual Analogue Scale (VAS) instruments for appetite have been validated[56] and accepted and generate reproducible results[149]. They are used in clinical research as a means of subjectively assessing study subjects' appetite, hunger, taste, and their enjoyment of and desire to consume food.

A four question Appetite VAS was used to subjectively assess the participants' sense of hunger, fullness (satiety), desire to eat (appetite) and prospective food consumption.

The four questions asked in the Appetite VAS were:

"1. *How hungry are you?*"

"2. *How full do you feel?"*

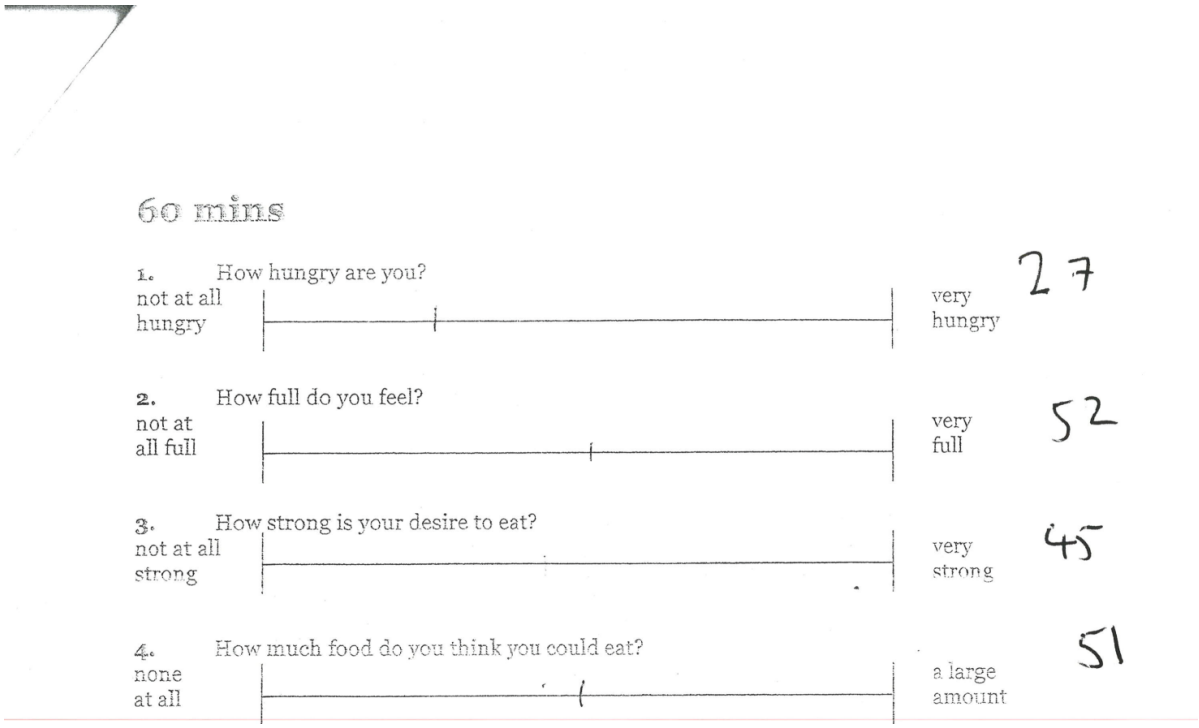
"3. *How strong is your desire to eat?"*

"4. *How much food do you think you could eat?"*

Below each question was a 100mm response line. Each response line was anchored at the 0 mm end with the one extreme (e.g. "not at all hungry") and at the 100 mm end with the opposite extreme (e.g. "very full").

Participants completed the Appetite VAS by placing a mark on each of the four response lines. They were instructed to complete the questionnaire before the start of breakfast and then at 30, 60, 120 and 180 minutes post-breakfast. A copy of a completed 60-minute Appetite VAS, representative of all the five Appetite VASs used in this experiment is presented in ***Figure 2.3*** below. Each set of Appetite VAS questions were completed immediately prior to the collection of participants' blood.

Figure 2.3 A completed 60-minute Appetite VAS



NB**:** The scoring of the completed Appetite VAS was done at the end of the experiment.

2.2.3.3 Phlebotomy

10 minutes before the start of breakfast, an intravenous cannula was inserted into each participant's antecubital space or posterior aspect of the hand. Sixteen mL of blood was collected within 5 minutes of commencing breakfast and the same volume again collected at 30, 60, 120 and 180 minutes. The experiment was terminated at 180 minutes after which participants had their intravenous cannula removed, an occlusive dressing applied and they were provided with a transport allowance to return home.

2.2.4 Collection and processing of blood samples

The 16 mL of blood collected was used for the measurement of the fasting and post-prandial levels of cholecystokinin, total ghrelin, active GLP-1, insulin, urea and glucose.

For plasma 4 mL of whole blood was placed into each of three K2EDTA BD Vacutainers (Becton, Dickinson and Company, Sydney, Australia) while for serum 4 mL of blood was placed into a SSTII *Advance* with gel separator BD Vacutainer.

The 4 mL of blood collected for CCK was immediately transferred into a BD K2EDTA Vacutainer that was pre-loaded with 2000 kIU of Aprotinin (500 kIU per 1mL whole blood; as *Trasylol*; Bayer HealthCare, NJ, USA). This was added to the tube to prevent the rapid degradation of CCK.

The 4 mL of blood collected for GLP-1 was immediately placed in a BD K2EDTA Vacutainer that contained 40 *μL* of dipeptidyl peptidase-IV inhibitor (10 *μL* per 1mL whole blood; Millipore Corp, Billerica, MA, USA). This was previously added to the BD K2EDTA tube to prevent rapid GLP-1 degradation.

The BD vacutainers containing additives were kept frozen at -20^{o}C until the morning of the experiment and removed from the freezer only immediately before phlebotomy.

Collected samples were immediately stored at 4^{o}C. Within 10 minutes of collection, samples were centrifuged for 15 minutes at 3000*g* 4^{o}C. Collected serum and plasma

were aliquotted into 1.5mL micro-centrifuge tubes (Eppendorf South Pacific Pty Ltd, Sydney, Australia) that were kept frozen at -80^{o}C until analysis. All serum and plasma samples were subjected to fewer than five freeze/thaw cycles and all of the collection and preparation of blood samples was carried out by the author.

2.2.5 Biochemistry measurements

Stored serum was used to measure fasting and post-prandial glucose, serum urea, total cholesterol and triglycerides by an immuno-chemiluminometric technique on an automated Architect ci16200 platform (Abbott Diagnostics, Il, USA). Stored plasma was used to measure fasting and post-prandial levels of free fatty acids by enzymatic colorimetric technique on the automated COBAS-MIRA platform (Roche Diagnostics Ltd, West Sussex, UK). These tests were kindly performed by scientists in the Biochemistry Laboratory of Royal Prince Alfred Hospital, NSW.

2.2.6 Radioimmunoassays

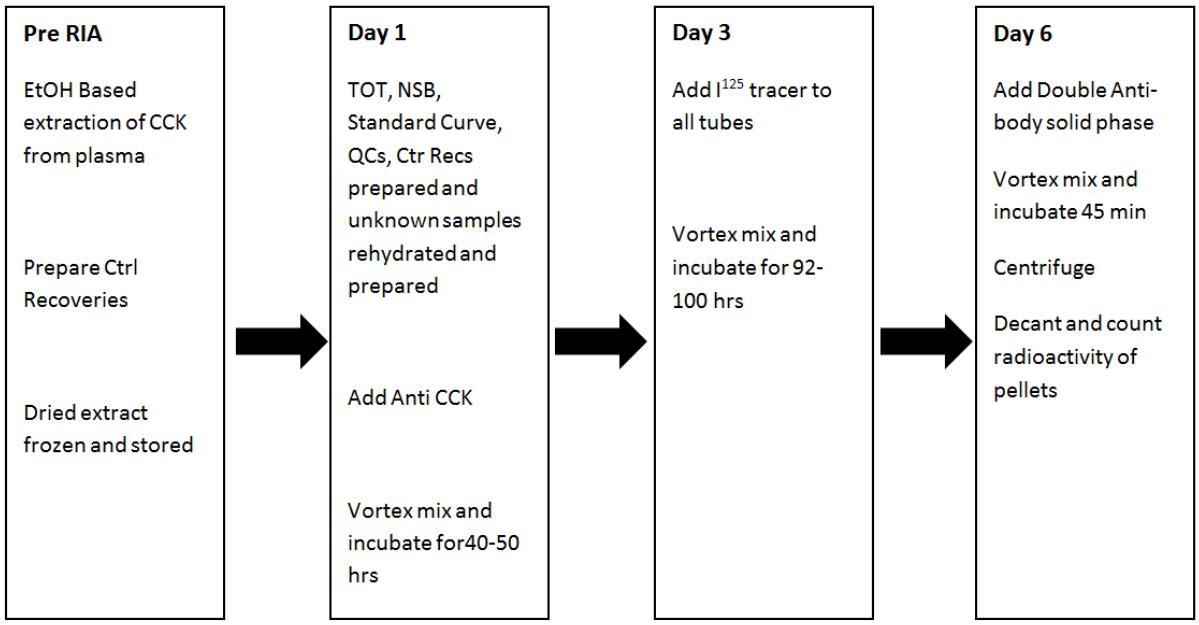
Fasting and post-prandial levels of CCK, total ghrelin, GLP-1 and insulin were all measured by manual radioimmunoassay (RIA) technique. All control and unknown samples were measured in duplicate. Over 12 months from early 2010, the author performed all of these RIAs in the Endocrinology Laboratory of Royal Prince Alfred Hospital, NSW.

The principle of the RIA technique and list of the equipment use is presented in the **Appendix: Item A.**

2.2.6.1 The cholecystokinin RIA

The Euria CCK RB 302 radioimmunoassay kit (Euro-Diagnostica AB, Malmo, Sweden) was used to measure levels of CCK from the stored plasma containing *Trasylol*. As per the Euria-Diagnostica CCK Product Information Statement (PIS)[150] the assay is based on antiserum with a very low cross-reactivity to gastrin and optimised to a very high sensitivity of 0.3 pmol/L. The kit has little cross-reactivity to non-sulphated forms of CCK or to gastrin (0.01-0 .5%). The CCK assay requires two steps; the first being the extraction of CCK from the frozen plasma samples, the second being the RIA of the unknown CCK extracts. As per the PIS, the intra assay coefficient of variation was 5.5% for 4.4 pmol/L CCK and 2.0% for 20.6 pmol/L CCK while the inter assay coefficient of variation was 13.7% for 4.2 pmol/L CCK and 4.1% for 20.6 pmol/L CCK. ***Figure 2.4*** outlines the CCK RIA procedure

Figure 2.4 *Outline of the CCK RIA*



Reagents

Anti-CCK, CCK 50mpl/L standard, Quality Controls (QCs) high and low and iodinated CCK were reconstituted as per the manufacturer's recommendations[150]. When not in use the reconstituted reagents were kept frozen at -20°C. The ready-to-use diluent and double-antibody solid phase solutions were kept refrigerated at 3.5°C. Unless specified antigen, standards, quality controls and iodinated CCK were thawed out at room temperature prior to use.

Extraction of CCK from stored Plasma

The ethanol-based extraction process was performed to the PIS. Frozen samples of plasma were thawed in an ice-bath kept below 4°C. 1000 μL of thawed plasma was then transferred into a borosilicate glass tube, which had been previously placed in the same ice-bath. 2000 μL of 96% EtOH was then added to each borosilicate glass tube with the mixture vigorously vortex mixed for 20 seconds. The EtOH - plasma mixture was removed from the ice bath and allowed to stand at room temperature for a further 10 minutes. This was then placed into a centrifuge at 2000g at 20°C for 15 minutes. The resulting supernatant was carefully decanted into another borosilicate glass tube and placed in a 36-well heating block kept constant at 37°C. The extract was evaporated to dryness under a continuous nitrogen stream with the dried extracts either immediately used in a CCK RIA run or kept frozen at -80°C until use.

Preparation of Recovery Controls

To assess the recovery of plasma CCK under our experimental conditions, two recovery controls (Ctr Rec) of known CCK concentration were prepared and assayed beside the extracts of unknown CCK.

A %Recovery of control of >50% was required to ensure assay validity. % Recovery of control was calculated by the following formula.

$$\% \text{ Recovery control} = \frac{(\text{pmol/L found with CCK standard} - \text{pmol/L found with diluent}) \times 100}{10}$$

The first Ctr Rec was made up of 200 *μL* of the 50 pmol/L CCK standard added to 800 *μL* of donor plasma that had been collected without the additive *Trasylol*. This Ctr Rec had a known concentration of CCK of 10 pmol/L. The second Ctr Rec was made up of 200 *μL* of diluent added to 800 *μL* of donor plasma (again without additive) that produced a sample with a known concentration of CCK of 0 pmol/L.

The mean recovery of CCK from the assay runs was 80%.

CCK Radioimmunoassay

The duration of a RIA run for unknown CCK extracts was 6 days. On the first day, dried plasma CCK extract was rehydrated with 1000 *μL* diluent, vortex mixed and left to stand on the bench for at least 30 minutes prior to the assay. Tubes were consecutively numbered, labelled and prepared for total count (TOT), non-specific binding (NSB), blank, the standard curve of known CCK concentrations (0.78, 1.56, 3.12, 6.25, 12.5, and 25 pmol/L), low QC (1.8-2.8 pmol/L CCK), high QC (9.9 – 13.1 pmol/L CCK), recovery controls and unknown samples. 700 *μL* of diluent was transferred into the NSB tube and 200 *μL* into the blank tube by manual pipette. 200 *μL* of standard, QCs, recovery controls and unknown samples were transferred into their corresponding tubes by manual pipette. 500 *μL* of Anti-CCK was added to all tubes by multi-pipette except to the total count and non-specific binding tubes. The tubes were vortex mixed, covered and placed in the 3.5°C refrigerator to incubate at 2.5°C for 44-48 hours.

On day three, 500 *μL* of tracer (Iodinated CCK) was added to all tubes using an *Eppendorf* multi-pipette. The tubes were then vortex mixed, covered and placed in the 3.5°C refrigerator to incubate for a further 92-96 hours.

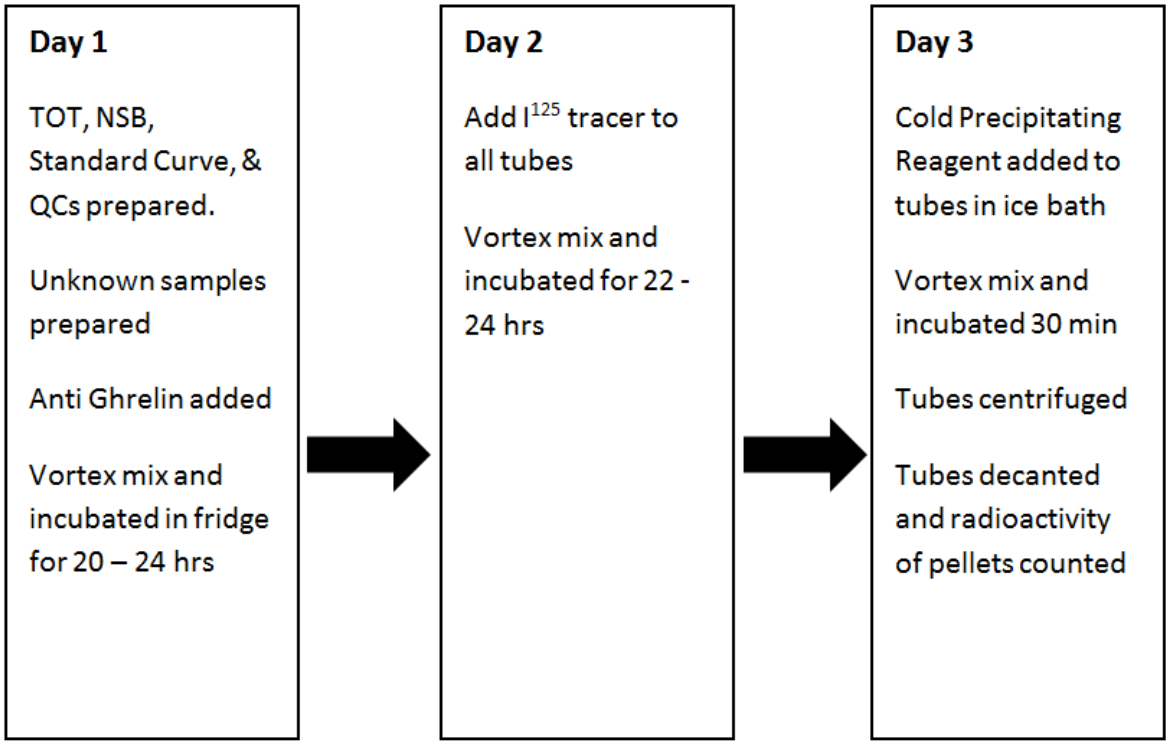
On day six, 100 *μL* of the double antibody solid phase solution was added to all tubes except the TOT tube by manual pipette. This reagent had to be placed onto a magnetic stirrer to ensure it was constantly mixed. The tubes were vortex mixed, covered and left to incubate in the 3.5°C refrigerator for 45 minutes. The TOT tube was placed aside and all remaining tubes put into the HD Scientific / Hettich Zentrifuger Rotixa 50RS refrigerated centrifuge and centrifuged at 4000*g* for 20 minutes at 4°C. The

supernatant was decanted from the centrifuged tubes by inverting them onto absorbent paper. Great care was taken not to dislodge the pellet at the bottom of each tube. All tubes were then placed into the automatic gamma counter and the CCK specific program (#18), which produced standard curve, QC and Ctr Rec and the assay results used. The % recovery control was calculated with all runs exceeding 50%.

2.2.6.2 The total ghrelin RIA

The GHRT- 89HK Total Ghrelin RIA kit (Millipore Corp, Billerica, MA, USA) was used to measure total Ghrelin in stored plasma. As per the manufacturer's PIS[151], the kit utilises a highly specific antibody for total ghrelin that has no cross-reactivity to glucagon, leptin or insulin. With a 100 μL unknown sample, the assay achieves a sensitivity of 27.6 pmol/L and recoveries of >90% are obtained when known concentrations of ghrelin are added to plasma. The coefficient of variation was 3.3 - 10% (intra-assay) and 14.7 – 17.8% (inter-assay) for concentrations of (total) ghrelin of 296.7 – 889.9 pmol/L. Each RIA run was carried out as a continuous procedure over three consecutive days. ***Figure 2.5*** outlines the ghrelin RIA

Figure 2.5 The outline of the ghrelin RIA



Reagents

When not immediately used, rehydrated reagents were kept frozen at -20°C. Prior to their use the reagents of iodinated Ghrelin, quality controls, and standards were thawed at room temperature. The ready-to-use assay buffer, antibody and precipitating reagent were all stored at 3.5°C when not in use.

Total Ghrelin Radioimmunoassay

On the morning of Day 1 of the RIA, the frozen plasma samples were thawed at 3.5°C together with the stored standard curve samples and QCs. Pointed polypropylene

tubes were consecutively numbered and labelled for TOT, NSB, blank, the standard curve of known (total) ghrelin concentrations (32.5, 64.9, 129.8, 259.6, 519.1, 1038.3 and 2076.5 pmol/L), the low QC (110.7 – 229.6 pmol/L), the high QC (260.8 – 541.4 pmol/L) and unknown samples. Assay buffer was transferred into the NSB tube (300 μL) and blank tube (200 μL). 100 μL of assay buffer was transferred into each of the tubes for the standard curve, QCs and unknown samples. 100 μL of standard, QCs or unknown samples were transferred into their corresponding tubes by manual pipette. 100 μL of ghrelin antibody was added to all tubes except the TOT and NSB. Tubes were vortex mixed, covered and incubated in the refrigerator at 3.5°C for 20 - 24 hours.

On the second day, 100 μL of iodinated Ghrelin was added to all of the tubes. The tubes were then mixed, covered and incubated at 3.5°C for 22 - 24 hours.

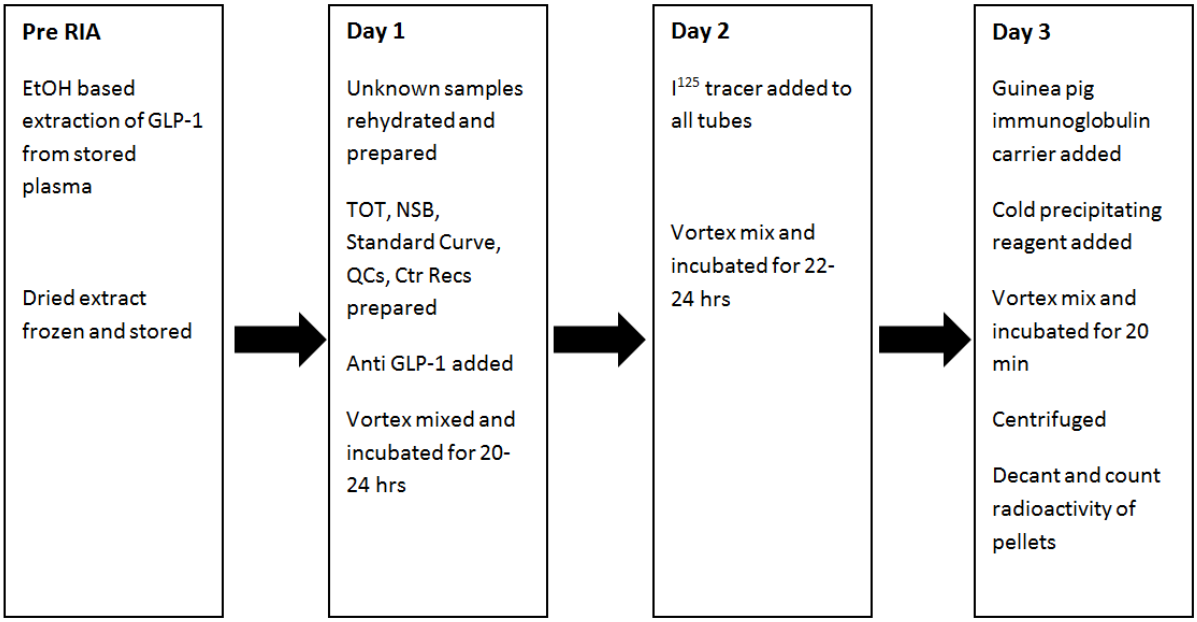
On the third day, 1000 μL of cold precipitating reagent was added to all tubes, which had been placed in an ice bath at <4°C, except for the TOT, which was placed aside. The tubes were vortex-mixed and incubated for 20 minutes at 3.5°C. These tubes were then centrifuged at 3000*g* for 20 minutes at 4°C (HD Scientific / Hettich Zentrifuger Rotixa 50RS refrigerated centrifuge). The supernatant was decanted by inverting the tubes onto absorbent paper. All of the tubes were then placed into the automatic gamma counter and the total ghrelin specific program (#19) run, which produced results for the standard curve, QCs and the unknown samples.

2.2.6.3 The active glucagon-like peptide-1 RIA

The Millipore GLP-1A-35HK kit (Millipore Corp, Billerica, MA, USA) was used to measure the levels of active GLP-1 from stored samples of plasma containing the additive: *DPP-IV inhibitor*. As per the manufacturer's PIS[152], when 300 *μL* of extracted plasma is processed the assay achieved a sensitivity of 3 pmol/L. The assay is highly specific for both of the biologically active forms of GLP-1 (7-36 $_{amide}$ and 7-37$_{amide}$) but does not detect GLP-2, glucagon, insulin or gastro-intestinal-peptide. As per the PIS, the intra-assay coefficient of variation was 27 - 30% and the inter-assay coefficient of variation 12 - 34% for concentrations of GLP-1 of 14 - 40 pmol/L.

The first stage of the assay was an ethanol-based extraction of GLP-1 from stored plasma and the second stage was the RIA of the extract. Following the addition of known concentrations of GLP-1 to donor plasma, the extraction process resulted in 60-80% recovery of GLP-1. The extraction process was carried out over a single day and the RIA of the extract over 3 consecutive days. ***Figure 2.6*** outlines the active GLP-1 RIA.

Figure 2.6 The outline of the active GLP-1 RIA



Reagents

Iodinated GLP-1 was rehydrated with the provided assay buffer and iodinated GLP-1 and the ready-to-use reagents of anti-GLP-1, standards and QCs were all frozen at -20°C when not in use. The ready-to-use assay buffer, precipitating reagent, guinea pig carrier and sample rehydrating solution were stored at 3.5°c when not in use.

Extraction of GLP-1 from frozen plasma

Stored plasma was thawed in an ice bath (kept at <4°C) and 300 *µL* of plasma used for each extraction. 1100 *µL* of 96% EtOH was added to each microfuge tube, which were then capped, inverted and vortex mixed before being incubated in the ice bath for 30 minutes. At the end of incubation the microfuge tubes were again inverted and centrifuged at 5800*g* for 10 minutes at room temperature. For the final step of the

extraction process, the supernatant was carefully decanted into pre-labelled borosilicate glass tubes and then placed into a 36-well heating block at 37°C. Under a continuous nitrogen stream the extracts were dried completely. Any dried extract not immediately used in a GLP-1 RIA run was stored at -80°C until use.

Active GLP-1 Radioimunoassay

On the first day dried extract was rehydrated by the addition of 300 *µL* of sample rehydrating solution. These tubes with rehydrated samples were incubated in an ice bath (at <4°C) for 30 minutes and then gently vortex mixed.

Borosilicate glass tubes were labelled for TOT, NSB, blank, for the standard curve (5, 10, 20, 50, 200, 500 pmol/L), low QC (29-61 pmol/L), high QC (175-364 pmol/L) and for the rehydrated samples with unknown levels of GLP-1. Assay buffer was added to the following tubes as follows: TOT and NSB 400 *µL*, blank 300 *µL* and standards and QCs 200 *µL*.

100 *µL*s of standards, 100 *µL*s of high and low QCs and 300 *µL*s of unknown GLP-1 extract were transferred to their corresponding borosilicate glass tubes. 100 *µL* of anti-GLP-1 was added to all of the tubes except for the TOT and NSB tubes. Afterwards, all of the tubes were vortex mixed, covered and incubated at 3.5°C for 20 – 24 hours.

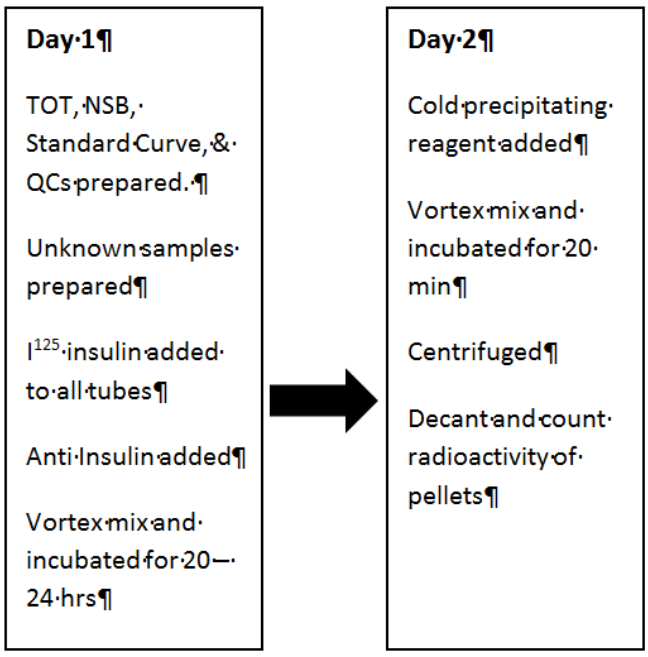
On the second day, 100 *µL* of iodinated GLP-1 was added to all of the tubes. All tubes were vortex mixed, covered and incubated in the 3.5°C refrigerator for 22 – 24 hours.

On the third day, 10 μL of guinea pig immunoglobulin carrier was transferred to all tubes except for the TOT. All tubes except for the TOT were placed into a ice bath (at <4°C) and 1000 μL of cold precipitating reagent added. All of the tubes were vortex mixed, covered and incubated at 3.5°C for 20 minutes. The TOT tube was put aside and the remaining tubes placed into the refrigerated centrifuge and spun at 4500*g* for 30 minutes at 4°C. After centrifuging, these tubes were carefully inverted and decanted before being placed onto absorbent paper. Great care was taken to not dislodge the pellet located at the bottom of each tube. All of the tubes were then placed into the automatic gamma counter and the active GLP-1 program (# 20) selected to run. This generated a standard curve, QCs and results for the unknown samples.

2.2.6.4 The insulin RIA

The Millipore HI-14K RIA kit (Millipore Corp, Billerica, MA, USA) was used to measure the levels of insulin from stored serum. According to the manufacturer's PIS[153] the assay sensitivity with 100 μL of sample was 12 pmol/L. The kit is highly selective for insulin and does not detect pro-insulin, glucagon, or insulin-like-growth factor 1. For insulin concentrations of 42 – 325 pmol/L, the intra-assay co-efficient of variation was 2.2 - 4.4% and inter-assay co-efficient of variation 2.9 – 6% with the measured recovery after the addition of known concentrations of insulin into serum from 93 - 100%. Each insulin RIA run was conducted over two consecutive days and ***Figure 2.7*** illustrates the insulin RIA.

Figure 2.7 The outline of the insulin RIA



Reagents

Prior to use the kit-provided iodinated insulin was rehydrated and together with the ready-to-use reagents of anti-insulin, standards and QCs kept frozen at -20°C when not in use. The ready-to-use reagents of the assay buffer and cold precipitating reagent were stored at 3.5°C when not in use.

Insulin Radioimmunoassay

100 μL of stored participant serum was thawed in the 3.5°C refrigerator while plastic pointed tubes labelled for TOT, NSB, blank, the standard curve (12, 30, 60, 120, 300, 600 and 1200 pmol/L), low QC (40.8 – 84.6 pmol/L), high QC (156 – 324 pmol/L) and for samples containing unknown levels of insulin. Assay buffer was added to the

following tubes as follows: 300 μL to the NSB tube, 200 μL to the blank tube and 100 μL for each of the standards, QCs and unknown tubes. Following this 100 μL of standards, QCs and unknown samples were added to their respective tubes. Afterwards 100 μL of iodinated insulin was added to all tubes followed by 100 μL of anti-insulin to all tubes except for the TOT and NSB tubes. All of the tubes were vortex mixed, covered and incubated at room temperature (22-25^{o}C) for 20-24 hours.

On the second day 1000 μL of cold precipitating reagent was added to all of the tubes except for TOT. The tubes were vortex mixed, covered and incubated at 3.5^{o}C for 20 minutes. The TOT tube was placed aside and the remaining tubes centrifuged at 4000g for 30 minutes at 4^{o}C. After centrifuging, the tubes were decanted by inversion and placed onto absorbent paper with great care taken to not dislodge the pellet at the bottom of each tube. All tubes were then placed into the automatic gamma-counter with the insulin specific program selected (#15) to run. Results were generated for the standard curve, QCs and samples which were then used.

2.3 DATA ENTRY AND STATISTICAL ANALYSES

Data was entered into a series of spread sheets using Microsoft Excel for Mac 2008 (version 12.3.1 build 110725). Following data entry a cleaning cycle was undertaken that involved cross-referencing the entered data with values from the original source material. Statistical analyses were performed with the IBM SPSS Statistics Version 19 software package (IBM Corp, NY, USA). All data entry and statistical analyses were carried out by the author.

Data was analysed for normative distribution by the Kolmogorov–Smirnov test. Parametric data is reported as mean ± Standard Error Mean (SEM) while non-parametric data as median with the inter-quartile range (IQR). All graphed data is presented as mean ± SEM unless specified. Missing data for CCK and GLP-1 was corrected by multiple imputation data analyses using the Marcov Chain Monte Carlo method. Results were considered significant if $p < 0.05$ except when noted. Twenty participants gave 80% power ($\alpha = 0.05$) to detect a 10% change in EI after 4 days of ad libitum diet treatments.

The Area Under the Curve or AUC (a superior comparator of serial time points than point-to-point or peak to trough[154]) is calculated by the trapezoidal rule, which approximates the region under a graph of the function *f(x)* as a trapezoid and calculates the area of the trapezoid. As the length of the Fixed Energy Meal experiment was 180 minutes, the AUC was defined as the sum of the trapezoids between 0 to 180 minutes.

$$\int_a^b f(x)\,dx \approx \frac{h}{2}\sum_{k=1}^{N}\left(f(x_{k+1}) + f(x_k)\right).$$

AUCs were calculated by using a user-defined formula add-on package within Microsoft Excel corresponding to the above equation.

AUCs were calculated for each of questions from the Appetite VAS; for each of the following hormones: CCK, total ghrelin, GLP-1 and insulin, and for the following biochemistry measures: urea, Blood Glucose Level (BGL), total cholesterol, triglycerides and free fatty acids. Comparisons were made between the results whilst on the 10% P, 15% P and 25% P diet treatments.

For parametric data, within-subject analysis was carried out using one-way within subject analysis of variance with repeated measures (ANOVA-RM). Data was checked with sphericity using the Mauchlys sphericity test and if sphericity was violated, the *Greenhouse-Geiser* correction was applied to the *F* and p-values. *Post-hoc* analyses used the *Bonferroni* correction method for multiple, pair-wise comparisons.

For non-parametric data, within-subject differences were analysed using the Freidman test. *Post-hoc* analyses were performed on significant results through multiple pair-wise Wilcoxon signed-rank tests with the *Bonferroni* correction applied to these results. They were considered significant only if they met their revised alpha level.

To evaluate the relationship between insulin and total ghrelin, Spearman rank order coefficients were calculated based on 15 pairs of data (5 time points, 3 treatments) and between the AUCs for each variable based on 22 observations (22 subjects).

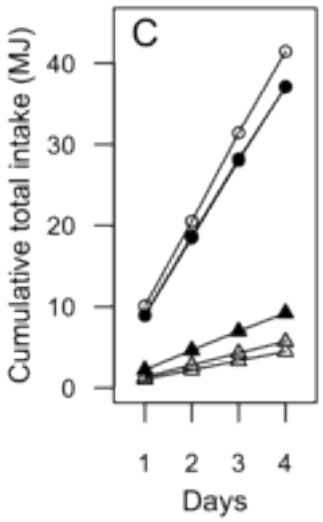
2.4 RESULTS

2.4.1 Four days of ad libitum feeding of diets differing in % P

The Fixed Energy Meal Experiment was a component of the larger project designed to test the *Protein Leverage Hypothesis* in humans with this author a sub-investigator in that larger project. This author's duties included: assisting in the recruitment and screening of subjects, supervising participants in the long-stay facility, implementing experimental protocols, collecting blood samples and performing hormone analyses; manuscript drafting, editing and revision. This author is recognised as a co-author of the initial article arising from the larger project: *"Testing Protein Leverage in Lean Humans"*[148]. The results from the overall project are essential in providing context for the results of the Fixed Energy Meal Experiment and are summarised below.

The total EI and specific macronutrient intake over the four-day study period differed according to the diet treatments. EI was highest on the 10% P diet while P intake was lowest. ***Figure 2.8*** demonstrates the changes to total energy intake and P intake on the 10%, 15% and 25% P diet treatments.

Figure 2.8 Lean humans increase energy consumption on a lower percent protein diet



Cumulative P (triangles) and total energy (circles) intake (MJ) for participants during the 4-day 10% P (white), 15% P (grey) and 25% P (black) *ad libitum* study periods.

NB: From *Gosby et al*[148] with the kind permission of the author.

The four-day EI (as Megajoules MJ) for the 10% P | 15% P | 25% P diet treatments were 41.5 ± 2.4, 37.1 ± 2.1, 37.1 ± 2.2 MJ and significantly different (F (2,42) = 6.7; p = 0.002). *Post-hoc* analyses demonstrated that the greatest difference was between the 10% P vs 15% P diet treatments (p <0.0001) though 15% P vs. 25% P was also significantly different (p = 0.0003).

The Four day Protein Intake (as MJ) for the 10%P | 15%P | 25%P diets were: 4.5 ± 0.3, 5.7 ± 0.3, 9.2 ± 0.5 MJ and also significantly differed (F (2,42) = 125.6; p <0.0001). *Post-hoc* analyses showed differences between the intakes of 10% P vs 15% P, 15% P vs 25% P and 15% P vs 25% P (all p <0.0001).

When the 10% P diet treatment was compared to the 15% P diet treatment, participants consumed 4.34 MJ more energy with a net result of a 3% reduction in the intake of P (-1.24 MJ EI from P) and a 15% increase in the intake of carbohydrates and fat (+5.59 MJ EI from CHO+F).

Comparing the 10% P diet treatment to the 25% P diet treatment, participants consumed 4.38 MJ more energy with this the net result of a 12% reduction in the intake of protein (-4.47 MJ EI from P) and a 25% increase in the intake of carbohydrates and fat (+9.15 MJ EI from CHO+F).

With the increase from the 15% P to 25% P diet treatment, the macronutrient intake altered with an increase in the EI by P of +3.5 MJ (15% P vs. 25% P: p <0.0001) that was balanced by a reduction in the EI by CHO+F of -3.57 MJ (15% P vs. 25% P: p <0.0001). This resulted in no change to the net total EI (15% P vs. 25 P%: $p = 1$).

Changes in food intake were evident by the third day, but did not reach significance until the fourth day. No changes to body weight were noted.

In summary the results from the overall experiment demonstrated increased energy intake on the diets that contained lower % P, which was consistent with the *Protein Leverage Hypothesis*. However, no differences in the EI between the medium and high P diets nor changes to body weight and satiety were found and the duration of the study may have been insufficient to test these aspects of the *PLH*.

2.4.2 Fixed Energy Meal Experiment: Appetite Visual Analogue Scores

For Appetite VAS Questions 1 – 4 the results according to diet treatments are presented in ***Figures 2.9.1 - 2.9.4***. All graphed data is presented as mean ± SEM unless specified otherwise.

The results of the baseline and AUCs analyses of VAS Question 1 - 4 are presented in **Table 2.2**.

Figure 2.9.1 Appetite VAS Question 1 (Hunger) per %P diet treatment

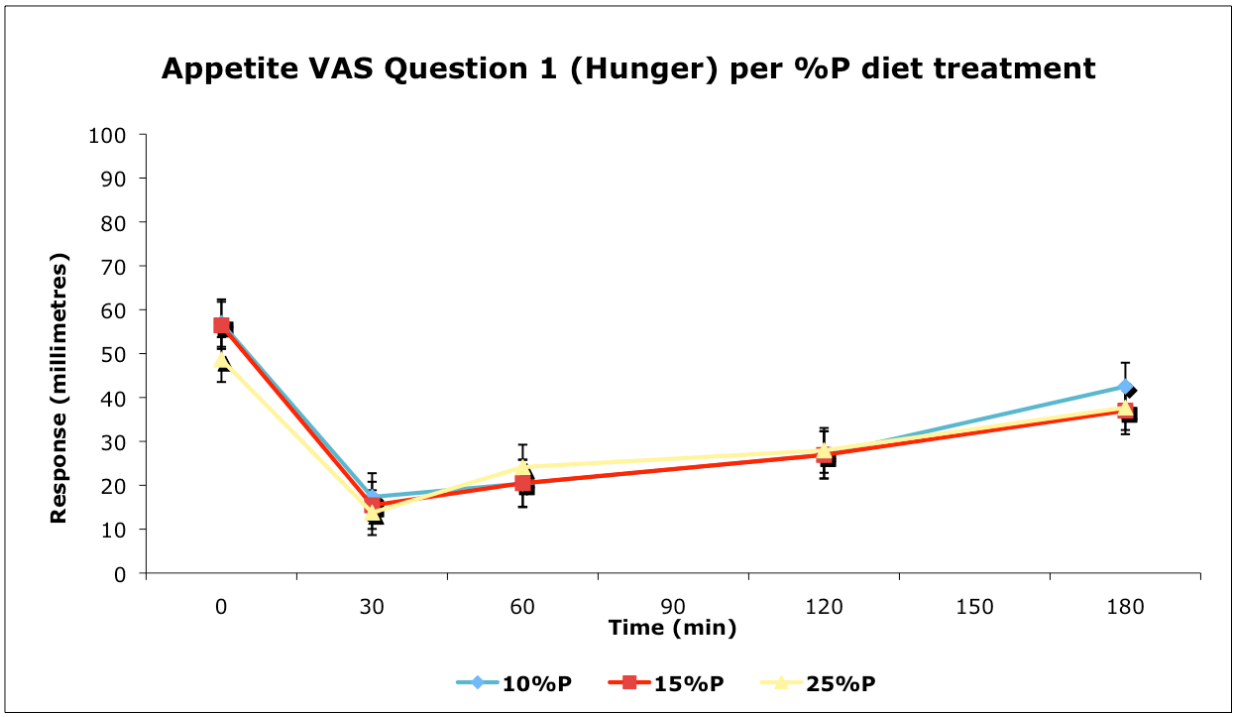


Figure 2.9.2 Appetite VAS Question 2 (Fullness) per % P diet treatment

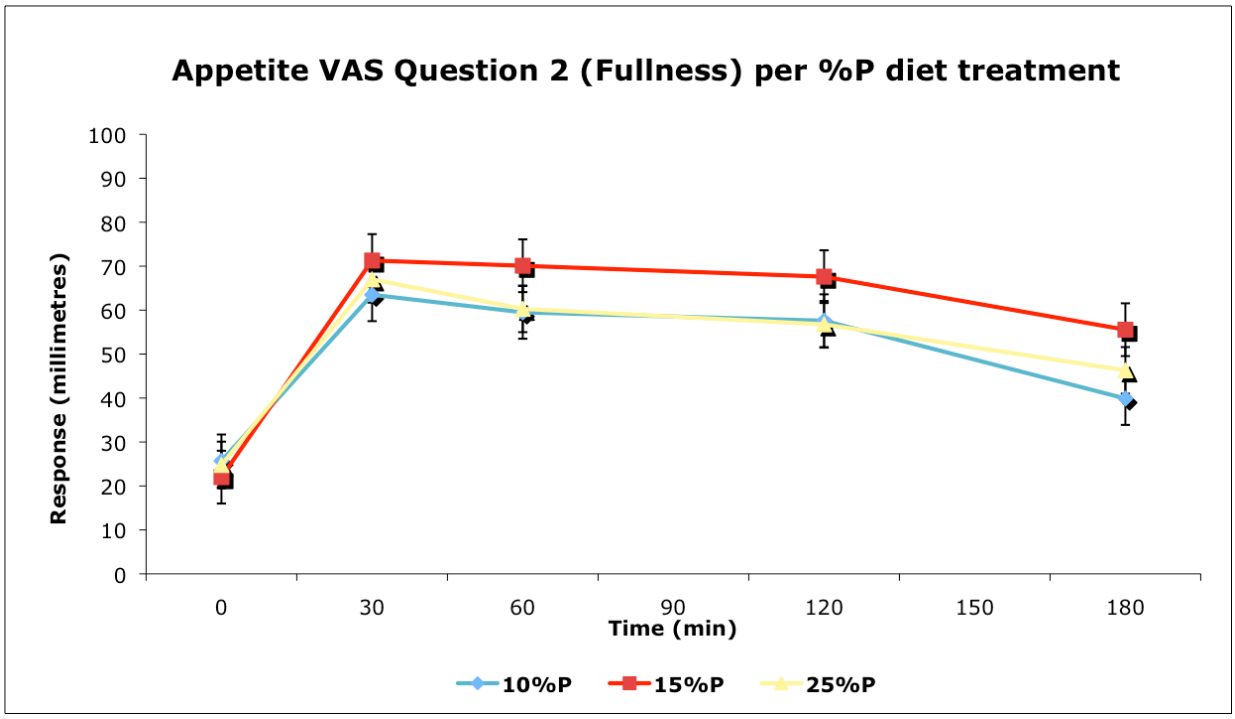


Figure 2.9.3 Appetite VAS Question 3 (Desire to eat) per % P diet treatment

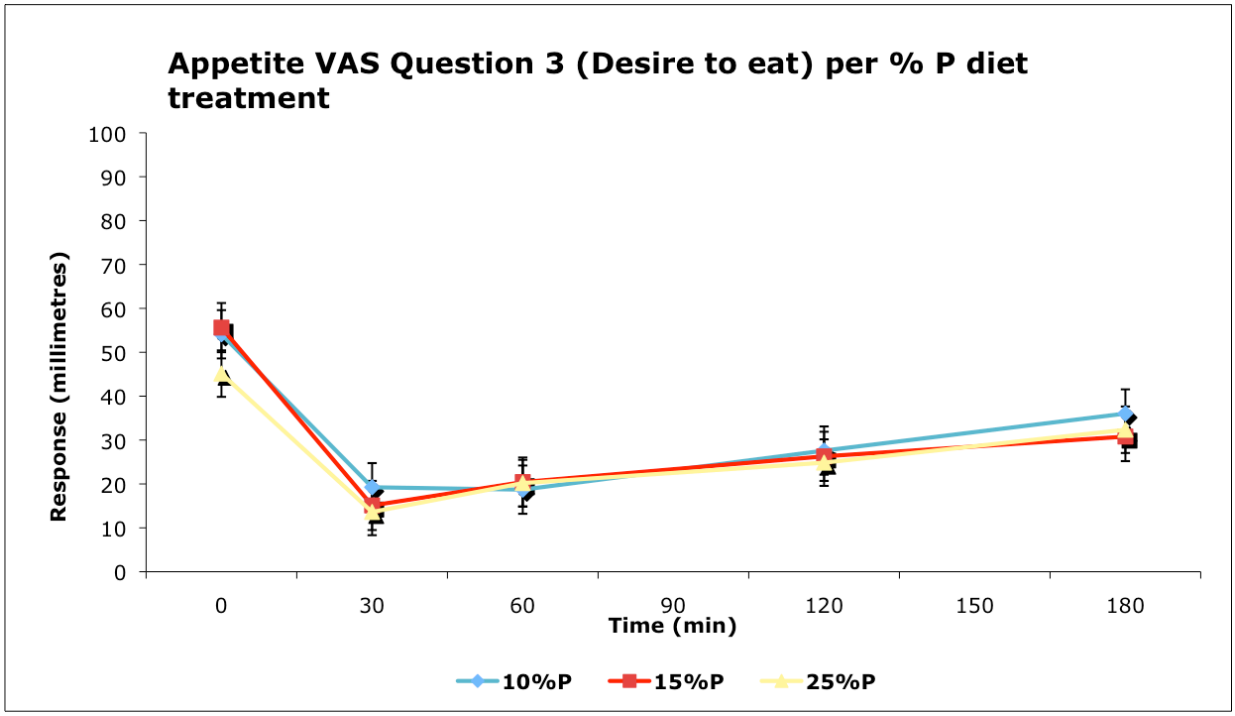


Figure 2.9.4 Appetite VAS Question 4 (Prospective Consumption) per % P diet treatment

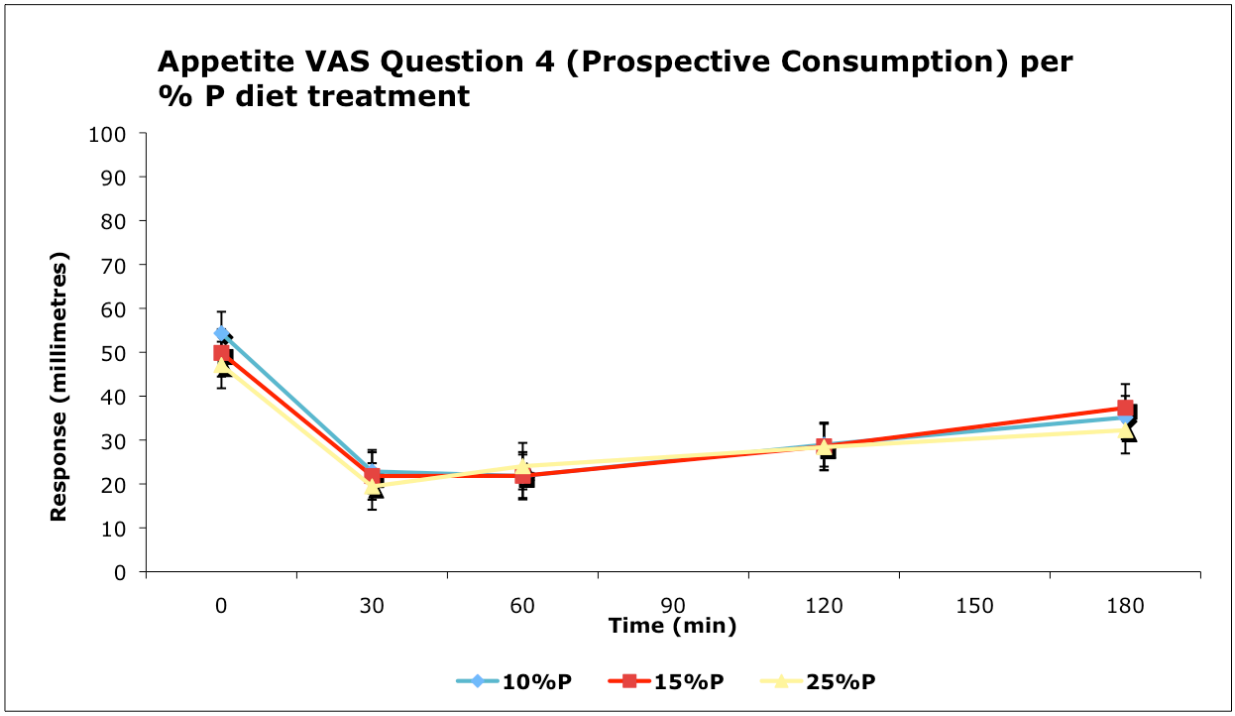


Table 2.2 Appetite VAS Q1 - 4: Baseline and AUCs per % P diet treatment

Appetite VAS per % P diet treatments	Baseline (mm)	Analysis	AUC (mm.min)	Analysis
Question 1				
10% P	57 ± 6.9*	$F_{(2,40)}$=0.78	4095 (2482.5, 6772.5)^	X^2 (2) = 0.38
15% P	56.4 ± 6.4	p = 0.47 +	3915 (2467.5, 6592.5)	p = 0.83 ‡
25% P	48.6 ± 7		5055, (1867.5, 6255)	
Question 2				
10% P	20 (8, 34) ^	X^2 (2) = 1.51	8709.6 ± 941.1 *	$F_{(2, 40)}$ = 2.37
15% P	19 (4,25)	p = 0.47 ‡	10755.7 ± 935.6	P = 0.10+
25% P	16 (6.3, 40.8)		9393.4 ± 846.7	
Question 3				
10% P	54.1 ± 7 *	$F_{(2, 40)}$ = 1.49	3907.5 (2366.3, 5298.8)^	X^2 (2) = 0.71
15% P	55.6 ± 6.7	p = 0.24+	2955 (1230, 6397.5)	P = 0.7 ‡
25% P	45.1 ± 6.6		3367 (1293.8, 4638.8)	
Question 4				
10% P	54.3 ± 5 *	$F_{(2, 40)}$ = 1.18	3547.5 (2508.8, 6930) ^	X^2 (2) = 1.54
15% P	49.9 ± 5	p = 0.31 +	4057.5 (1901.3, 7477.5)	p = 0.46 ‡
25% P	47.1 ± 5.3		3742.5 (2381.3, 5325)	

* mean ± SEM; ^ median, IQR

\+ ANOVA with RM ‡ Freidman's Test

There were no significant baseline differences for any of the Appetite VAS questions, nor significant differences for any of the Appetite VAS AUCs. However, a non-significant trend towards difference for the AUC of Q2 (fullness) was noted.

2.4.3 Fixed Energy Meal Experiment: Cholecystokinin

The levels of CCK at baseline to 180-minutes and the CCK AUCs per diet treatment are presented as ***Figures 2.10 – 11***. All graphed data is presented as mean ± SEM unless specified otherwise. CCK descriptive data and the results of analyses are presented in **Table 2.3.**

Figure 2.10 CCK: Baseline to 180 minutes per % P diet treatments

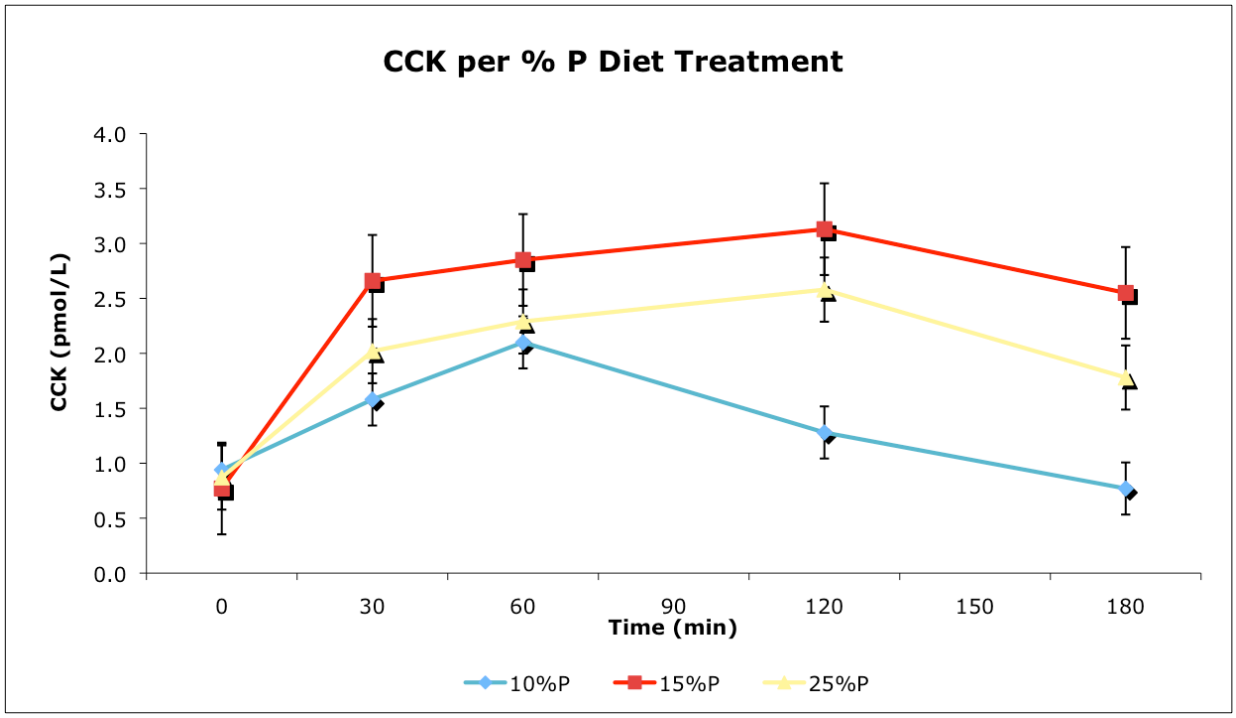


Figure 2.11 CCK AUCs per % P diet treatments

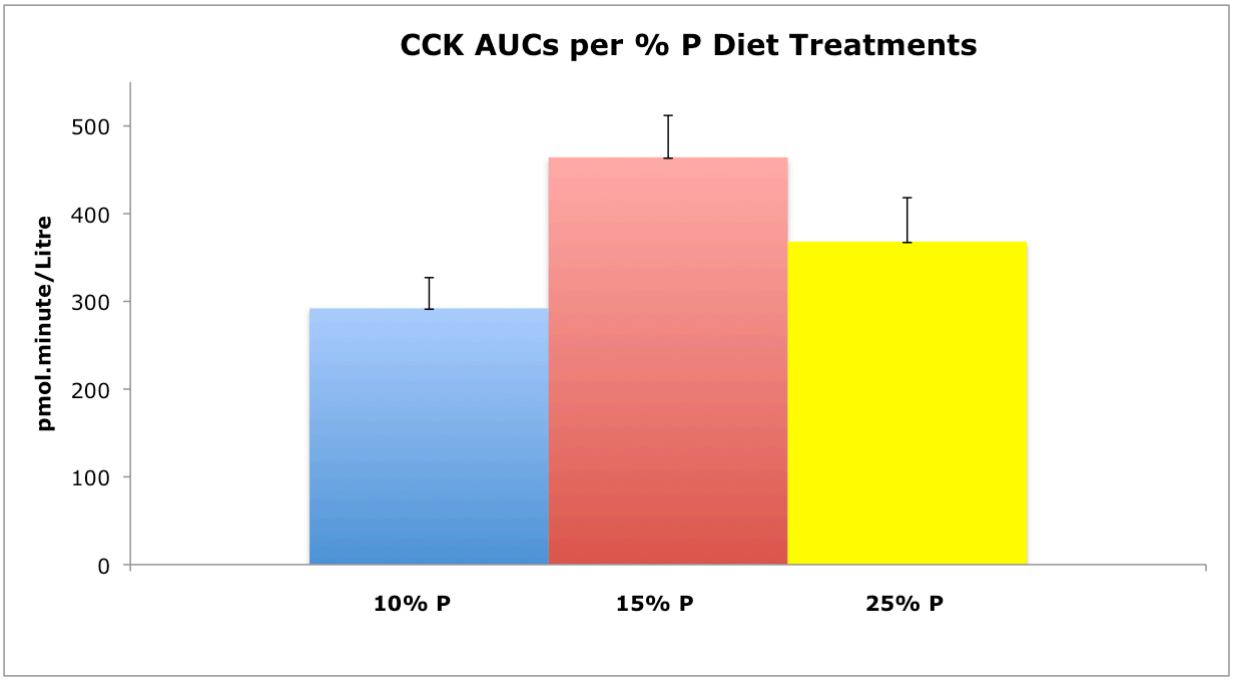


Table 2.3 Descriptive data and statistical analyses for CCK per %P diet treatments

CCK per % P diet treatments	Baseline (pmol/L) ^	Analysis ‡	AUC (pmol.min/L) *	Analysis +
10% P	0.37, 0.30 - 1.29	X^2 (2) = 0.69	292.12 ± 34.96	$F_{(2,44)}$ = 7.29
15% P	0.33, 0.29 – 0.67	p =0.71	464.18 ± 47.79	p =0.002
25% P	0.39, 0.31 - 0.76		368 ± 50.09	

* mean ± SEM; ^ median, IQR

\+ ANOVA with RM ‡ Freidman's Test

No significant differences were found when baseline CCK was ranked according to diet treatment (p = 0.71).

A significant difference was found when CCK AUCs were assessed according to the diet treatments (p = 0.002). *Post-hoc* testing demonstrated a significant mean difference between 10% P vs. 15% P diet treatments (I – J = -172.06, p = 0.001) but not between 15% P vs. 25% P (p = 0.12) or 10% P and 25% P diet treatments (p = 0.44).

Concentrations of CCK rose after the consumption of the meal. For 10% P, levels peaked at 60 minutes while for 15% P and 25% P, they peaked at 120 minutes. and when AUCs were compared, there was at least a 23% difference in the AUC for the 10% P and other diet treatments.

2.4.4 Fixed Energy Meal Experiment: total Ghrelin

The baseline to 180-minutes levels and AUCs of total ghrelin per diet treatment are presented in ***Figures 2.12 – 13***. All graphed data is presented as mean ± SEM unless specified otherwise. Total ghrelin descriptive data and the results of analyses are presented in **Table 2.4**.

Figure 2.12 Total ghrelin to 180 minutes per % P diet treatments

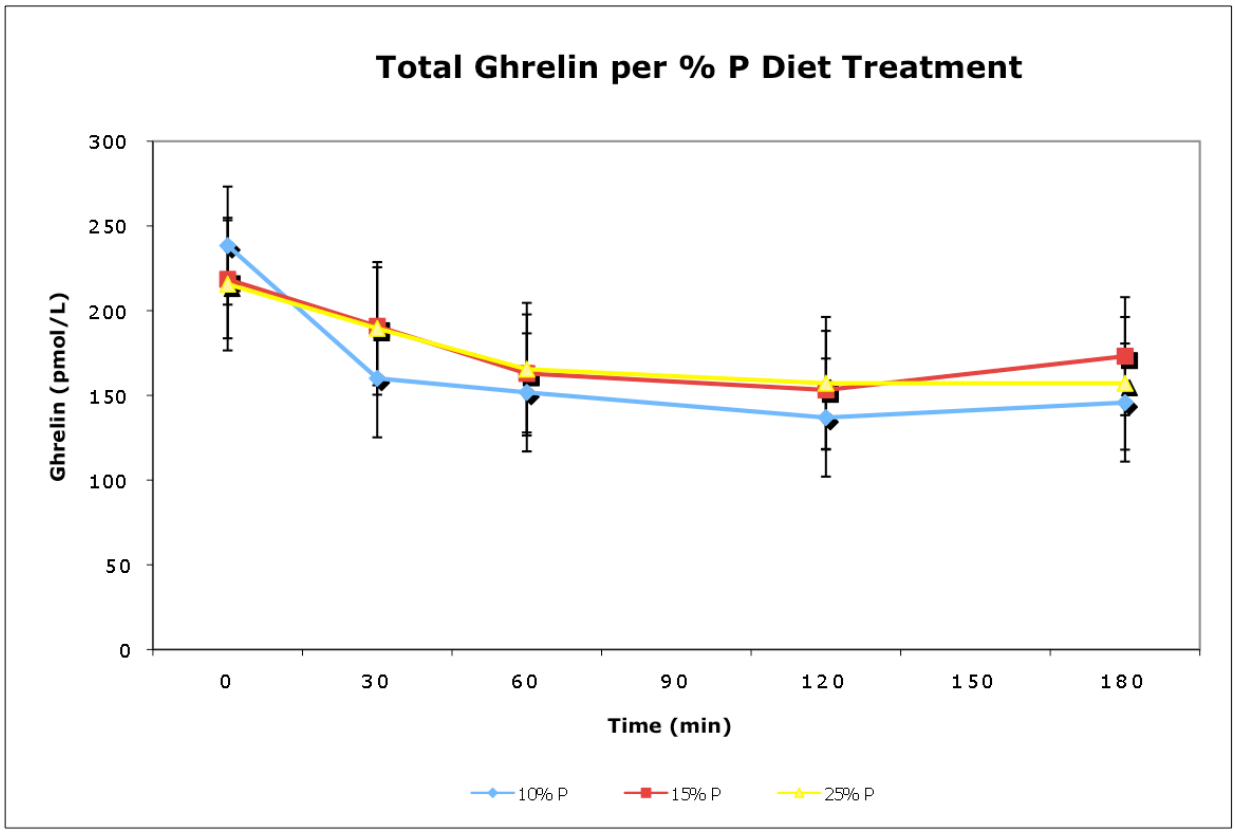


Figure 2.13 Total ghrelin AUCs per % P diet treatments

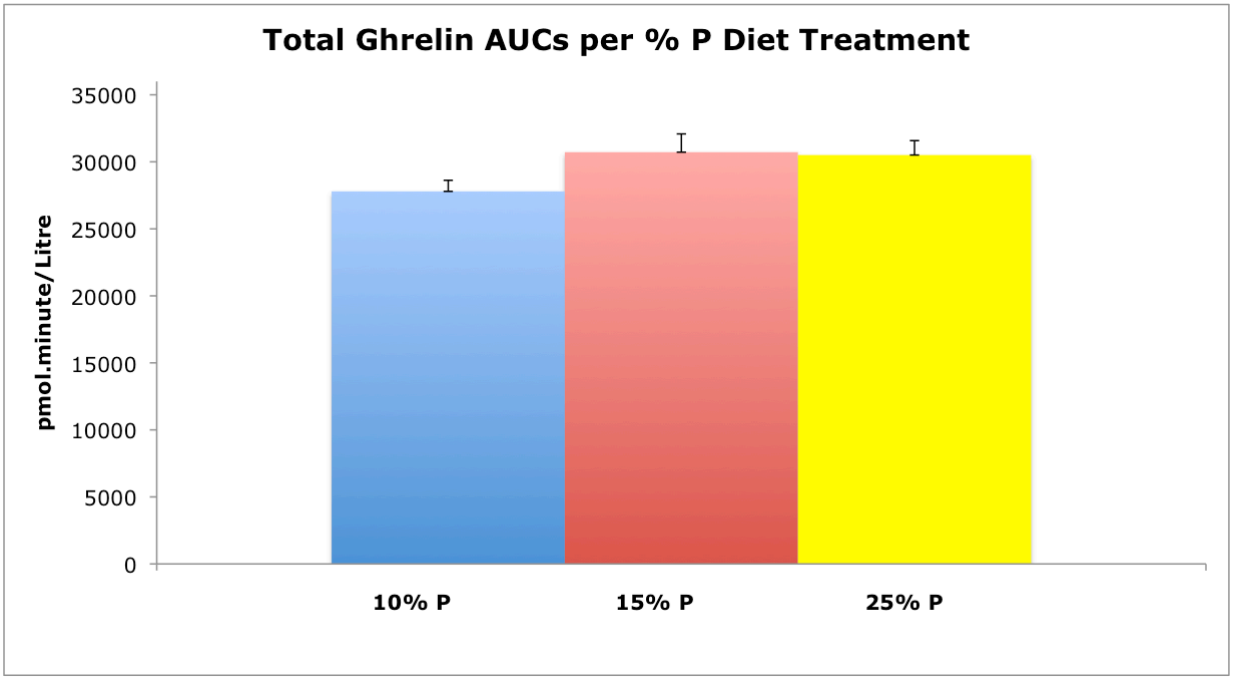


Table 2.4 Total ghrelin descriptive data and statistical analyses per % P diet treatments

Total ghrelin per % P diet treatments	Baseline (mmol/L) *	Analysis +	AUC (mmol.min/L) *	Analysis +
10% P 15% P 25% P	238.39 ± 25.75 160.07 ± 5.38 151.77 ± 4.77	$F_{(1.05, 23.10)} = 9.89$ $p = 0.004$	27794.52 ± 825.10 30719.88 ± 1361.50 30508.13 ± 1074.40	$F_{(2, 44)} = 4.15$ $p = 0.02$

* mean ± SEM; + ANOVA with RM

A significant difference was found for baseline total ghrelin per diet treatments (p=0.004 with the *Greenhouse-Geisser* correction). *Post-hoc* analyses demonstrated the mean differences were between the 10% P vs. 15% P treatments (I – J = 78.2, p = 0.026) and the 10% P vs. 25% P treatments (I – J = 86.6, p = 0.007) but not between the 15% P vs. 25% P diets (p = 0.38).

Post-prandial total ghrelin concentrations decreased on all three diet treatments. For the 10% P and 15% P treatments, levels reached their nadir at 120 minutes while for the 25% P treatment; nadir was reached at 180 minutes.

There was at least 10% different between the highest and lowest AUCs for total ghrelin per % P diet treatments. Analyses showed significant differences between the AUCs according to the diet treatments (p = 0.02) with *post-hoc* analyses demonstrating a mean difference between the 10% P vs. 25% P treatments (I – J= -2713.61, p = 0.017)

but not between the 10% P vs. 15% P diets (p = 0.11) nor between the 15% P vs. 25% P diets (p = 1).

2.4.5 Fixed Energy Meal Experiment: Glucagon-Like Peptide-1

Levels of GLP-1 at baseline to 180-minutes and AUCs are presented in ***Figures 2.14 – 15.*** All graphed data is presented as mean ± SEM unless specified otherwise. The descriptive data and results of analyses for GLP-1 are presented in **Table 2.5**.

Figure 2.14 Baseline to 180 minutes levels of GLP-1 per % P diet treatments

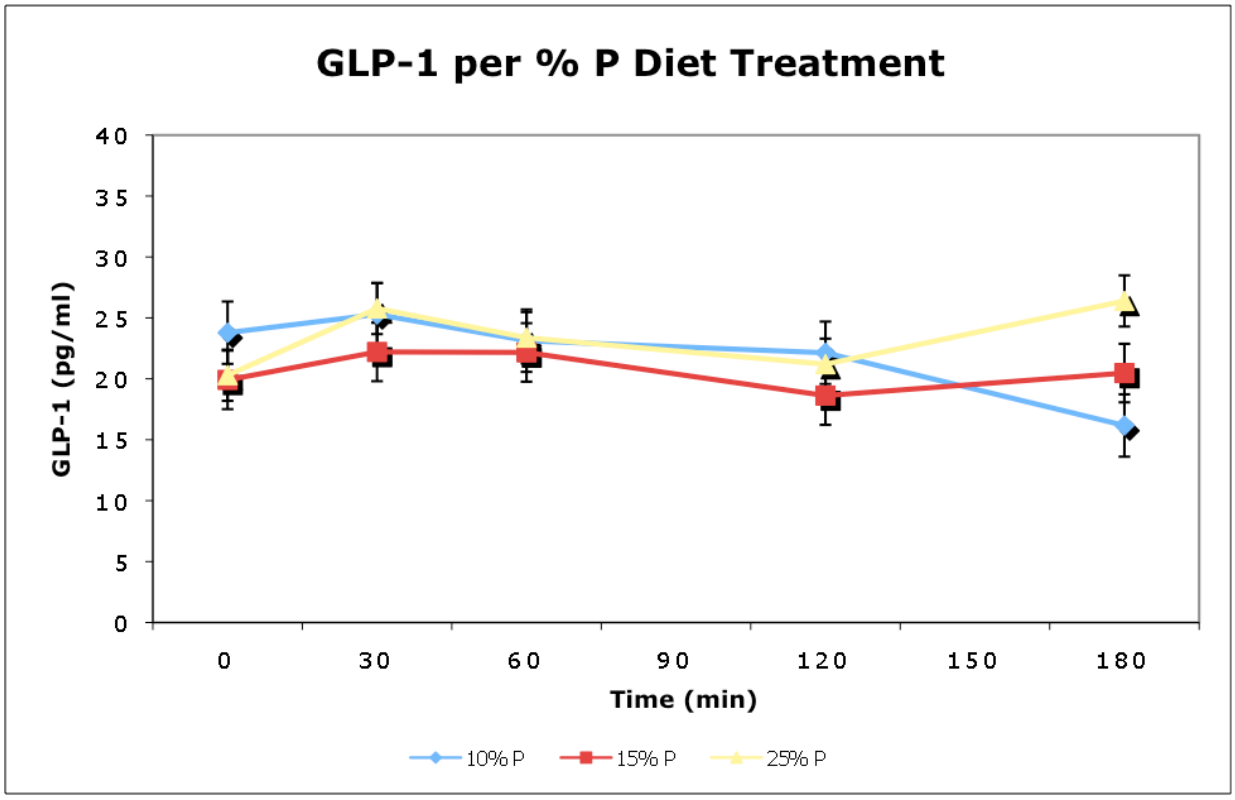


Figure 2.15 GLP-1 AUCs per % P diet treatments

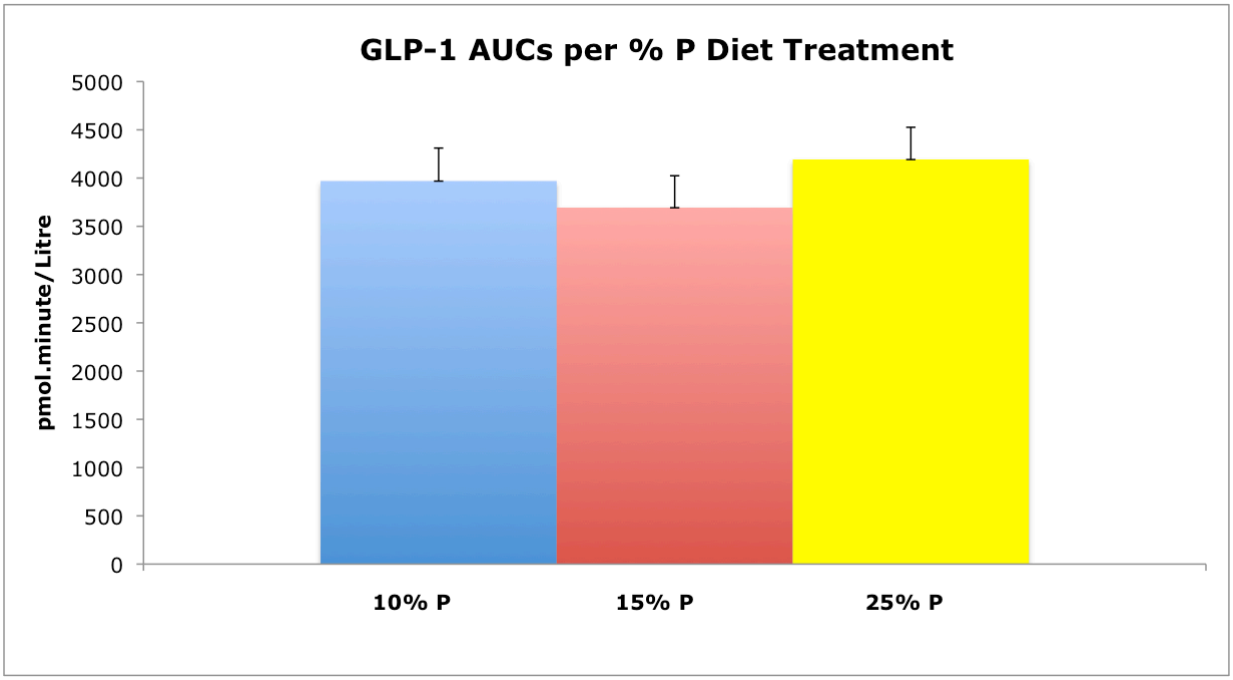


Table 2.5 GLP-1 descriptive data and analyses per % P diet treatments

GLP-1 per % P diet treatments	Baseline (pg/mL)^	Analysis ‡	AUC (pg.min/mL) ^	Analysis ‡
10% P	19.60 (11.4, 33.14)	X^2 (2) = 0.35	3642.6 (2906.7, 4558.95)	X^2 (2) = 0.78
15% P	19.13 (13.10, 23.28)	p = 0.84	3670.20 (2548.35, 4176.15)	p = 0.68
25% P	19, (11, 25)		4058.4 (3165.3, 5250)	

^ median, IQR ‡ Freidman's Test

No significant differences were found for baseline levels of GLP-1 ranked according to the diet treatments, (p = 0.84).

For all the diet treatments, post-prandial concentrations of GLP-1 increased and then fell. For the 10% P treatment, levels fell below baseline while for the 15% P and 25% P treatments at 180 minutes, they remained above baseline. On the 10% P treatment GLP-1 concentrations peaked by 30 minutes while on the 15% P and 25% P treatments the post-prandial levels of GLP-1 followed a bimodal pattern, peaking at 30 minutes then falling at 60 minutes and then rising again to a secondary peak by 180 minutes.

There was only a small difference of ~1% between the GLP-1 AUCs per diet treatment and when ranked accordingly, GLP-1 AUCs did not significantly differ (p = 0.68).

2.4.6 Fixed Energy Meal Experiment: Insulin

The baseline to 180-minutes levels and AUCs of insulin per diet treatments are presented in ***Figures 2.16 – 17***. Insulin descriptive data and the results of analyses are presented in **Table 2.6**.

Figure 2.16 Baseline to 180-minute levels of insulin per % P diet treatments

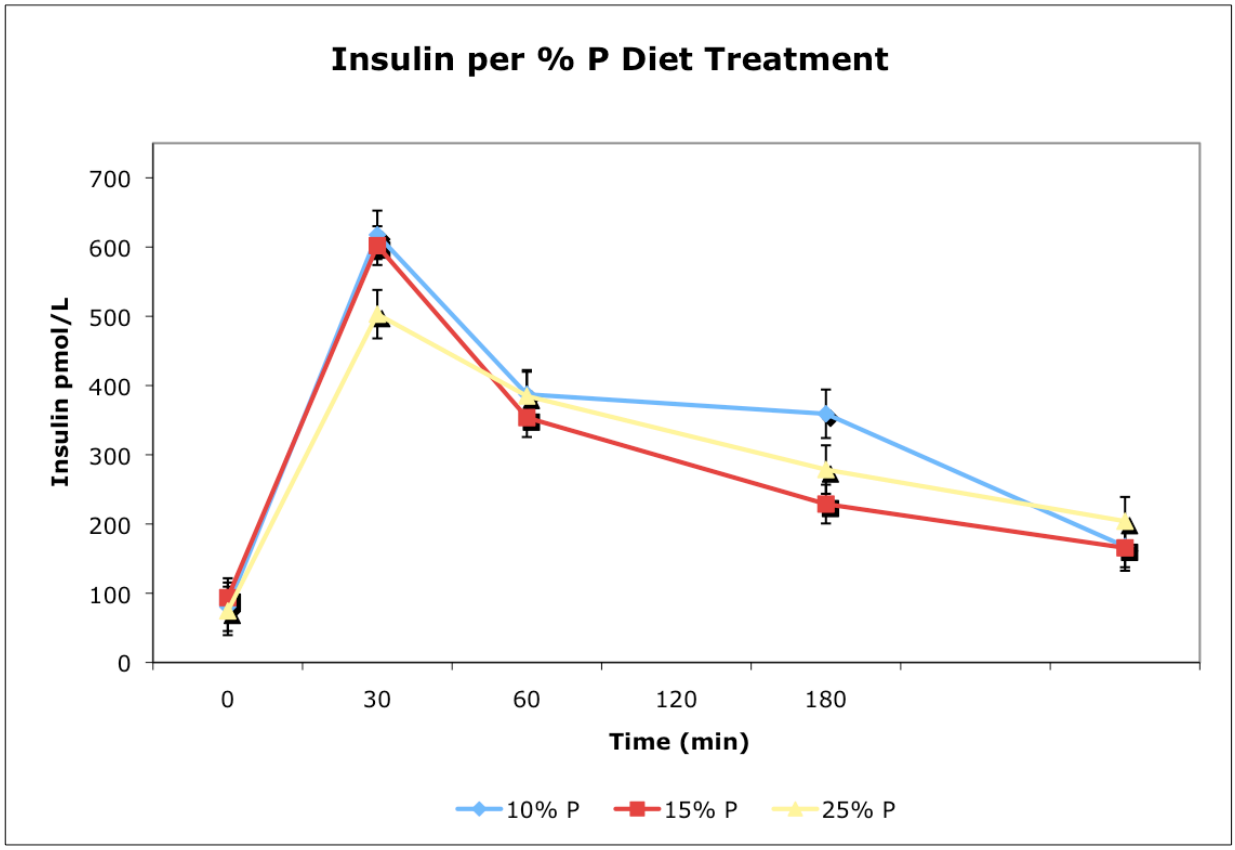


Figure 2.17 Insulin AUCs per % P diet treatments

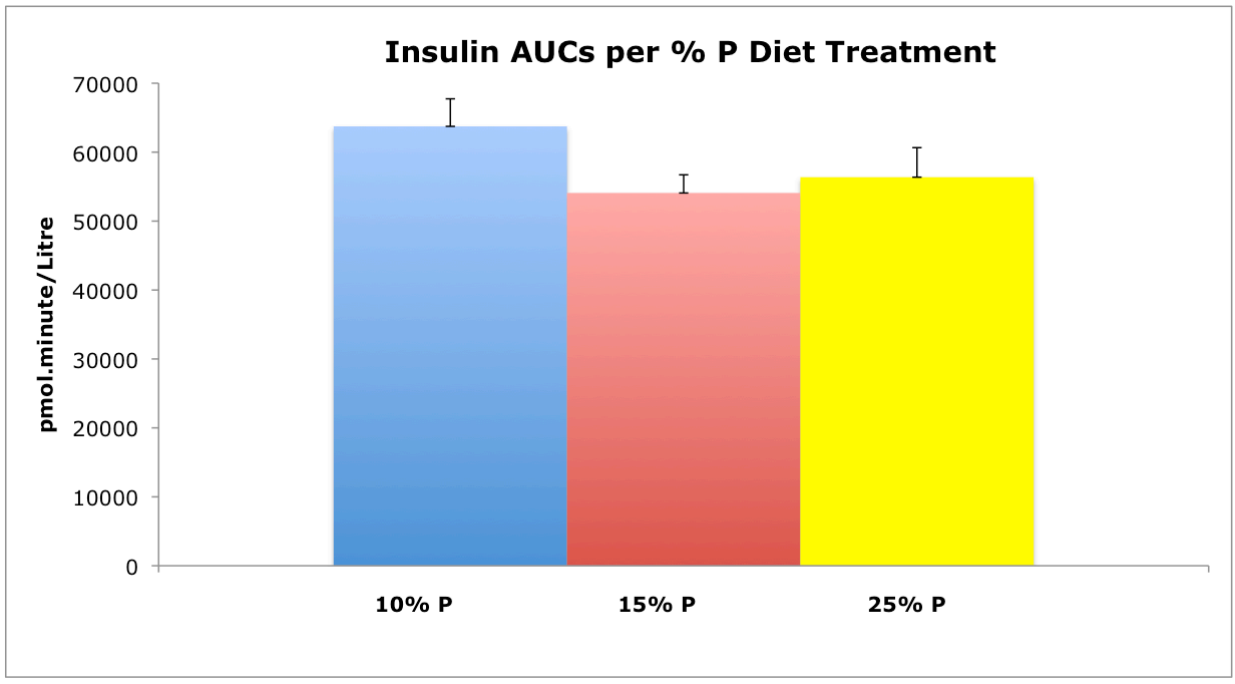


Table 2.6 Descriptive data and results of analyses for insulin per % P diet treatments

Insulin per % P diet treatments	Baseline (mmol/L) *	Analysis +	AUC (mmol.min/L) *	Analysis +
10% P 15% P 25% P	80.44 ± 3.64 93.7 ± 6.42 74.49 ± 5.67	$F_{(2, 44)} = 5.20$ $p < 0.01$	63744.5 ± 19145.4 54079.8 ± 12645 56370.2 ± 20555.2	$F_{(2, 44)} = 4$ $p = 0.03$

* mean ± SEM; + ANOVA with RM

Significant differences were found between levels of baseline insulin according to the diet treatments (p = 0.009). *Post-hoc* analyses demonstrated a mean difference between the 15% P vs. 25% P treatments (I – J = 19.21, p = 0.04), a non-significant trend towards a mean difference between the 10% P vs. 15% P treatments (I – J= -13.26, p = 0.09) but no difference between the 10% P vs. 25% P diets (p = 0.77).

For all diet treatments post-prandial levels of insulin rose and peaked by 30 minutes before declining for the rest of the experimental period. Insulin AUCs were highest on the 10% P treatment and there was at least an 18% difference between the 10% P and the other treatments.

Insulin AUCs also differed according to the diet treatments (p = 0.03) with *post-hoc* analyses demonstrating a mean difference between 10% P vs. 15% P treatments (I – J = 9664.76, p = 0.03), a non-significant trend between the 10% P vs. 25% P treatments

(I – J = 7374.33, p = 0.10) but no mean differences between the 15% P vs. 25% P treatments (p = 1.0).

2.4.7 Fixed Energy Meal Experiment: Total Ghrelin x Insulin

Spearman's rank correlation coefficients were calculated to examine for any interaction between levels of total ghrelin and insulin. Pair-wise analyses were made for the five measured time points (0, 30, 60, 120 and 180 minutes) and the AUCs per diet treatment.

With the exception of the 120 minute point on the 10% P diet treatment (which just met significance at p = 0.049), no significant correlations, nor trends towards significance were found between total ghrelin and insulin for any paired observations on any diet treatment (all p values >0.11). In addition, when the total ghrelin and insulin AUCs for each diet treatment were compared, no significant correlations were found (10% P diet treatment p = 0.14; 15% P diet treatment p = 0.23; 25% P diet treatment p = 0.36).

2.4.8 Fixed Energy Meal Experiment: Urea and Glucose

The results to 180 minutes of testing for urea and glucose according to the diet treatments are presented in ***Figures 2.18*** and ***2.19*** respectively.

Descriptive data and analyses for the baseline and AUCs are presented in **Table 2.7** for urea and **Table 2.8** for glucose.

Figure 2.18 Urea per % P diet treatment

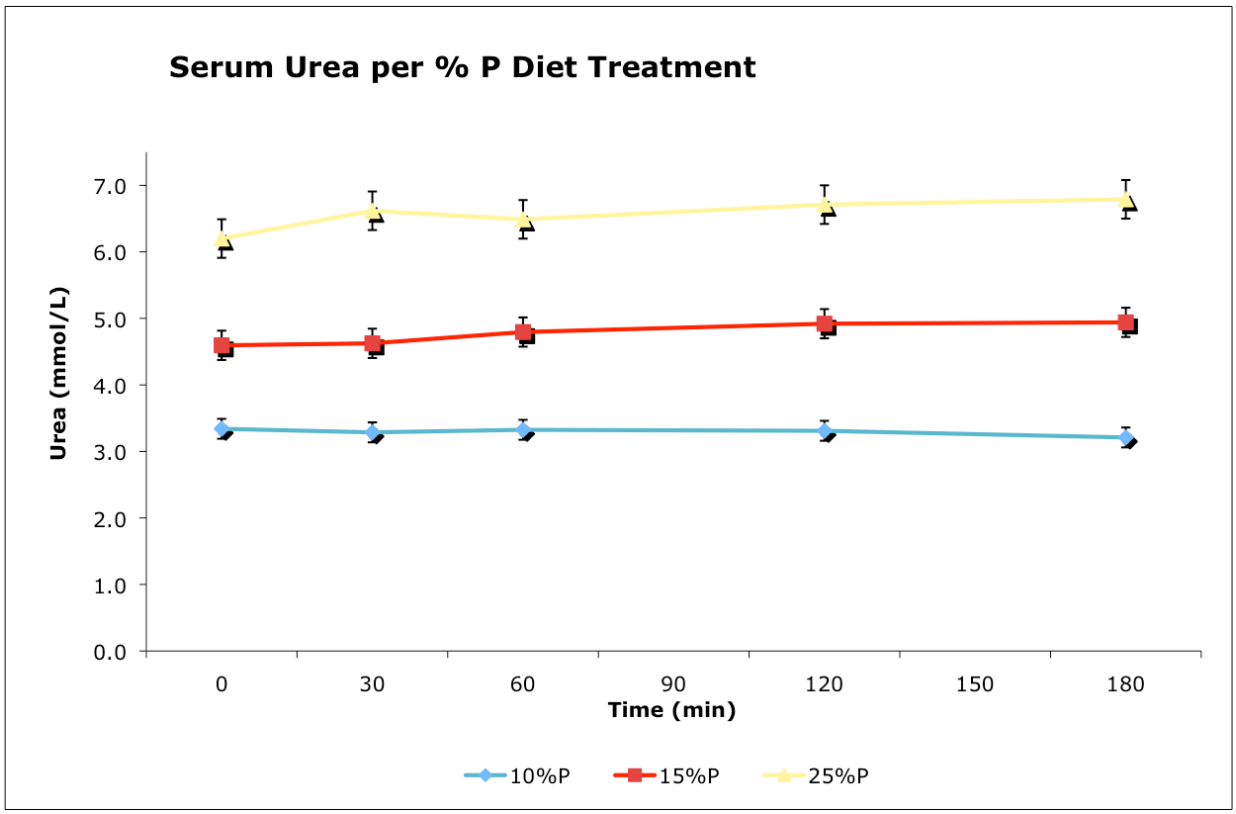


Table 2.7 Baseline, AUCs and analyses for Urea per % P diet treatments

Urea per % P diet treatments	Baseline (mmol/L) *	Analysis +	AUC (mmol.min/L) ^	Analysis ‡
10% P	3.34 ± 0.14	$F_{(2, 36)} = 76.32$	583.5 (400.5, 618)	X^2 (2) = 23.68
15% P	4.6 ± 0.21	P<0.0001	802.5 (714, 907.5)	P<0.0001 ‡
25% P	6.32 ± 0.29		1249.5 (897, 1360.5)	

* mean ± SEM; ^ median, IQR

\+ ANOVA with RM ‡ Freidman's Test

For urea, baseline and AUC levels increased very significantly according to % P diets (both p <0.0001).

For fasting differences, *post-hoc* analyses confirmed significant mean fasting differences between all the diet treatments [10% P vs. 15% P (I - J= -1.44, p <0.0001); for 15% P vs. 25% P (I-J=-1.69; p<0.0001); and for 10% P vs. 25% P (I-J= -3.13; p<0.0001)].

For differences between the AUCs according to the diet treatment *post-hoc* analyses (with a *Bonferroni* correction that generated a revised *alpha* significance level of p = 0.018) indicated the main difference was between the 10% P vs. 25% P diets (Z = -3.74, p <0.0001) and near significant differences existed between 10% P vs. 15% P and 15% P vs. 25% P (both p = 0.02).

Figure 2.19 Serum glucose per %P diet treatment

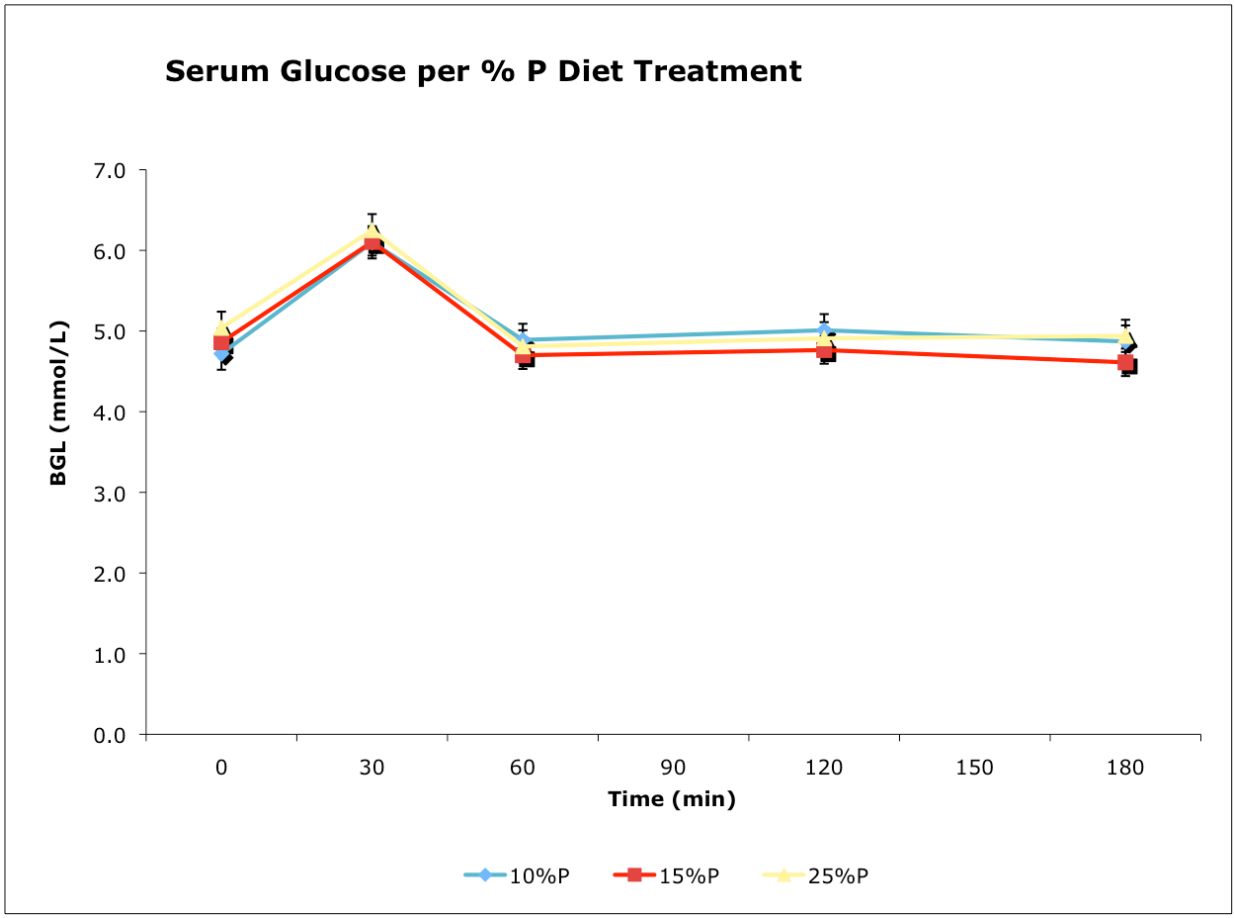


Table 2.8 Serum glucose: Baseline and AUCs per %P diet treatments

Serum glucose per %P diet treatments	Baseline (mmol/L) *	Analysis +	AUC (mmol.min/L) ^	Analysis ‡
10% P	4.72 ± 0.09	$F_{(2,21.75)} = 3.45$	923.25 (670.1, 1038.8)	X^2 (2) = 0.8
15% P	4.87 ± 0.07	$p = 0.07$	849 (780, 905.6)	$p = 0.67$
25% P	5.09 ± 0.15		861 (817.9, 961.1)	

* mean ± SEM; ^ median, IQR

\+ ANOVA with RM ‡ Freidman's Test

For glucose, baseline measures did not significantly differ (although a non-significant trend for glucose to increase according to % P diet was noted, p = 0.07) while AUCs ranked according to the diet treatments also did not differ (p = 0.67).

2.4.9 Fixed Energy Meal Experiment: Triglycerides, free fatty acids and total cholesterol

The serum levels to 180 minutes for free fatty acids (FFAs), triglycerides (TG) and total cholesterol (TC) according to diet treatments are presented in ***Figures 2.20 – 22*** respectively. Descriptive data and the results of analyses for fasting levels and the AUCs for FFAs, TG and TC are presented in **Table 2.9 – 11.**

Figure 2.20 Serum Free Fatty Acids per % P diet treatment

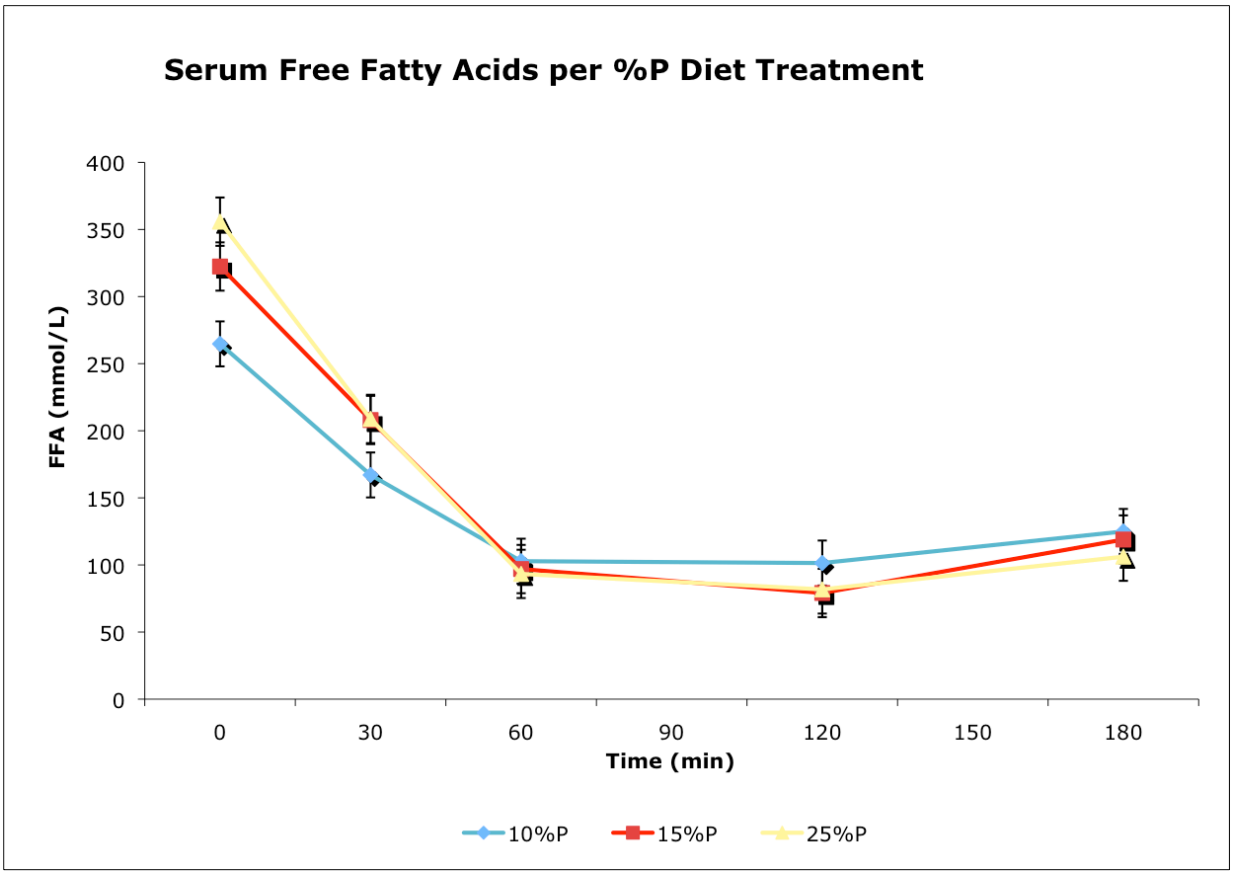


Table 2.9 Free Fatty Acids: Baseline and AUCs per % P diet treatment

FFAs per % P diet treatments	Baseline (umol/L) *	Analysis +	AUC (umol.min/L) *	Analysis +
10% P	218.7 ± 35.08	$p = 0.03$	16610.22 ± 2721.59	$p = 0.58$
15% P	280.35± 40.88		18730.44 ± 23356.48	
25% P	324.87 ± 44.91		19043.48 ± 2165.55	

* mean ± SEM; + ANOVA with RM

For FFAs, the fasting baseline measures significantly increased according to % P diet treatments ($p = 0.03$). AUCs did not differ according to % P diet treatments (using the *Greenhouse-Geisser* correction $p = 0.58$).

The fasting *post-hoc* analyses demonstrated a significant mean difference between the 10% P vs. 25% P diets (I - J= -106.17, $p = 0.04$) but not for the other diets (10% P vs. 15% P: $p = 0.18$, 15% P vs. 25% P: $p = 0.92$).

Figure 2.21 Serum triglycerides per % P diet treatment

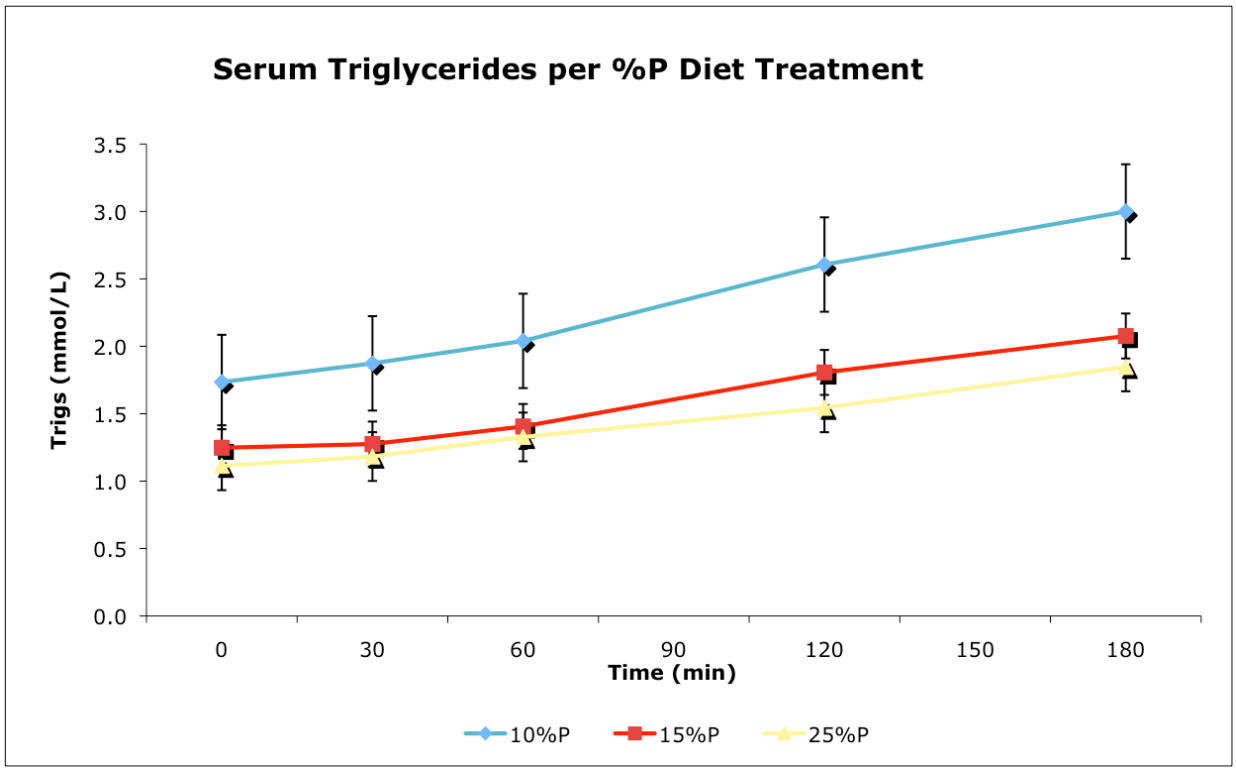


Table 2.10 Serum triglycerides: Baseline and AUCs per %P diet treatment

TGs per % P diet treatments	Baseline (mmol/L) *	Analysis +	AUC (mmol.min/L) *	Analysis +
10% P	1.51 ± 0.26	$F_{(1.57,44)} = 3.45$	283.67 ± 60.61	$F_{(2,42)} = 2.80$,
15% P	1.14 ± 0.14	$p = 0.06$	229.73 ± 36.61	$p = 0.07$
25% P	1.07 ± 0.11		196.89 ± 32.43	

* mean ± SEM; + ANOVA with RM Test

For TGs, neither the fasting baseline measures nor the post-prandial AUCs significantly differed, although non-significant trends for levels of TGs to be inversely associated with % P were noted for both (baseline using the *Greenhouse-Geisser* correction: $p = 0.06$ / AUCs: $p = 0.07$).

Figure 2.22 Serum total cholesterol per % P diet treatment

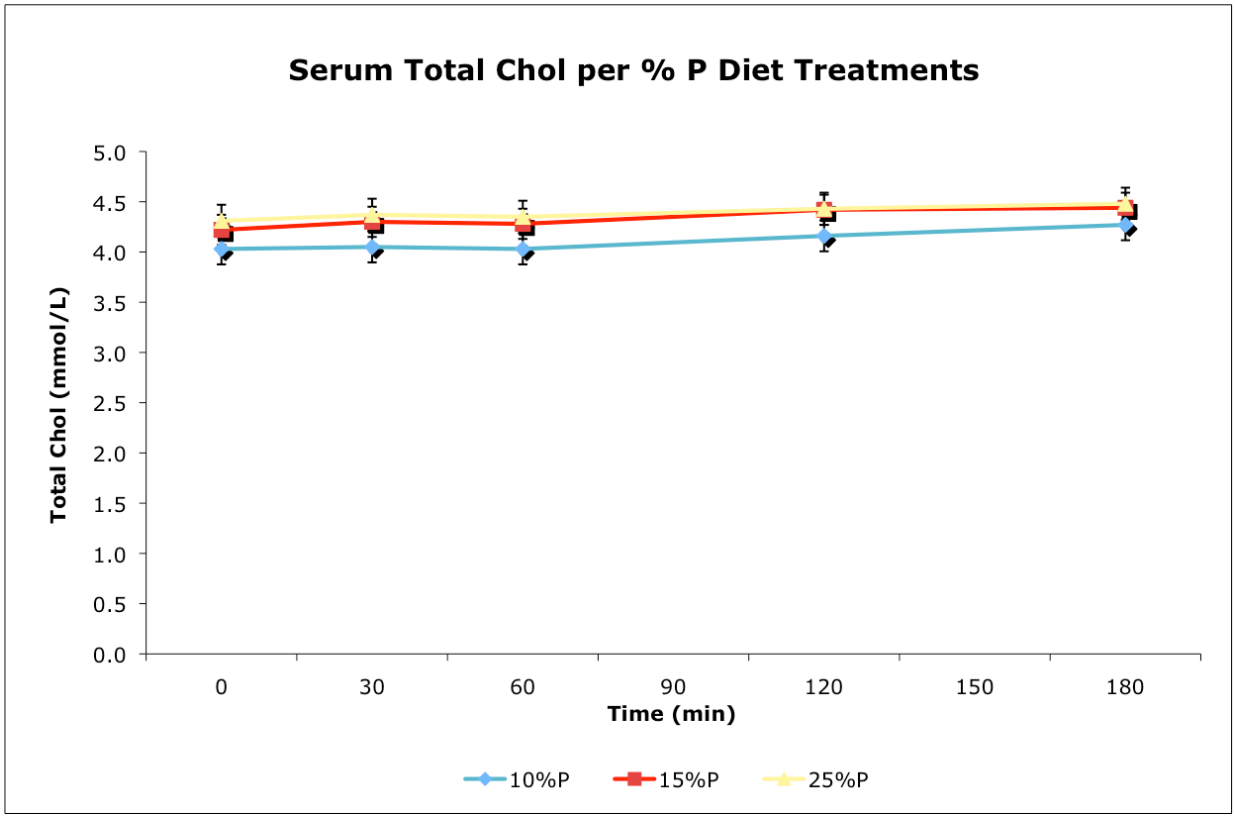


Table 2.11 Total Cholesterol: Baseline and AUCs per %P diet treatment

TC per % P diet treatements	Baseline (mmol/L) ^	Analysis ‡	AUC (mmol.min/L) ^	Analysis ‡
10% P	3.85 (3.05, 4.38)	p =0.10	642 (76.5, 742.5)	p = 0.045
15% P	4 (3.7, 4.5)		724.5 (279, 849)	
25% P	4.2 (3.8, 4.9)		726 (270, 885)	

^ median, IQR ‡ Freidman's Test

For TC the fasting baseline measures ranked according to the diet treatments did not differ, although a non-significant trend for levels to increase with % P was noted (p = 0.10) while AUCs significantly increased according to % P diet treatments (p = 0.045).

The *post-hoc* AUC analyses used a *Bonferroni* correction that set a revised *alpha* value to $p<0.017$ and showed a significant difference between the 10% P vs. 25% P diets ($Z = 2.72$, $p = 0.006$) but not the other two diet combinations (10% P vs. 15%: $p = 0.19$, 15% P vs. 25% P: $p = 0.42$).

2.5 DISCUSSION

2.5.1 Protein Leveraging

When compared historically to dietary CHO and F, the contribution of P to the development of overweight and obesity has been relatively under explored. Insights gained from research into the diet of invertebrates and mammals led to Simpson and Raubenheimer developing the *Geometric Framework*[84], which placed animals in a model that accounted for nutrient intake and the dietary environment. This in turn led to the *Protein Leverage Hypothesis*[42], which postulated that food consumption is regulated to maintain a target intake for dietary P and that relatively small changes in the availability of dietary P will result in large changes to the intake of CHO and F. As CHO and F are more energy dense than P what follows is that, if the increased energy intake is not offset by an increase to energy expenditure, weight gain is inevitable. To date, the evidence supporting the *Protein Leverage Hypothesis* in humans has been inferred indirectly from observational studies and the mechanisms behind protein leveraging remain unknown.

"*Testing the Protein Leverage hypothesis in humans*" was rigorously designed to determine whether humans would leverage their intake of dietary P as predicted by the *Protein leverage Hypothesis*[42]. The study demonstrated that lean humans who followed four days of *ad libitum* diets that differed in their % P (10% P, 15% P or 25% P) in a random order had the highest EI on the lowest % P diet. The 4-day total EI on the 10% P treatment energy intake (see ***figure 2.8***) was made up of a significantly increased intake of CHO+F (+15 to +25% compared to 15% P and 25% P diets

respectively) and a decreased intake of P (-3 to -12 % compared to 15% P and 25% P respectively).

The results show that lean humans will, by the fourth day, react to a changed dietary environment by prioritising their intake of dietary P at the expense of increasing their total energy intake in a fashion as predicted by the *Protein Leverage Hypothesis*. While the results did not demonstrate that four-day leveraging is associated with changes to body weight nor that protein leveraging occurs over longer periods what can be inferred is that if subjects had been maintained on a 10% P diet without a sustained increase in energy expenditure, weight gain would have been inevitable.

When these results are reviewed in light of the secular trends reported locally and internationally, namely increased total EI from CHO and F and static or decreased intake of dietary P[79-83], the value of the *Protein Leverage Hypothesis* in explaining part of the aetiology of the global epidemic of overweight and obesity is hard to deny.

2.5.2 Subjective measures of appetite and satiety

In the current study, there were no fasting or post-prandial differences found for subjective hunger, fullness (satiety), desire to eat (appetite) and prospective food consumption between the three diet treatments as assessed by the Appetite VAS. The lack of differences in the fasting state was reported in the article "*Testing Protein Leverage in Lean Humans*"[148].

The lack of fasting differences was not unexpected for while *Weigle et al*[73] reported that higher P diets were associated with increased satiety and decreased hunger under fasting conditions using the same Appetite VAS instrument, their experimental protocol consisted of two weeks of a baseline weight maintaining diet (50% CHO, 15% P) followed by two weeks of an isocaloric diet (50% CHO, 30% P) and then 12 weeks of an *ad libitum* diet (50% CHO, 30% P) and as importantly, the authors also reported that during the *ad libitum* diet phase the Appetite VAS measures returned to baseline. This suggests that while higher P diets reduce subjective assessment of hunger and improve satiety while fasting, it may only become evident under non *ad libitum* experimental conditions and in dietary interventions that are longer than four days and in meals with % P greater than that found in the current study.

The lack of post-prandial differences is also in keeping with the inconsistent results that are reported in the current literature. At least two studies that used the single meal test diet protocol and same Appetite VAS instruments have demonstrated that higher P meals are associated with post-prandial changes of increased satiety and decreased hunger. *Smeets et al*[72] found that the AUC to 180-minutes on the Appetite VAS satiety question was significantly higher on the 30% P compared to the 10% P liquid/solid lunch while *Bowen et al*[74] demonstrated that 100% P compared to 100% CHO (glucose) liquid pre-loads were associated with reduced appetite on the Appetite VAS instrument and decreased food intake from the buffet lunch provided afterwards.

However in contrast to the above studies, others using the same Appetite VAS instrument have found no significant differences between diet treatments differing in %P. *Raben et al*[68] compared mixed meals of differing macronutrient compositions

(including a 32% P / High P) following an overnight fast and failed to demonstrate any changes to VAS satiety or hunger over the 300 minutes of post-prandial testing. *Blom et al*[125] compared 47% CHO vs. 58% P breakfasts that followed an overnight fast and also reported no significant post-prandial changes on any Appetite VAS measure.

When the positive and negative studies are compared, points of differences that emerge include the time of day chosen to assess appetite and hunger, the types of experimental meals chosen and their macronutrient composition. The current study's experimental protocol is more similar to *Raben et al* and *Blom et al* in the use of mixed meals over liquid pre-loads or meals and testing after an overnight fast and it may be that the effects of higher P diets on post-prandial appetite and satiety are stronger between meals rather than after an overnight fast.

Finally, while the Appetite VAS appetite instrument is a well-recognised, validated and widely used research tool to measure subjective experiences of satiety and hunger, several limitations that reduce the sensitivity of the instrument are well known[56,149].

One is that participants may score the 100mm scale according to a bi or tri-modal pattern, clustering their responses at the extremes and mid-point rather than treating the response line as a linear continuum[155]. This was a phenomenon seen in a minority of this study's Appetite VAS response score-sheets. Another issue is that the ideal period of time to score the Appetite VAS is still unclear with some suggesting a duration of four hours[149]. While this issue was considered during our protocol development stage, for logistical reasons that included the effects of a longer period on the storage and transfer of blood and tissue samples, maintaining participant co-

operation at the end of a four day experimental period and after reviewing the protocols from published studies such as *Bowen et al*[74] and *Smeets et al*[72], we chose to complete the Fixed Energy Meal Experiment in three hours. However this may mean that the sensitivity of the Appetite VAS instrument was reduced for our experiment.

In order to clarify whether higher % P diets are associated with decreased appetite and increased satiety, a future study design that assessed the over-night fasting state and then over several meal time inter-meal periods, whilst allowing participants to select and initiate their own meal time and providing a wide range of % P meals, would address all of the above issues.

2.5.3 Dietary P: Hormonal and metabolic markers

The relationships between dietary P, the hormones that modulate appetite & satiety and the biochemical markers of metabolism were assessed in *"Testing Protein Leverage in lean humans"* and the Fixed Energy Meal Experiment. We demonstrated that after four days of *ad libitum* feeding from diet treatments differing in % P, significant differences were found for the fasting levels of total ghrelin, insulin, FFAs and urea. None of the following results were published in *"Testing Protein Leverage in lean humans"*[148].

The fasting levels of ghrelin were highest and fasting levels of urea lowest on the 10% P diet treatment; fasting levels of insulin were highest on the 15% P diet treatment and fasting levels of FFAs and urea highest on the 25% P diet treatment. Non-significant trends were noted for the fasting levels of glucose (lowest on 10% P treatment) and

TGs (highest on the 10% P treatment). There were significant relationships found between dietary P and fasting levels of CCK, GLP-1 and TC.

The Fixed Energy Meal Experiment assessed the relationship between dietary P and the post-prandial secretion of the same hormones and metabolic markers. The study showed that diets differing in % P were associated with significant changes to the post-prandial AUCs (to 180 minutes) for CCK, total ghrelin, insulin, urea and TC.

The post-prandial AUCs for CCK, total ghrelin, urea and TCs were lowest following the 10% P diet treatment while the post-prandial AUC for insulin highest. The post-prandial AUC for TGs were also highest following the 10% P diet treatment, though this was a non-significant trend. There were no significant differences for the different diet treatments and the post-prandial AUCs for GLP-1, glucose or FFAs.

These results show that by the fourth day of *ad libitum* diets that differ in % P, significant differences are found in both the fasting and post-prandial secretion of several hormones involved in the regulation of appetite, satiety and food intake; and for several markers related to the metabolism of food and dietary P.

2.5.3.1 Cholecystokinin

When compared to higher P diet treatments the 10% P treatment was associated with the lowest post-prandial AUC and the most attenuated post-prandial rise of CCK (see ***figures 2.10*** and ***2.11***), suggesting that that diets higher in % P are associated with greater CCK secretion in the post-prandial period.

The post-prandial secretion of CCK is mediated by the breakdown products of the three macronutrients: glucose, amino acids and FFAs for respectively CHO, P and F. Among these nutrients the hierarchy that ranks their relative potency placed amino acids and FFAs above glucose but debate exists regarding the relative potencies of the first two nutrients[96].

As the % F in this study's diet treatments was fixed at 30%, the results from this study do not directly address this question, but as amino acids are more potent stimulators of post-prandial CCK secretion than glucose, we can conclude the observed differences in CCK secretion were probably attributable to the changes in dietary P rather than to the changes in dietary CHO.

While previous single meal test studies[67,74] demonstrated that high (80-100%) compared to no to medium (0- 20%) P meals are associated with increased and prolonged post-prandial CCK secretion, our study is among the first to demonstrate the relationship with solid mixed meals rather than with liquid pre-loads that preceded a standardised meal or liquid only meals as reported in the other studies.

Another strength of our experimental protocol is that compared to the studies listed above, the range of % P in our protocol (10 - 15- 25 % P compared to 0 - 51 – 70 - 100 % P) is much closer to the % P currently being consumed by the general population[80,81]. Furthermore the differences in % P between the diet treatments were smaller in our study than those in the above studies. When this is considered alongside the physical form of our experimental meals we are confident that our results are not only robust but more applicable to the general dietary setting.

In our study no differences were found for fasting CCK after four days of *ad libitum* diets differing in %P. Differences in fasting CCK have only been reported in a single study[156] by *Robertson et al.* of healthy participants who consumed a standardised dinner high either in F (62% F) or CHO (77% F) with a constant % P (7%) which was followed by an overnight fast. The authors reported higher fasting levels of CCK the morning after the high F dinner, which was attributed to differences in the % F of the evening meal.

When compared to *Robertson et al*, our protocol had a much lower magnitude of the difference in % EI by macronutrients (ΔP between high and low P diet treatment 15% compared to Δ F between high and low F treatments for *Robertson et al* 42%) and as we kept % F constant, it was not unexpected that differences in fasting CCK were not found. Differences in the levels of fasting CCK could be demonstrated when the diet treatments have a greater magnitude of difference in % P, however when extreme macronutrient ratios are used, whether the associated observed differences have any general applicability is debatable.

Studies have previously examined the clinical potential of CCK as a novel anti-obesity therapy. Levels of CCK have been increased by intravenous infusion[103] while studies have reported on agents that activate the CCK receptor[97,157]. However all of these studies had only modest effects on appetite and weight and to date, the development of an effective clinical agent that modulates level of CCK for the treatment of overweight and obesity has proven elusive.

Our findings may point to great value in exploring and understanding the relationship between dietary P and CCK as an alternative means of influencing CCK secretion and modulating its physiological actions. If future studies validate these findings it would confirm that relatively modest increases in the % P of a diet will lead to a significantly increased and prolonged post-prandial CCK secretion. Given what is known about CCK's and its physiology effects on reducing meal size and initiating meal termination[95,100], this may have important implications for the treatment of the overweight and obese.

2.5.3.2 Ghrelin

Comparing four days of *ad libitum* feeding from the different diet treatments, fasting ghrelin was found to be highest on the 10% P treatment (see ***figures 2.12*** and ***2.13***). This study would be among the first to demonstrate that fasting ghrelin is affected by several days of *ad libitum* feeding from diets differing in their % P.

To date only three studies[126,158,159] were identified that examined the longer-term effects of dietary manipulation on fasting ghrelin in the non-pathological state. The results from all three studies showed no link between their diet treatments and fasting levels of ghrelin. It is worth noting that these three studies significantly differed from our own on several grounds.

Firstly, while all studies used healthy subjects, the study populations differed on mean age, gender and whether the overweight were included.

Secondly studies differed in the duration and nature of their dietary interventions and the type of dietary intervention. In particular *Beasley et al*[126] examined three periods of 6-week isocaloric feeding from high CHO, high P or high F diets; *Zhang et al*[158] examined four weeks of a weight-maintaining low-glycaemic index or healthy eating diet while *Vortuba et al*[159] examined six days of a weight maintaining healthy diet that was followed by three days of *ad libitum* overeating from a multiple choice cafeteria diet.

Thirdly, while *Beasley et al* and *Zhang et al* assessed the effects on fasting ghrelin from changing the macronutrient composition of the diets, *Vortuba et al* examined the effects of both changed macronutrient composition and increased EI.

When these negative studies are compared to the current study, the macronutrient portions used by *Beasley et al* (% CHO |% P |% F: High P*:* 48% | 25% | 27%, High CHO: 58% | 15% | 27%; High F: 48% | 15% |37%) were those that most closely resembled the diet composition used in this study. While these many protocol differences make direct comparisons between the studies problematic, the novelty of our finding may be attributed to factors that include the inclusion of only lean subjects in our study and that the observed short-medium term changes in fasting ghrelin demonstrated by our study may become attenuated in studies with a longer duration of diet treatment.

As morning fasting ghrelin is known to play an important role in initiating meal consumption[109,110] the higher levels of fasting ghrelin seen with the lowest % P diet treatment may represent part of a physiological adaption to the reduced protein

content of the current dietary environment. The physiological effects that could arise from these changes may be earlier meal initiation and speculatively, an increase in the meal size but the fixed timing and EI of our dietary intervention prevented an assessment of the effects of increased fasting ghrelin on subjects' voluntary eating habits.

The finding that the highest levels of fasting ghrelin were associated with the lowest %P diet treatment is consistent with the *Protein Leverage Hypothesis,* as altered ghrelin secretion may be one of the mechanisms that contributes to the predicted increase in food seeking behaviour that is the consequence of placing an animal in a food environment low in dietary P.

This study also demonstrated that the lowest post-prandial AUC for ghrelin was associated with the 10% P diet treatment, the treatment that showed the greatest post-prandial suppression of ghrelin (38% reduction from baseline as compared to a 25% and 28% reduction for the 15% P and 25% P diets respectively). This raises the question of whether this was due to the P or CHO content of the diet treatments.

When the macronutrients are assessed for their effects on post-prandial ghrelin release, F has consistently been demonstrated to have the weakest effect[89-93] with the ranking between CHO and P still unclear. Several studies[122-124] in normal and overweight humans using single meal tests have found that CHO rather than P has the greatest effect on suppressing post-prandial ghrelin secretion. However this finding has not been consistently replicated as seen by the results of two studies in normal and overweight humans; one using a single mixed breakfast (% CHO| P | F: 55% | 10% |

35% vs. 55% | 25% | 20%)[71] and the other liquid pre-meal pre-loads (100% CHO or 100% P)[74]. Neither study reported any significant differences for post-prandial ghrelin suppression between the meals. A third study of normal weight men[125] compared a high P or high CHO mixed breakfast (47.3% | 19.3% | 33.4% vs. 14.1% | 58.1% | 27.8%) in a cross over fashion and reported that it was P that most powerfully suppressed post-prandial ghrelin.

These studies differed in their macronutrient ratios and as to whether %F was kept constant. In contrast, this study kept %F constant with macronutrient ratios differing only in % CHO vs. % P, adding to the robustness of our study protocols. The results of the current study also support those that found it is CHO rather than P which has the greatest effect on post-prandial ghrelin secretion[73-75].

Taken together, these results suggest that the relationship between dietary P and ghrelin is mediated more by changes to fasting ghrelin rather than by changes to post-prandial ghrelin secretion. Furthermore, these changes become manifest over a period of as little as four days and can be elicited by a relatively modest change to the % P in the diet. Whether the relationship between dietary P and ghrelin has any "real world" implications for the dietary advice given to the overweight and obese will require longer-term studies to confirm the strength and duration of the relationship, with specific focus on whether the relationship is attenuated in the overweight and obese.

2.5.3.3 Glucagon-Like Peptide-1

Following four days of *ad libitum* diet treatments differing in % P, no differences were found for fasting GLP-1, nor were there any differences in post-prandial GLP-1 AUCs following the fixed energy intake, varying in % P breakfasts of the Fixed Energy Meal Experiment (see ***figures 2.14*** and ***2.15***).

The lack of differences between the %P treatments and post-prandial GLP-1 secretion stands in contrast to three studies that have reported positive findings. The first study[70] assessed 24 hours of dietary interventions and found that the 30% P compared to the 10% P dinner (and for men lunch) was associated with increased GLP-1 secretion. The second study[67] compared 70% P vs. 87% CHO liquid preloads which were followed by *ad libitum* standardised buffet-style lunches and reported increased post-prandial GLP-1 secretion following the high P preload. Finally the third study[68] reported on 32% P vs. 65% CHO and 65% F breakfasts given as a single meal test experiment and found higher post-prandial GLP-1 secretion following the high P breakfast.

However the evidence for GLP-1 secretion and dietary P is not consistent with two studies reporting no relationship between high P diets and GLP-1 secretion. In the first study[69] 24 hours of a 10% P vs. 30% P diets were compared while in the second study 25% P vs. 10% P lunches as a single meal test experiment were compared[72]. Both studies reported no association between the higher P diets and changes to post-prandial GLP-1 secretion.

Among these five conflicting studies, the results from the study which used liquid pre-loads[67] can be discarded for three reasons: firstly it was the only study to include overweight and obese participants; secondly it was the only study to use liquid pre-meal pre-loads and thirdly, the diet interventions had significantly divergent macronutrient ratios when compared to all other studies. When the results of this study are removed, the balance of evidence is evenly poised as to whether higher P diets have effects on GLP-1 secretion.

When the results are considered collectively, the current study shifts the balance of evidence to suggest that higher P diets have little effect on fasting or post-prandial GLP-1 secretion. This conclusion is supported by the known increased potency of dietary CHO and F relative to P on the post-prandial secretion of GLP-1[137]. Another implication of this negative finding is that contrary to the findings presented in *Westerterp-Plangenta et al*[70], any increased satiety associated with higher P diets is unlikely to be mediated by the post-prandial secretion of GLP-1.

2.5.3.4 Insulin

Following four days of *ad libitum* feeding of the diet treatments differing in % P, the lowest levels of fasting insulin were associated with the 25% P diet. All fasting insulin results were within the normal range and not at levels consistent with insulin resistance (see ***figure 2.16***).

As the diet treatments in this study had fixed % F it follows that the treatment highest in % P is also the diet treatment lowest in % CHO. This means that another

interpretation of the insulin results is that fasting insulin was lowest following the diet lowest in % CHO, which is consistent with the known and central role of dietary CHO in influencing insulin secretion[143].

Studies in the overweight and obese comparing diets differing in their ratios of macronutrients have consistently reported that reductions in fasting insulin are proportional to the loss of body weight rather than to the macronutrient content of the experimental diets[77,160-162]. Uniquely among these four studies *Leidy et al*[162] focused on the role of dietary P by comparing isocaloric weight-maintaining diets differing in % P (14% P vs. 25% P) but fixed in % F, with the authors reporting that there were no diet related changes to fasting insulin.

Unlike our study, the above studies were conducted with overweight and obese subjects. It is well-known that overweight and obesity is associated with hyperinsulinaemia and insulin resistance, which in turn leads to an increased risk of metabolic co-morbidities such as diabetes mellitus and cardiovascular disease[163]. It has also been established that weight loss reduces insulin secretion and improves peripheral insulin sensitivity, which reduces the rates of these same metabolic complications[164,165]. It is likely that in studies with overweight and obese subjects, the powerful relationship between body weight and insulin secretion will overshadow the more subtle effects on fasting insulin secretion that arise from altered macronutrient ratios.

We also found that the 180-minute post-prandial AUC for insulin was highest following the 10% P breakfast, corresponding to the diet treatment with the greatest %

CHO (60% and see ***figure 2.17***). This result is consistent with the strongly validated finding that in both normal weight and overweight populations, post-prandial insulin secretion is strongly associated with the dietary CHO[71,121-123,125].

Intriguingly *Veldhorst et al*[71] was one of the few studies to assess the role of dietary P and post-prandial insulin secretion by fixing their experimental diets in % CHO (55%) but varying them in % P and % F. The authors reported that compared to the high % F & low % P breakfast, the high % P & low % F breakfast was associated with higher post-prandial insulin secretion and increased satiety, which suggests that between P and F, P has the greater effect on post-prandial insulin secretion.

However this finding has not been replicated in *Weigle et al*[73] where normal to overweight subjects were given two weeks of a weight maintaining diet (50% CHO| 15% P |35% F) followed by two weeks of an isocaloric diet and then twelve weeks of an *ad libitum* diet (both diets 50% CHO| 30% P| 20%F). This study also kept %CHO constant while %P and %F varied. Unlike *Veldhorst et al* these authors found no differences for on fasting and post-prandial AUC insulin measures between their diets treatments but as the EI of this study's experimental diets differed so considerably, a direct comparison between the two studies is problematic.

Overall these results buttress the evidence that it is CHO much more than P and F that influences the fasting and post-prandial secretion of insulin.

2.5.3.5 Ghrelin and Insulin

No significant correlations, either for AUC measurements or for the paired time points, were found between levels of total ghrelin and insulin across any of the diet treatments.

Soon after the discovery of ghrelin in 1999, it was noted that the post-prandial suppression of ghrelin closely mirrored the post-prandial elevation of insulin, which led to suggestions that insulin played a role as a post-prandial inhibitor of ghrelin secretion[109]. However the results from studies, conducted in both normal and overweight subjects, that have specifically examined this topic have thus far been mixed.

Two studies reported the presence of the inverse correlation between post-prandial ghrelin and insulin after the consumption of meals that were made up of all macronutrient groups in a conventional balanced ratio[121,128]. In contrast, one study found the correlation only after the consumption of meals dominant in CHO but not after meals dominant in P or F[123] while a fourth study that examined meals dominant in CHO and P found no evidence of post-prandial correlations between the two hormones[125].

The results from our study support the notion that for diet treatments modestly varying in % P and % CHO, no significant correlations exist between the post-prandial rise of insulin and the post-prandial suppression of ghrelin.

2.5.4 Dietary P and urea, glucose, free fatty acids, triglycerides and cholesterol

The changes to levels of fasting and post-prandial urea were linearly associated with the changes to the % P of the diet treatments, which is in keeping with the knowledge that for healthy subjects, the metabolism of urea linearly corresponds to the intake of dietary P (see ***figure 2.18***). The fact that a higher intake of P increases the metabolic pathways leading to increased levels of urea in the serum and in the urine has been well-established[166-168].

When compared to the subjects' habitual pre-study intake for P, which was assessed prior to each four-day *ad libitum* diet intervention by the completion of a four-day food recall dairy[148] (mean %P: 18.5%), the changes to serum urea provide biochemical confirmation that the manipulation of the subjects' diets (and P intake) was being achieved.

No significant changes were found for fasting and post-prandial glucose for any diet treatment (see ***figure 2.19***). This is unsurprising as all the fasting and post-prandial glucose (and levels of fasting insulin) levels remained in the non-pathological range and all of the subjects were lean and healthy. The glucose results are entirely consistent with the known effects of post-prandial digestion and absorption on serum glucose[169].

It was demonstrated that fasting levels of FFAs were highest after four days of *ad libitum* feeding on the 25% P diet and lowest after four days of *ad libitum* feeding on the 10% P diet (see ***figure 2.20***).

A 1966 study[170] was the first to demonstrate that prolonged fasting was associated with progressively higher levels of FFAs, which in turn are correlated with a decrease in urinary nitrogen excretion that measures the metabolism of urea. The suggested relationship between FFAs and P metabolism, with higher levels of FFAs altering the metabolism of P to favour anabolic pathways and suppress catabolic pathways has been confirmed in more contemporary studies[171].

While the current study's period of fasting was shorter than the over 24 hours of fasting in both studies, the direction of our findings, which showed that levels of FFAs were correlated with the %P in the diet, remain consistent with the direction of the earlier studies.

Intriguingly, the changes to levels of FFAs lends support to the *Protein Leverage Hypothesis*. As predicted by the *PLH,* the over-consumption of CHO and F in a low dietary P environment should lead to changes in the metabolic pathways that regulate body protein stores to favour processes that increase plasma levels of amino acids at the expense of breaking down stores of protein. If this were to occur, it would be marked by suppressed fasting levels of FFAs, as was demonstrated in our study.

The post-prandial levels of FFAs did not significantly differ across the diet treatments. This was not unexpected as levels of FFAs have traditionally been assessed under

conditions of fast or after the infusion of metabolically active agents[170-172] with little evidence supporting significant changes following the consumption of food. It is only in T2DM, where post-prandial FFAs are increased by the underlying metabolic derangement from the condition, that there is a suggestion that post-prandial FFAs play a role in pathogenesis of cardiac disease[173]. However there is no evidence to show that this process has any clinical significance in the absence of T2DM.

No differences were found between the three diet treatments for fasting TC. However post-prandial levels of cholesterol significantly differed according to diet treatment with the lowest levels on the 10% P diet (see **table 2.11**). The 18% difference between the lowest to highest cholesterol AUC cannot be explained by any dietary variation in the breakfast meals as they did not vary according to food types or in the % F content (all 20%). In addition, variation in the ingestion of food can be excluded as all of the participants consumed the entirety of their breakfasts within 15 minutes of commencing the Fixed Energy Meal Experiment.

Studies[174,175] that have examined the effects of consuming low fat meals (defined as ≤35% F) on post-prandial fat and lipid metabolism have found generally reported that the fat content of the meal is correlated with post-prandial triglycerides rather than with post-prandial levels of total cholesterol. However in a 2005 study[176] that compared 6 weeks of feeding from diets that were isocaloric and fixed in % F (30%) but varying in % P (14% P vs. 25% P) the low-P diet was shown to be associated with a significantly higher (85%) post-prandial release of chylomicrons.

While our experiment did not measure cholesterol sub-fractions, the finding of higher post-prandial levels of total cholesterol on the lowest % P diet is not inconsistent with the results of the 2005 study. However the strength of this putative relationship remains unknown as does whether the finding is associated with any clinical significance, although it should be noted that there exists a strong and well validated relationship between levels of total cholesterol and cardiovascular disease[177].

2.5.5 Additional comments: strengths, limitations and other issues

It is inherent in their design that despite the best efforts of researchers, experimental dietary interventions do not reflect eating conditions experienced in the *real world* and food consumption is modified accordingly[154]. Many aspects of our experimental protocols such as *ad libitum* food consumption, variety and the choice built into the food menu and the organising of subjects into small groups for the purposes of food consumption closely mimicked normal eating behaviours and this was done so that the obtrusiveness of the experimental protocols was reduced. However, despite these efforts, for most of this study's subjects it remained very obvious that they were involved in a scientific experiment.

Compared to the large majority of single meal test studies that have been discussed in this chapter, one of the strengths of our protocol was the utilisation of same % P dietary treatment for 4 days prior to the start of the test. This allowed for the standardisation of the pre meal-test dietary environment and improved the robustness of the findings.

One additional limitation to our study worth highlighting was the lack of overweight and obese subjects. As can be seen in the baseline participant demographics, the subjects in our study were younger and leaner than the general population and whether our findings can be generalised to older, overweight and obese populations still needs to be determined.

Related to the time intensiveness of the experimental protocol, we found that this aspect was a deterrent to potential subjects in paid employment and had the practical result of limiting recruitment to subjects with larger amounts of unstructured or leisure time, favouring the young (and lean). Over the process of recruitment, it became evident that the numbers of potential subjects who were overweight and obese was much smaller than lean subjects, which would have reduced the statistical power to assess food intake in the overweight and obese so a joint decision was made by the investigators to not enrol overweight and obese subjects into the study.

2.6 CONCLUSIONS

The design of our experimental protocols allowed for a rigorous comparison of diet treatments that were made up of four days of *ad libitum* feeding and a fixed energy intake breakfast, which varied in their % energy intake by protein. We found that the low protein diet treatment was associated with the highest levels of fasting ghrelin and lowest post-prandial levels of cholecystokinin. Furthermore, changes to post-prandial levels of total ghrelin and to the fasting and post-prandial levels of insulin were demonstrated, which corresponded to the meal's macronutrient composition.

These results demonstrate that relatively modest increases to dietary protein are associated with changes to hormone secretion that broadly favour reduced hunger and increased post-prandial satiety, though it should be noted that no subjective changes to measures of hunger and appetite were found.

Whether such changes are present, whether similar hormonal changes are present in the overweight and obese, and whether these hormonal changes are associated with alterations to body weight can only be answered with future research.

CHAPTER 3

Testing the *Protein Leverage Hypothesis* in lean and obese mice

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3.1 INTRODUCTION

3.1.1 Animal studies in the development of the Geometric Framework and Protein Leverage Hypothesis

Simpson and Raubenheimer's research in aphids[178], locusts[179,180] and grasshoppers[86] demonstrated that many different invertebrate species consumed food to meet targets for total energy intake and macronutrients. Using this research, they developed a model of food intake which they termed the *Geometric Analysis of Nutrient Intake* later shortened to the *Geometric Framework*.

In a series of articles[181,182,87,183] they argued that their schematic representation of an animal's food intake and dietary environment, which incorporated macronutrients as the vertical and horizontal axes, provided a unifying schema that not only took into account energy intake and dietary composition but also had the power to predict how changes to the availability of macronutrients would affect the animal's food intake and body weight. They concluded that the *Geometric Framework* model provided the theoretical underpinning to the evidence presented in their invertebrate studies, which

demonstrated that the intake of food was regulated not just by the energy content but also by the macronutrient content in the diet, with the priority being the intake of dietary protein (P).

In 1997 Simpson and Raubenheimer published the *Geometric Analysis of macronutrient selection in the rat*[87], the first vertebrate-based study to incorporate these ideas. The authors re-examined previously published rat and food intake studies within the context of the *Geometric Frameworks*. Two studies examined 12 isocaloric but differing macronutrient ratio food pairings[184] and 12 low P vs. high P food pairings[185] in young rats while a third study examined young to mature rats given a choice of diets that contained 100% pure macronutrients[186]. Reviewing the data through the *Geometric Framework*, the authors demonstrated that *ad libitum* food intake is regulated by the intake of P and non-P macronutrients rather than by total energy content.

In 2005, they published the *Protein Leverage Hypothesis*[42] which developed these ideas further to posit that firstly, the intake of P was prioritised and regulated above the other macronutrients; secondly, that changes to the availability of dietary P resulted in changes in food intake and thirdly, a relatively small reduction in the availability of dietary P would lead, as the animal tries to meet their P target, to a significant increase in total food intake that in turn predisposes the animal to weight gain. While the *PLH* remains a theory, if it is validated, there would be important implications for the study of the decades-old global trend of increased overweight and obesity.

More detailed information on the *Geometric Framework* and *Protein Leverage Hypothesis* can be found in ***Chapter 2:*** *Section 2.1.2.1-2* and ***Figure 2.1*** for an illustration of the *Framework*.

3.1.2 A mouse model for human obesity and testing the PLH

The mouse has long been used as an experimental model for many human diseases and as it shares 99% of the same genetic material as humans this relationship remains just as relevant today[187]. In the current context the C57BL/ 6J mouse has been identified as a strain that readily develops obesity, hyperinsulinaemia, hyperglycaemia and hypertension[188] in response to the ready availability of a high fat diet[189,190]. This Diet Induced Obesity (DIO) C57BL/ 6J mouse model has become a validated and widely used tool for the study of human obesity and the metabolic syndrome[191].

In the first published study that tested the *Protein Leverage Hypothesis* model prospectively, *Sorensen et al*[192] carried out two concurrent *proof of concept* dietary experiments in young and lean C57BL/6J mice. In the first experiment, the mice were provided with 20 days of an *ad libitum* diet that was made up of paired isocaloric foods which varied in the ratio of dietary P to non-P (9% P vs. 49% P, 9% P vs. 31% P, 17% P vs. 48% P and 23% P vs. 48% P). The results showed that the mice regulated the consumption of their food by balancing the intake of the two different chows to reach a protein target of ~23% energy intake by P (hence abbreviated to *X*% P).

In the second experiment separate groups of mice were fed one of the 5 diets varying in %P in a no-choice, *ad libitum* format for 32 days. The results of this experiment

demonstrated that lower the ratio of P in the diet, the higher the intake of food, suggesting that mice consumed their food not to reach a caloric target but to maintain a target for P. Taken together, these experiments demonstrated that food was eaten to prioritise dietary P and not caloric content, consistent with and predicted by the *Protein Leverage Hypothesis*.

For the secondary outcomes of the study, no significant relationships between % P intake and body composition were found except for one in a subgroup of mice from the second experiment fed the 9% P diet, who developed a significantly higher % body fat than their littermates fed the higher % P diets.

Previous studies in obese rats have demonstrated they balance their macronutrient intake under free choice conditions[193] and that their body composition is affected by the macronutrient composition of fixed diets, with adiposity being related to the diet's fat (F) and carbohydrate (CHO) content[194]. However these studies did not focus on the role of dietary P, nor were they designed to test for protein leveraging carefully or to test whether the phenotype of the animal had effects on their macronutrient intake or body composition.

Given the potential implications of the *Protein Leverage Hypothesis* in explaining the aetiology of the recent exponential increase in global rates of overweight and obesity, a carefully designed study to assess these issues in the obese animal is warranted.

3.1.3 The DIO mouse and the secretion of gut hormones and adipokines

As the DIO C57BL/6J mouse is one of the major animal models for studying obesity, understanding the abnormalities that develop in the DIO C57BL/ 6J mouse to the secretion of key obesity related gut hormones and adipokines may shed light on the associated hormonal abnormalities in obese humans.

In a longitudinal study by *Bullen et al*[195] in C57BL/6J mice initially aged 3 - 5 weeks provided with *ad libitum* access to either standard chow (SC) (SC: %CHO| %P| %F: 56%| 27%| 17%) or high fat chow (HFC) (HFC: 43%| 15%| 42%), it was demonstrated that HFC fed mice developed significant and progressive weight gain compared to their baseline weight and to the SC fed mice. Changes to the circulating levels of adiponectin, insulin, and leptin were assessed over the course of the study.

For adiponectin, by week 8 serum levels significantly differed, increasing two and three fold in the SC and HFC fed mice respectively, before falling (more rapidly in the HF group) back towards baseline. Serum insulin levels doubled in HFC fed mice and by 8 weeks were significantly higher than levels in the SC fed mice. Finally for serum leptin, both HF and SC fed mice developed increased serum leptin levels compared to baseline and by week 6, the levels in the HFC group were significantly greater than the SC fed group. Interestingly there were no time or chow related changes of significance in the non-fasting levels of glucose, which remained relatively stable through the 10-week study.

Perreault et al[196] conducted a 20 week study in C57BL/6J mice aged 4 weeks fed either SC or HFC, for 16 weeks to create lean (LE) and obese (OB) phenotypes. The mice were then fed either a low F or a high F diet and fasting and random levels of ghrelin were measured. The authors reported that obesity was associated with reduced levels of fasting ghrelin and a reduced response to exogenously administered ghrelin but that weight loss in the OB mice restored their sensitivity to the effects of ghrelin.

In another 10-week longitudinal study by *Moesgaard et al*[197], female C57BL/6J mice aged 3 weeks at the start of the study were fed SC (%CHO| %P| %F: 63% | 26% | 11%) or HFC (27% | 16% | 59%) with changes to fasting and random levels of ghrelin, glucose and insulin the study end points. While the HFC fed mice gained significantly more weight than SC fed mice, there were no differences found between the groups for fasted levels of ghrelin, glucose and insulin. However, the HFC fed group had significantly lower random levels of ghrelin and higher levels of glucose.

Varady et al[198] examined alternative day caloric restriction vs. DIO feeding in C57BL/6J mice aged 3 weeks at start of the study. After 7 weeks of intervention feeding, adiponectin levels were found to be inversely related to adiposity in all mice.

In the first of two studies examining serum leptin in C57BL/6J mice, *Van Heek et al*[199] demonstrated that serum leptin was highest in the heaviest mice. In the second study *Shi et al*[200] showed that HFC fed C57BL/6J mice had significantly higher levels of leptin compared to littermates fed HFC but who then lost weight after being placed on a SC diet and a control group of SC fed mice. The results of both studies clearly

demonstrated that DIO fed C57BL/6J mice develop hyperleptinaemia in response to weight gain, as seen in humans[201].

Studies utilising other rodent models of obesity such as the HFC fed obese Zucker rat[202-204] and the OB/OB mouse[205] have also consistently reported that obese phenotypes are associated with elevated levels of fasting leptin and reduced levels of fasting ghrelin.

Given what is known about human physiology and the secretion of these same hormones (See ***Chapter 2*** *Section 2.1.3.2* for ghrelin, *Section 2.1.3.4* for insulin and ***Chapter 4*** *Section 4.1.4.1* for adiponectin and *Section 4.1.2.3* for leptin) it is apparent that the DIO fed obese C57BL/6J mouse exhibits the same hormonal response to the secretion of adiponectin, leptin, insulin and glucose as humans (though this may be less so with ghrelin).

This suggests that it is valid to draw conclusions from the changes in the secretion of these hormones in response to an intervention in DIO C57BL/6J mice that may be of use in predicting responses in obese humans.

It is hypothesised that obese mice, when presented with diets differing in percent P, will display the same protein prioritising behaviour as lean mice and preserve energy intake by protein over total energy intake. As a secondary aspect in the study, it will be examined whether body composition and the secretion of glucose, adipokines and gut hormones are affected by diets that vary in P and whether these changes are modified by phenotype.

3.2 MATERIALS AND METHODS

This experiment had two phases. Phase I was the induction of obesity in half the available mice by six weeks of a high fat chow conditioning diet. The primary aim of the study was examined in Phase II, which was to compare in obese and lean mice, their daily food intake of experimental diets that differed in % P. The following secondary outcomes were assessed: body weight, body composition and fasting levels of glucose, ghrelin, adiponectin and leptin.

3.2.1 Animals and Ethical Considerations

50 inbred 8-week old C57 Black/ 6J male mice were ordered from the Animal Resource Centre, Canning Vale Western Australia. However within the timeframe available to conduct the experiment, only 6-week old male mice were available and thus, these mice were used. This strain of mice has been used in the previous *PLH* mouse study[192] and develops obesity on feeding with a high F diet[189].

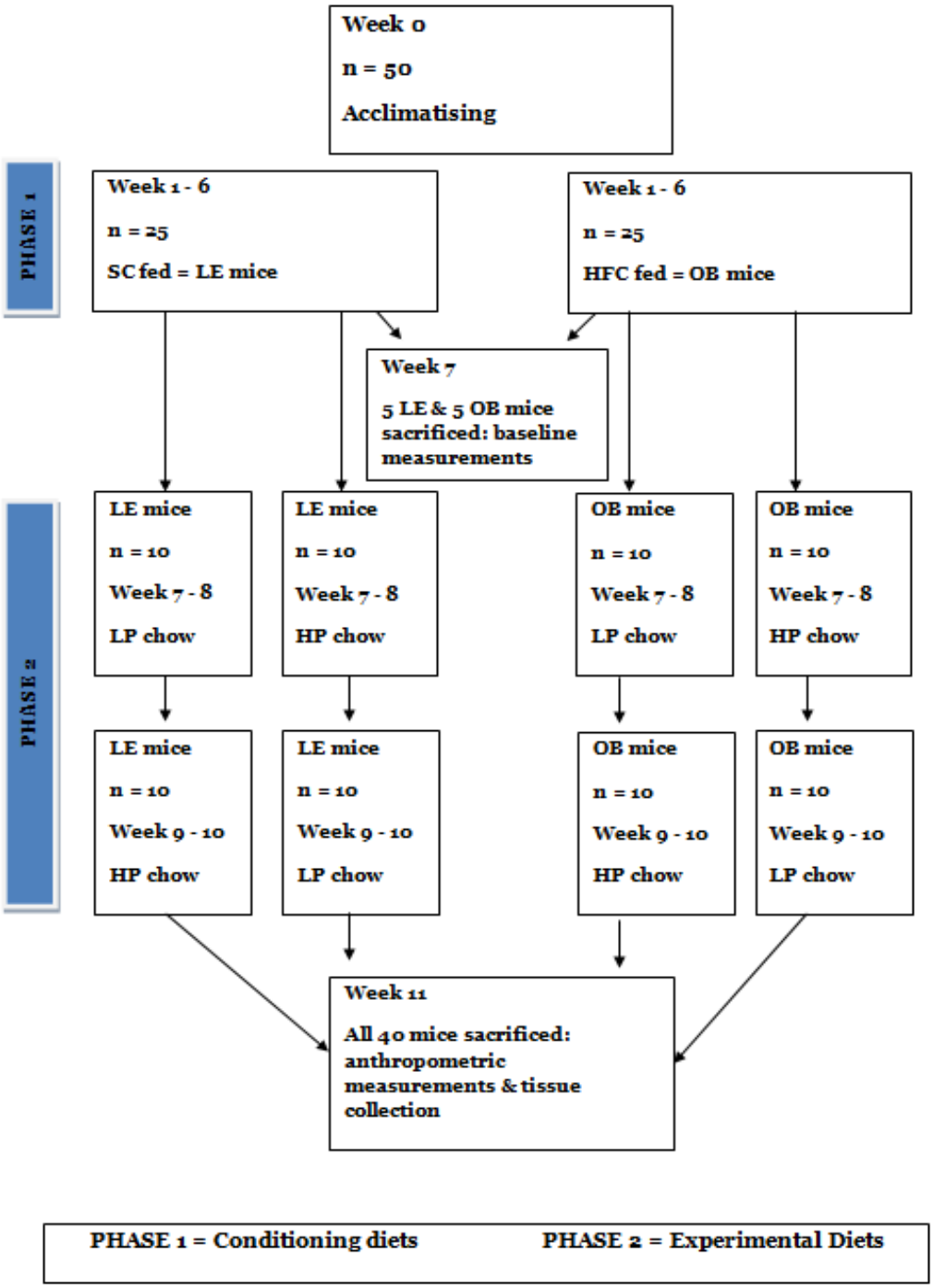
The number of mice used in this study was calculated paying attention to the principle of reduction, to ensure an adequately powered study while minimising animal wastage through the unnecessary processing of a larger number of animals. Power calculations demonstrated that 10 mice would be sufficient to detect a 10% difference in food intake.

The study was carried out under the auspices of the Animal Ethics Committee, University of Sydney as Protocol Number: L02/10-2010/1/5375. Resource and

equipment funding was provided by the grants awarded by the Sydney University Nutrition Research Foundation and Small Equipment Scheme of the School of Molecular Biosciences.

Figure 3.1 presents the experimental protocol in diagrammatic form.

Figure 3.1 Overview of the study of the *Protein Leverage Hypothesis* in Obese mouse



Legend **LE** = lean, **OB** = obese, **HFC** = high fat chow, **SC** = standard chow, **LP** = low protein, **HP** = high protein

3.2.2 *Animal housing and husbandry*

The experiment was conducted in the Animal House of the School of Molecular Biosciences, University of Sydney. Throughout the experiment, the mice were maintained at an ambient temperature of 24 – 26^{0} C and a relative humidity of 53 – 56% under a 12: 12 hour light-dark photoperiod. Lights were turned on at 0730. All the mice were housed on two shelves located in the same room with the position of their cages rotated randomly on a weekly basis.

Each mouse was individually housed in a standard Macrolon Cage Type 2 (Length: 23 cm, Weight: 17 cm, Height: 14 cm) and had *ad libitum* access to water. A double layer of elephant filter (48 x 58 cm^{2}) was provided for bedding material. This was changed twice weekly. Small metal rings, tissue paper and small cardboard tubes were attached or added to the cages to provide environmental enrichment and material for nesting.

A purpose built food spillage collector made of clear plastic was placed into each cage. The collector allowed for an accurate assessment of food intake. Food was placed into the grooves beside the water bottle and in order to eat, mice had to jump into the insert. Food was otherwise out of reach. This design allowed for the author to collect and measure all spilled or incompletely eaten food. See ***Figures 3.2*** **(a)** and **(b)** below.

Figure 3.2 (a) Macrolon Cage Type 2 with insert and environmental enrichment.

NB: The red arrows show where mice sat inside of the insert in order to feed

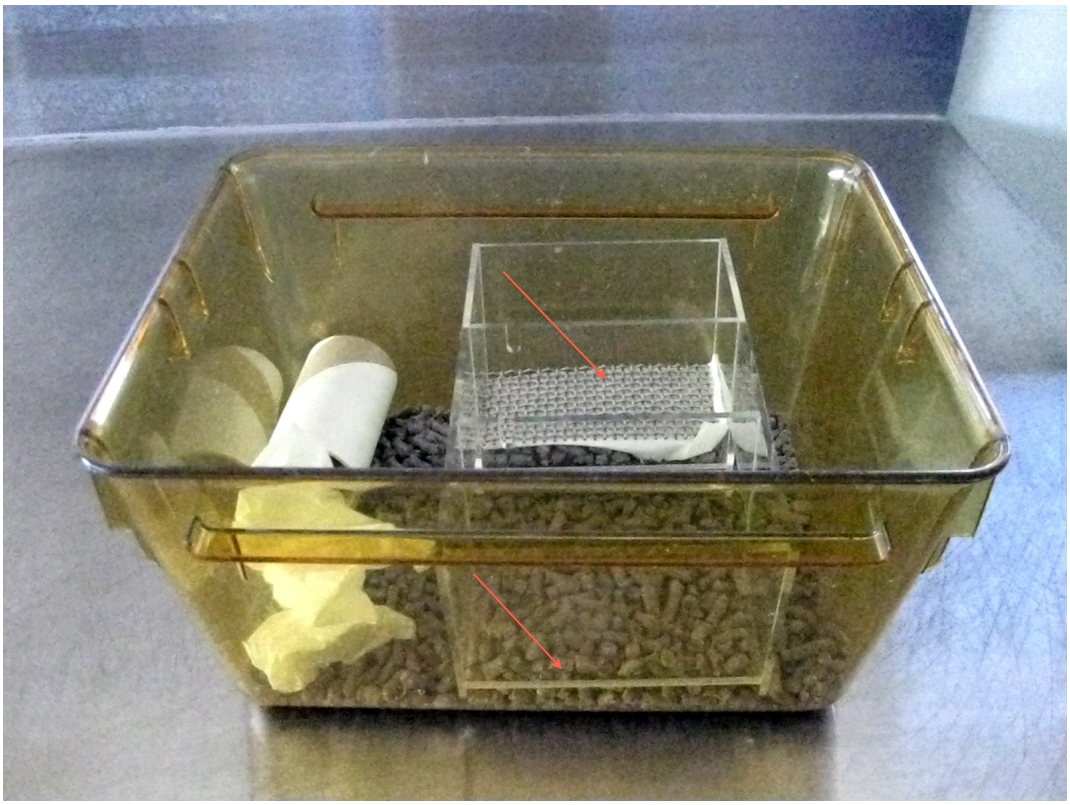


Figure 3.2 (b) Macrolon Cage Type 2 with insert, chow and water bottle

Before the intervention began, the mice had a seven-day period of acclimation during which they were kept under experimental conditions and provided with *ad libitum* access to standard chow. Throughout the entire experiment, only two Animal House staff or the author handled the mice.

3.2.3 Phase I: Conditioning diets and the induction of obesity

At the beginning of the first phase of the experiment, the 50 mice were randomly split into equal groups of 25 prior to the commencement of the conditioning diets. Randomisation was performed according to an open sourced HTML based program. One group of mice was provided with Standard Chow (SC) while the other group was fed High Fat Chow (HFC). This was done to generate two phenotypes of mice: Lean (LE) and Obese (OB). During the Phase I/ conditioning diet period, all of the mice had *ad libitum* access to their chow, which was changed and refilled on a second daily basis.

The SC conditioning diet was Irradiated VS Meat free Rat and Mice Diet by Speciality Feeds, Western Australia. This diet had the following nutritional parameters: Energy (E): 14KJ per gram with the content: %(E) CHO 59.4%, % P 23%, % F 12%.

The HFC used was SF03-002 36% Fat Modification of AIN93G by Speciality Feeds, Western Australia. This diet had the following nutritional parameters: E: 22.8 KJ per gram with the content: % CHO 17.6, % P 15%, % F 59%.

During Phase I, the mice were weighed every seven days, which was performed by placing each mouse in a small wire-cage on top of a set of Mettle Toledo scales (PL602-S, max load 601 g (grams); readability 0.01 g; repeatability 0.008g). To ensure standardisation of weighing and to minimise the distress of the mice, the procedure was carried out in the morning and on the same day of the week in staggered small groups of mice.

At the beginning of the final week of Phase I (week 6) the special plastic feeding inserts (see ***Figure 3.2***) were added to each cage in order to give mice an opportunity to familiarise themselves with the new feeding apparatus prior to the beginning of the experimental diets.

At the end of the Phase I five mice fed SC and five mice fed HFC were randomly selected to be euthanised and their anthropometric data and body tissues collected (see *Section 4.2.5* below for details). This provided the opportunity for baseline measures (body composition, adiposity and fasting levels of glucose and hormones) to be established for both LE and OB mice prior to the experimental diets.

3.2.4 Phase II: Experimental diet treatments differing in percent Protein

At the conclusion of the conditioning diets (end week 6) the experiment moved to Phase II, which involved two 2-week feeding periods where mice were provided with *ad libitum* access to one of two experimental diet treatments in a random order. The 1st Feeding period was weeks 7 and 8 while the 2nd Feeding period was weeks 9 and 10.

Prior to commencing Phase II, the remaining 20 mice from the LE group and 20 mice from the OB group were randomly split into two subgroups of equal size (10 mice) (i.e. the 20 LE mice into two sub-groups of n=10; the 20 OB mice into two subgroups of n=10) to allow for a comparison of the effect of the order of the experimental diet treatments.

The experimental diet treatments consisted of custom manufactured High Protein (HP) or Low Protein (LP) chow. The two experimental diet feeds were isocaloric but differed in % E by dietary P and were prepared according to the recipes reported by Sorenson *et al*[192]. Manufacture was by Gordon's Speciality Stock Feeds (Bargo NSW), who had prior experience in making the chow in 2007, when they prepared the same feeds following the same recipes for a published experiment that tested the *Protein Leverage Hypothesis* in mice (*Personal correspondence;* Simpson SJ 15th Dec 2009[206], Huang X, 29th March 2011).

The experimental feeds were a semi-synthetic chow manufactured in the form of dried pellets and made up of the following ingredients: casein, cornstarch, wheat starch, sucrose, sunflower oil, cellulose powder, a mineral mix and a vitamin mix. Both isocaloric chows had a similar composition except for the ratio of casein to starch, which determined the % P content.

The nutritional composition of the LP diet treatment was E: 3.8 kcal per gram with the content: % CHO: 71.8%, % P: 9% and % F: 7.8%. The nutritional composition of the HP diet treatment was E: 3.8 kcal per gram with the content: % CHO: 57.4%, % P: 23%, %F: 7.8%.

For both LE and OB mice, now split into two equally sized sub-groups of 10 mice, one sub-group was randomly allocated to start their 1st feeding period (14 days) with HP chow to be followed in their 2nd feeding period by LP chow. The other sub-group was allocated the LP for their 1st feeding period followed by HP chow for their 2nd feeding period (see ***Figure 3.1*** for details).

During this phase of the experiment, the mice were weighed at the beginning and end of each week. Fresh, pre-weighed portions of chow were placed into each mouse's cage on a 2nd daily basis with enough food provided to ensure that mice had access to chow at all times. The plastic feeding inserts were changed on a twice-weekly basis except during the cross-over period from HP to LP chow or *vice versa*. At these times, before dispensing the new chow, all of the uneaten old chow was discarded (after weighing) and both the Macrolon cage and plastic insert changed.

During each distribution of food the remaining chow (uneaten and spilled) was collected from inside the plastic insert and its weight tallied (Mettler Toledo PL602-S scales) before being discarded. The intake of food for each mouse was calculated as the difference between food given and food remaining with the daily intake calculated by averaging food intake over 7 days (1/2 feeding period).

At the end of Phase II and the experimental diet feeding (end week 10) all of the mice were euthanised and blood and body tissues collected (see *Section 3.2.5* below).

3.2.5 Assessment of glucose, euthanasia and the collection of blood and body tissues

After an overnight fast where food but not water was removed by 1700 of the preceding day, the mice were euthanised. At 0800, immediately prior to euthanasia, the mouse's fasting blood glucose level was measured by the method of tail docking. At least 0.6 uL of blood was applied to an Accu-Chek Performa Electrode strip attached to an Accu-Chek Performa blood glucose meter (Roche Diagnostics Pty Ltd, Australia). At the beginning (week 0), during the middle (week 6) and at end of the experiment (week 11), the performance of the Accu-Chek Blood Glucose meter was assessed with reference to the Accu-Check Performa Control solution.

The mice were individually euthanised in a room separated from their littermates to minimise their potential distress. The author performed the euthanasia of all the mice and also performed all the procedural work to collect their blood and body tissues.

Each mouse was killed by cervical dislocation. Their weight was promptly recorded and immediately thereafter at least two millilitres of whole blood was collected by intra-cardiac puncture. For plasma collection at least 1 mL of blood was placed into a K2-EDTA containing micro-container and for serum collection, at least 1 mL of whole blood was placed into a SST Gel micro-container (BD Micro-containers with micro-guard closure; Becton, Dickson & Company Diagnostic Pty Ltd, Australia). The BD micro-containers were stored in a refrigerator (set at 2^{o}C) for no longer than 20 minutes before being centrifuged in an unrefrigerated device for five minutes at 10,000RPM. The supernatant plasma or serum was transferred into individual

eppendorfs that were then placed into the freezer (-20°C) prior to their same day transfer to a specialised -80°C sample refrigerator. Samples were kept in this unit until they were removed for biochemical or hormone analysis.

For the final step of this process, each mouse was dissected following the validated *Reed 1* project protocol[207] to assess body composition and adiposity. Firstly, the abdominal cavity was exposed. Secondly the bilateral retro-peritoneal and peri-renal fat pads were identified and removed. No attempt was made to isolate and discard vessels, ureters or the adrenal gland from the adipose tissue. Thirdly, the gonadal fat pads were identified with reference to their proximity to the epididymis and vesicular gland and then resected. Both groups of fat pads were weighed with total gonadal fat pad mass, total peri-renal fat pad mass and total abdominal adiposity recorded (the latter the sum of gonadal fat and peri-renal fat).

3.2.6 Measuring Adiponectin, Ghrelin, Insulin and Leptin

Adiponectin, total ghrelin, insulin and leptin were all measured by manual radioimmunoassay (RIA) technique. The RIAs were performed over consecutive days with samples subjected to 4 or fewer freeze / thaw cycles. All of the mice RIAs were kindly performed by senior hospital scientists with the Endocrinology Laboratory of Royal Prince Alfred Hospital, NSW (Ms. Marilyn Brown for adiponectin and leptin and Mr. Kim Lee for ghrelin and insulin).

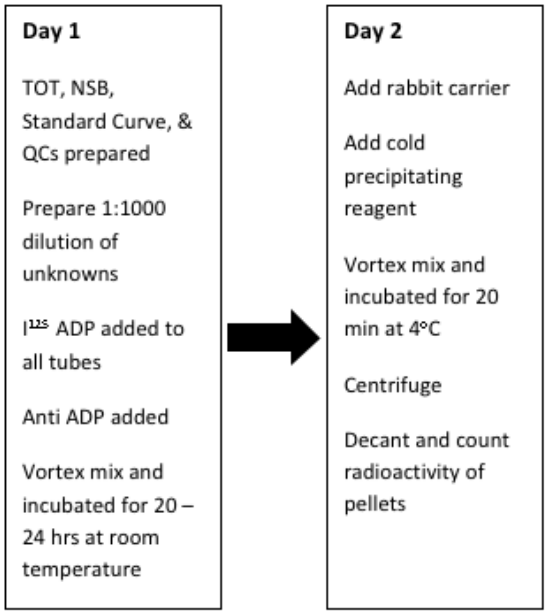
Controls and QCs were assayed in duplicate while unknowns were assayed as singletons. However to assess the precision of the assays, at least 5% of the unknowns

were assayed in duplicate. The intra-assay CV% from these duplicate samples were all <10%. Further details regarding the methodology of RIAs can be found in ***Chapter 2***, *section 2.2.6.*

Adiponectin

Mouse adiponectin was measured with the MADP-60HK kit (Millipore, Billerica, MA, USA). As per the manufacturer's product information statement (PIS)[208] the minimal volume of plasma or serum for this assay is 5 µL of sample, which must be diluted prior to being processed. 4995 µL of assay buffer was added to 5 µL of the unknown sample, which prepared a 1:1000 dilution. 100 µL of the diluted unknown sample was then assayed. Using 100 µL of a 1:1000 dilution, the stated recovery is 97-114% and the lowest level of adiponectin detectable by the assay 1 ng/mL. The MADP-60HK assay has a 100% specificity for mouse and rat adiponectin and has an intra-assay precision of 3.73- 4.43 %CV and an inter-assay precision of 8.24 – 7.13 %CV (for sample concentrations of 3 and 12 ug/ml). ***Figure 3.3*** outlines the procedure for the mouse adiponectin RIA.

Figure 3.3 Outline of the Adiponectin RIA



Plastic pointed tubes were prepared TOT, NSB, blank, the standard curve (0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 ng/mL), low QC, high QC and for unknown samples. Assay buffer was to the following tubes as follows: 300 *u*L to the NSB tube, 200 *u*L to the blank tube and 100 *u*L for each of the standards, QCs and unknown tubes. Following this 100 *u*L of standards, QCs and 100 *u*L of the 1:1000 dilution of unknown serum samples were added to their respective tubes. Next, 100 *u*L of iodinated ADP was added to all tubes followed by 100 *u*L of anti-ADP to all tubes except for the TOT and NSB tubes. Finally, all of the tubes were vortex mixed, covered and incubated at room temperature (22-25^{o}C) for 20-24 hours.

On the second day 10 *u*L of rabbit carrier was added to all tubes except for TOT. Following on, 1000 *u*L of cold precipitating reagent was added to all of the tubes except for TOT. The tubes were then vortex mixed, covered and incubated at 3.5^{o}C for 20 minutes. The TOT tube was placed aside and the remaining tubes centrifuged (HD

Scientific / Hettich Zentrifuger Rotixa 50RS refrigerated centrifuge) at 4000g for 30 minutes at 4°C. After centrifuging, the tubes were decanted by inversion and placed onto absorbent paper with care taken to not dislodge the pellet at the bottom of each tube. All tubes were then placed into the automatic gamma-counter and the ADP specific program selected to run.

(Total) Ghrelin

Mouse (total) Ghrelin was measured using the GHRT-89HK kit (Millipore, Billerica, MA, USA). As per the manufacturer's PIS[151] this kit has a 100% specificity for rodent ghrelin. 50 µL of plasma is the minimal volume of unknown sample that can be assayed while still ensuring assay validity. Further details regarding the RIA of ghrelin can be found in ***Chapter 2,*** *Section 2.2.6.2.*

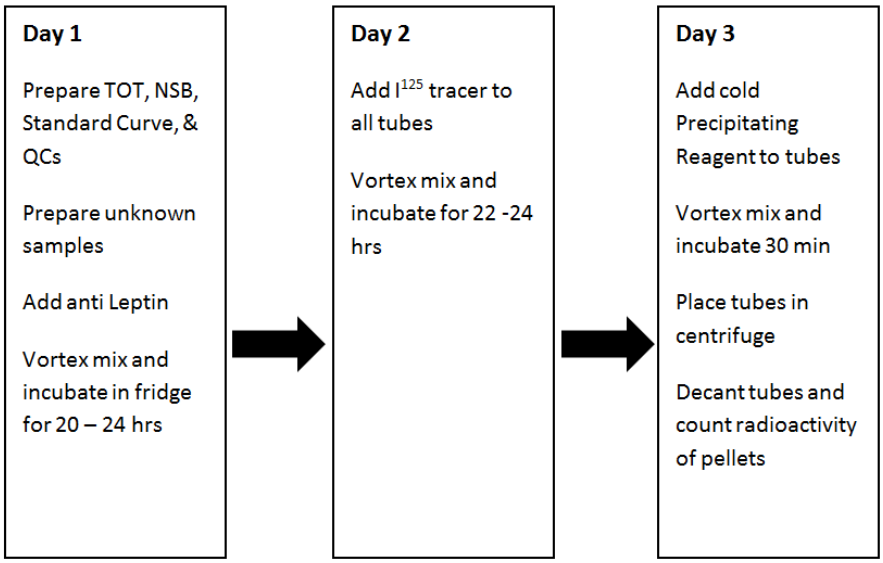
Insulin

Mouse insulin was measured with the RI-13K kit (Millipore, Billerica, MA, USA). As per the manufacturer's PIS[209], 50 µL of plasma or serum is the minimal volume that can be assayed to ensure assay validity. The assay has an 100% specificity for mouse insulin with the lowest sensitivity at 0.1 ng/mL. The intra-assay precision is 1.4 – 4.6 %CV with the inter-assay precision 8.5 – 9.4 %CV using concentrations of insulin of 0.5 – 3.7 ng/mL. The normal fasting range was reported to be 0.5-2 ng/mL. Further details regarding the RIA of insulin can be found in ***Chapter 2****, section 2.2.6.4.*

Leptin

Mouse leptin was measured using the ML-82K kit (Millipore, Billerica, MA, USA). As per the manufacturer's PIS[210], 50 µL of plasma or serum is the minimal volume that can be assayed while ensuring assay validity. The intra-assay precision is 4 – 11.2 %CV with the inter-assay precision 3.3 – 14.6 %CV using concentrations of leptin of 0.4 – 5.4 ng/mL. ***Figure 3.4*** outlines the procedure for the mouse leptin RIA.

Figure 3.4 Outline of the mouse leptin RIA



Pointed polypropylene tubes prepared for TOT, NSB, blank, the standard curve of known concentrations of leptin (0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 ng/mL), the low and high QCs and for the unknown samples. Assay buffer was transferred into the NSB tube (300 *u*L), blank tube (200 uL) and into each of the tubes for standard curve, QCs and unknown samples (100 *u*L). 100 *u*L of standards, QCs or unknown samples were transferred into the corresponding tubes followed by 100 *u*L of mouse leptin

antibody to all tubes except the TOT and NSB. The tubes were then vortex mixed, covered and incubated in the refrigerator at 3.5°C for 20 - 24 hrs.

On the second day, 100 *u*L of iodinated mouse leptin was added to all of the tubes. The tubes were then mixed, covered and incubated at 3.5°C for 22 - 24 hrs.

On the third day, 1000 *u*l of cold precipitating reagent was added to all tubes except for the TOT, which was placed aside. The tubes were vortex-mixed and incubated for 20 minutes at 3.5°C. These tubes were then centrifuged at 3000g for 20 minutes at 4°C (HD Scientific / Hettich Zentrifuger Rotixa 50RS refrigerated centrifuge) with the supernatant decanted by carefully inverting the tubes onto absorbent paper. All of the tubes were then placed into the automatic gamma counter and the mouse leptin specific program run.

3.3 DATA ENTRY AND STATISICAL ANALYSES

Data was entered into a series of spread-sheets using Microsoft Excel 2008 for Mac (version 12.3.1 build 110725). Following data entry, the computerised data underwent a cleaning cycle that consisted of cross-referencing spreadsheet figures back to the original source material. All of the statistical analyses were performed using the IBM SPSS Statistics Version 19 software package (IBM Corp, NY, USA). The author performed all of the data entry and all of the statistical analyses.

Data was checked for normality through the Kolmogorov–Smirnov test and is reported as mean ± Standard Error Mean (SEM) unless otherwise stated. Statistical results were considered significant if $p < 0.05$ except when noted. A sample size of 10 mice was calculated to provide 80% statistical power ($\alpha = 0.05$) to detect a 10% difference in the primary end point, which was total daily energy intake.

For the RIAs of adiponectin, ghrelin, insulin and leptin three data points (6% of the data) were missing due to insufficient volume of samples. One data set for adiposity (2% of the data) was incomplete due to difficulties with dissection of the animal. All the missing data sets have been treated as reversion to the mean. There were no other examples of missing data.

Between group differences in body weight, food intake, glucose, adiponectin, ghrelin, insulin and leptin were assessed by independent sample t-tests. In addition, for food intake, within-group analyses were carried out using one-way within-subject repeat measures analysis of variance (ANOVA-RM).

Data was initially checked for sphericity using the Mauchlys sphericity test and if this was violated, the *Greenhouse-Geiser* correction was applied to the *F* and *p* values. When relevant, *post-hoc* analyses were calculated with the *Bonferroni* correction method applied for the multiple, pair-wise comparisons. To assess for the effects of the order of the experimental diets, ordering was added as a co-factor to the ANOVA-RM.

As the mice supplied were younger than that originally planned and as the age of a mouse affects the degree of weight gain (see Section 3.5.2), a sub-group analysis was conducted out of body weight and food intake data from the top and bottom quartile of mice for weight. This was carried out to ensure that the direction of the findings for the overall cohort remained consistent to that of mice at the extremes of body weights.

3.4 RESULTS

3.4.1 Phase I: The conditioning diet and the induction of obesity

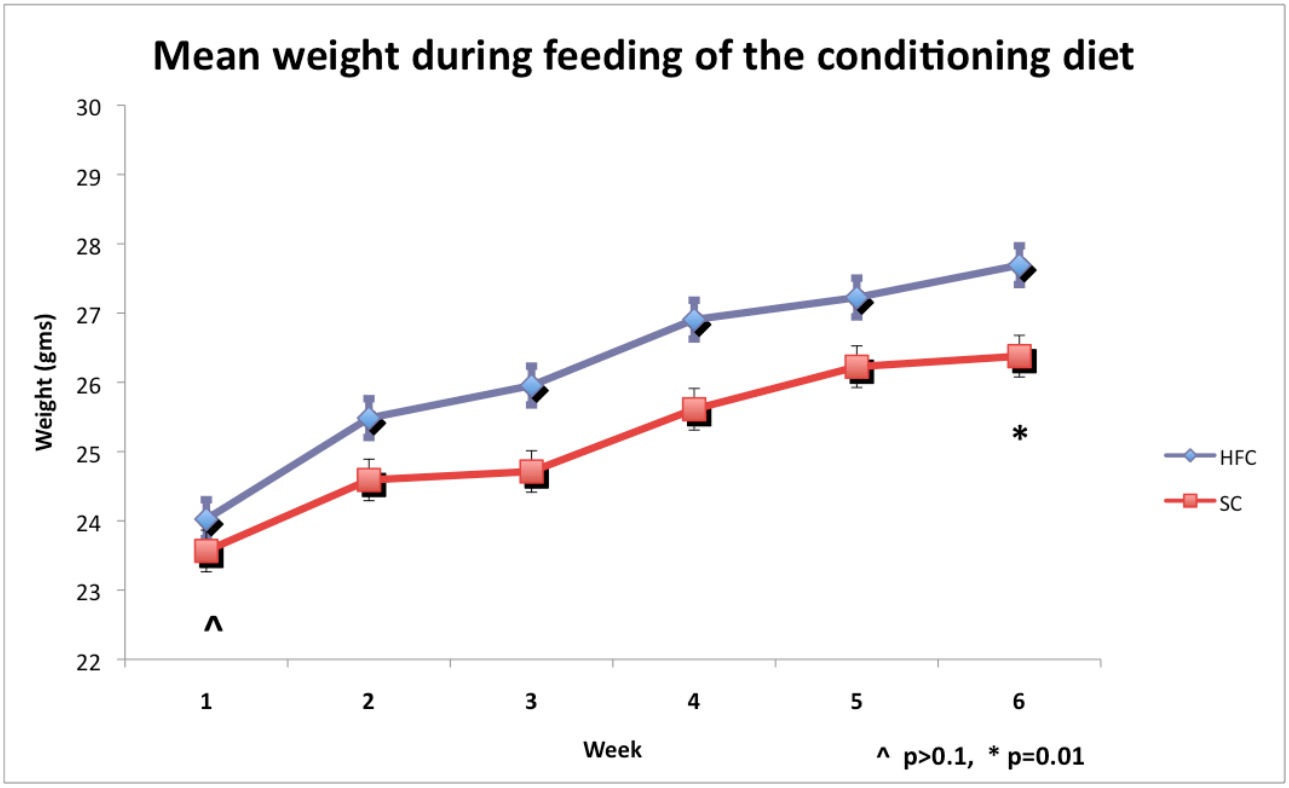
Table 3.1 shows results for weight at the beginning and conclusion of the conditioning diets.

Figure 3.5 displays the mean weekly weight during the period of the conditioning diets. All figures presented as mean + SEM.

Table 3.1 Body weight before and after conditioning diets for LE and OB mice

Weight (gms)	LE mice	OB mice	Analyses
Pre conditioning diet (week 0)	23.57 ± 0.27	24.02 ±0.28	$p = 0.25$
Post Conditioning diet (week 6)	26.37 ± 0.3	27.70 ± 0.36	$p = 0.001$

Figure 3.5 Mean weekly weight during Phase I/ conditioning diets



Mean weight was assessed at the beginning and end of Phase I. At the beginning there were no significant differences for weight mice (Feed/Group: Weight (gms); SC/LE: 22.8 ± 0.27; HFC/OB: 23.5 ±0.28; p=0.25). By the end of Phase I the OB mice were significantly heavier than the LE (+5% or +1.33 gms, p = 0.01).

Significant within-group differences were also found for both LE and OB mice when weight at the beginning was assessed against weight at the end of the conditioning diets. (Δ Wgt (gms): LE: +3.6 ± 0.21, p <0.0001; OB: +4.2 ± 0.16, p <0.0001).

3.4.2 Phase I baseline measures: body composition, fasting glucose, adiponectin, total ghrelin and leptin

Baseline measures

These results were derived from the 5 LE and 5 OB mice sacrificed at the end of the conditioning diet. The LE mice were significantly lighter and (non-significantly) increased total-abdominal adiposity with significantly lower levels of fasting leptin and a trend towards higher levels of adiponectin. **Table 3.2** shows baseline results for LE and OB mice.

Table 3.2 Baseline characteristics at the end of the conditioning diet: LE and OB mice

Group / Measures	**LEAN N=5**	**OBESE N=5**	**Analyses**
Length (mm)	80.8 ± 0.8	84.4 ± 1.7	$p > 0.1$
Weight (gms)	24.7 ± 0.7	27.9 ± 0.7 *	$p = 0.01$
Abdominal adiposity (gms)	0.6 ± 0.1	0.7 ± 0.1	$p > 0.1$
Glucose (mmol/L)	6.0 ± 0.4	6.5 ± 0.3	$p > 0.1$
Adiponectin (µg/mL)	44.6 ± 9.0	17.9 ± 1.7	$p = 0.07$
Total ghrelin (ng/mL)	5.9 ± 1.5	4.9 ± 1.1	$p > 0.1$
Insulin (ng/mL)	0.9 ± 0.2	1.4 ± 0.3	$p > 0.1$
Leptin (ng/mL)	2.9 ± 0.4	4.8 ± 0.2	$p = 0.006$

3.4.3 Phase II: experimental diets and food intake

Table 3.3 presents Food Intake (FI) and Energy Intake per diet treatment per mouse for LE and OB mice.

Table 3.4 presents mean daily FI according to diet treatment order for the subgroups of LE and OB mice.

Table 3.5 presents total FI per chow per feeding period for LE and OB mice.

Figure 3.6 displays total FI per feeding period for the LE and OB mice.

Figure 3.7 shows FI per ½ week and according to diet treatment order for LE and OB mice.

Over the 2 feeding periods (each 2 week duration) of Phase II / experimental diet feeding, both phenotypes consumed a greater intake for LP chow compared to HP chow.

Mean Food Intake per feeding period per mouse for LP chow vs. HP chow was:

LE mice: 53.8 vs. 47 gms / +14% LP chow/ 204.4 vs. 178.6 kcals; p <0.0001.

OB mice: 56.3 vs. 51.2 gms/ +10% LP chow/ 213.9 vs. 197.0 kcals; p <0.001.

Table 3.3 Phase II Experimental Diets: Food and Energy Intake per mouse

Food Intake (gms)/ Energy Intake (kcal)	**LEAN N=20**		**OBESE N=20**	
	LP chow (N=20)	**HP Chow (N=20)**	**LP Chow (N=20)**	**HP Chow (N=20)**
Mean FI per mouse 1st Feeding period	24.3 ± 1.6	23.3 ± 1.6	27.6 ± 1.6	24.9 ± 1.5
Mean FI per mouse: 2nd Feeding period	29.5 ± 1.5	23.7 ± 1	28.7 ± 1.7	27 ± 1.4
Total FI per mouse	53.8 ± 2.7	47.0 ± 2.8§	56.3 ± 3.6	51.2 ± 3.2¥
Total EI per mouse	204.4 ± 10.4	178.6 ± 10.6	213.9 ± 13.6	197.0 ± 12.2

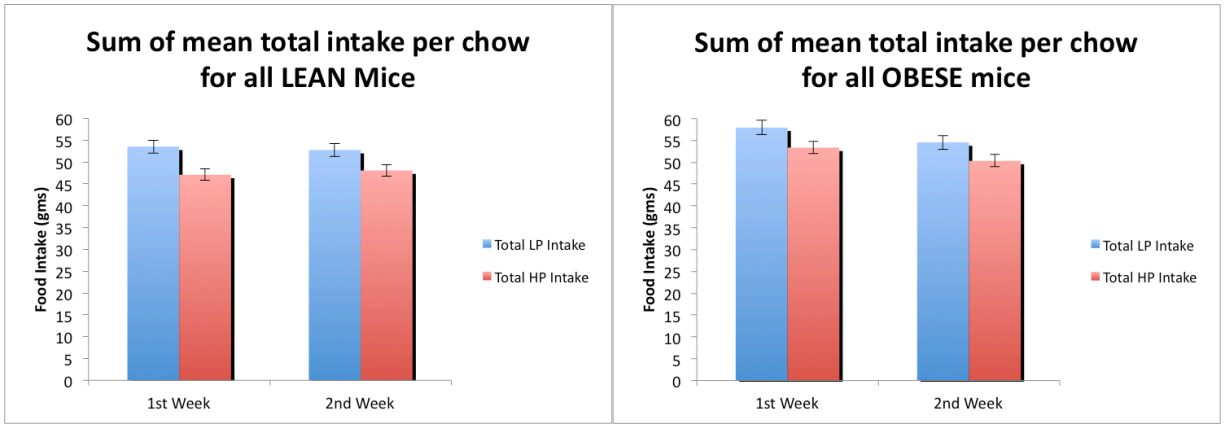
Statistical Differences: **¥** = p <0.001 **§** = p<0.0001

Table 3.4 Mean daily FI by week per diet treatment order for LE and OB mice

Mean Daily Food Intake (gms) by week	LEAN N=20		OBESE N=20	
	Subgroup LP –> HP N=10	Subgroup HP –> LP N=10	Subgroup LP –> HP N=10	Subgroup HP –> LP N=10
Week 7	^{LP} 3.3 ± 0.1	^{HP} 3.1 ± 0.1 §	^{LP} 4.0 ± 0.1	^{HP} 3.7 ± 0.1 §
Week 8	^{LP} 3.5 ± 0.1	^{HP} 3.4 ± 0.1 §	^{LP} 3.8 ± 0.1	^{HP} 3.4 ± 0.1 §
Week 9	^{HP} 3.4 ± 0.1	^{LP} 4.3 ± 0.1 §	^{HP} 3.9 ± 0.1	^{LP} 4.2 ± 0.1 §
Week 10	^{HP} 3.3 ± 0.1	^{LP} 4.1 ± 0.1 §	^{HP} 3.7 ± 0.1	^{LP} 4.4 ± 0.1 §

Statistical Differences: For both groups: Within-group weight vs. Time § = p <0.0001

Figure 3.6 Total FI per ½ feeding period (1 week) per chow for **(a)** LE and **(b)** OB mice



(a)LE mice **(b) OB mice**

For LE mice in detail: Analyses indicated significant differences for the total intake of LP vs. HP chow and for averaged daily FI per week. The latter remained significant after the order of diet treatments was added as a co-factor to the analyses: F (3, 1.2) = 20.9, p <0.0001.

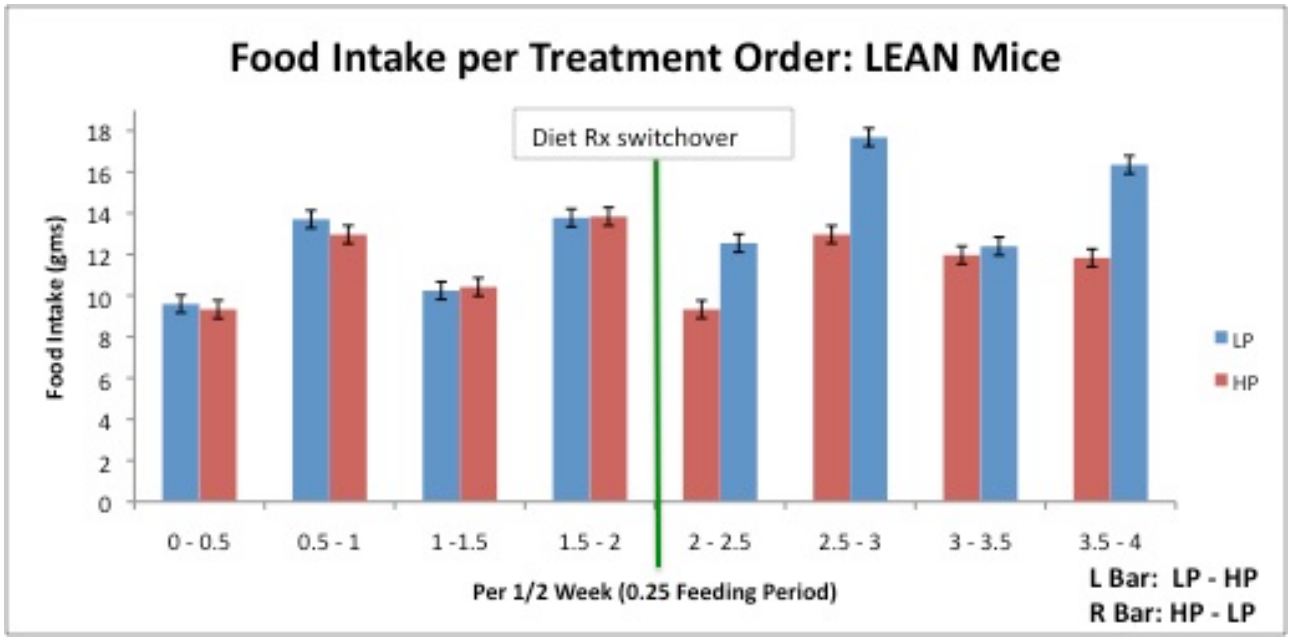
For OB mice in detail: Analyses also indicated differences for total intake of LP vs. HP chow and for averaged daily FI per week, which also remained significant after the order of diet treatments was added as a co-factor to the analyses: F (3, 0.7) = 24.26, p <0.0001.

Effects of the order of the diet treatments

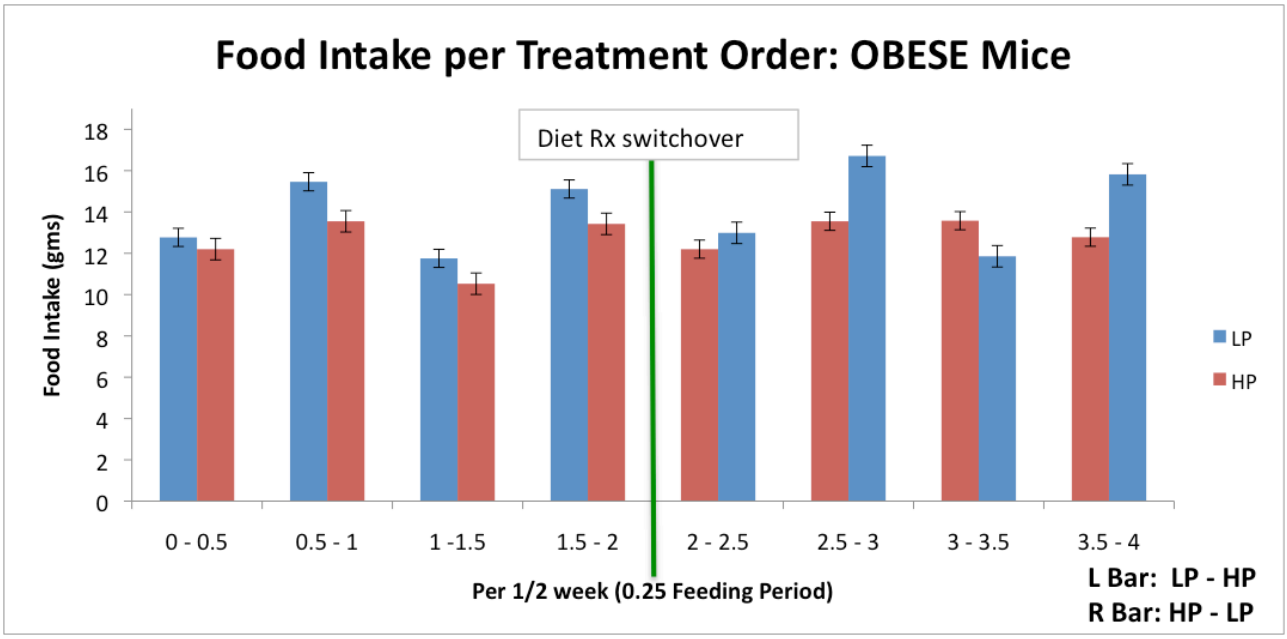
Table 3.5 Total FI per chow according to feeding period for all LE and OB mice

Total Food Intake (gms)	**LEAN N=20**		**OBESE N=20**	
	LP	**HP**	**LP**	**HP**
1st Feeding Period	485.3	465.6	551	497
2nd Feeding Period	589.8	473.2	573.8	539.6
Total FI (2 feeding periods)	1063.1	951.3	1124.8	1036.6

Figure 3.7 Food Intake per 1/2 week according to treatment order for **(a)** LE and **(b)** OB mice



(a) LE mice



(b) OB mice

The magnitude of the difference between the intake of LP and HP chow was affected by the order in which the feeds were presented. In particular, a greater increase in LP chow was seen when the diet treatment order was HP then LP than *vice versa*. This also meant that when either LP or HP chow were given in the 2nd Feeding Period (i.e. weeks 9 and 10), a greater amount was consumed.

However, the differences in total food intake and mean averaged daily intake per week remained significant even after the order of the diet treatments was added as co-factor to analyses.

This phenomenon can be seen in **Tables 3.4** and **3.5** and with particular reference in ***Figure 3.7*** by comparing the mean FI per diet treatment by feeding periods.

3.4.4 Phase II: Experimental diets and body weight

Table 3.6 presents weight over time during the period of experimental diets.

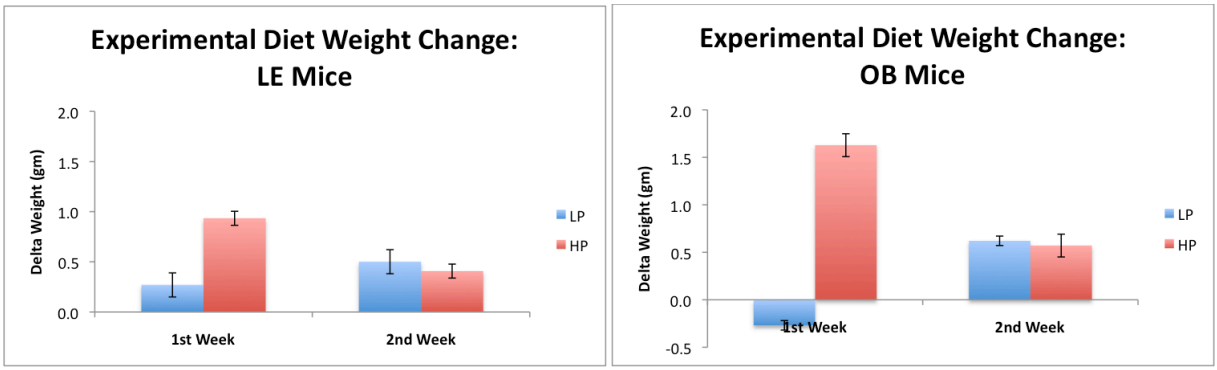
Figures 3.8 and ***3.9*** display the changes in weight per subgroup of LE and OB mice according to the experimental diet.

Table 3.6 Phase II: mean weight per week for LE and OB mice

Phase II per week: Weight (gms)	**LEAN N=20**	**OBESE N=20**
Week 7	25.7 ± 0.4 §	27.1 ± 0.4 §
Week 8	25.8 ± 0.4 §	27.1 ± 0.4 §
Week 9	27.0 ± 0.4 §	28.6 ± 0.4 §
Week 10	27.3 ± 0.4 §	29.1 ± 0.5 §

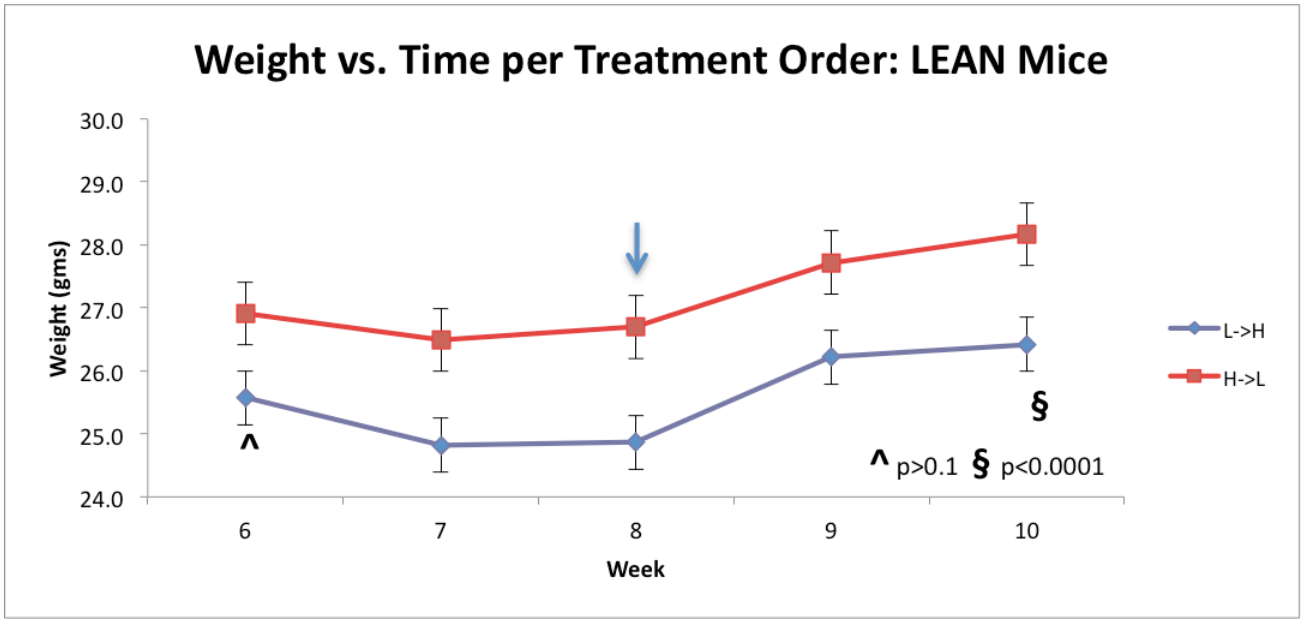
Statistical Differences: For both phenotypes within group weight vs. time **§** = $p<0.0001$

Figure 3.8 Phase II: Δ Weight per week per chow for **(a)** LE mice and **(b)** OB mice

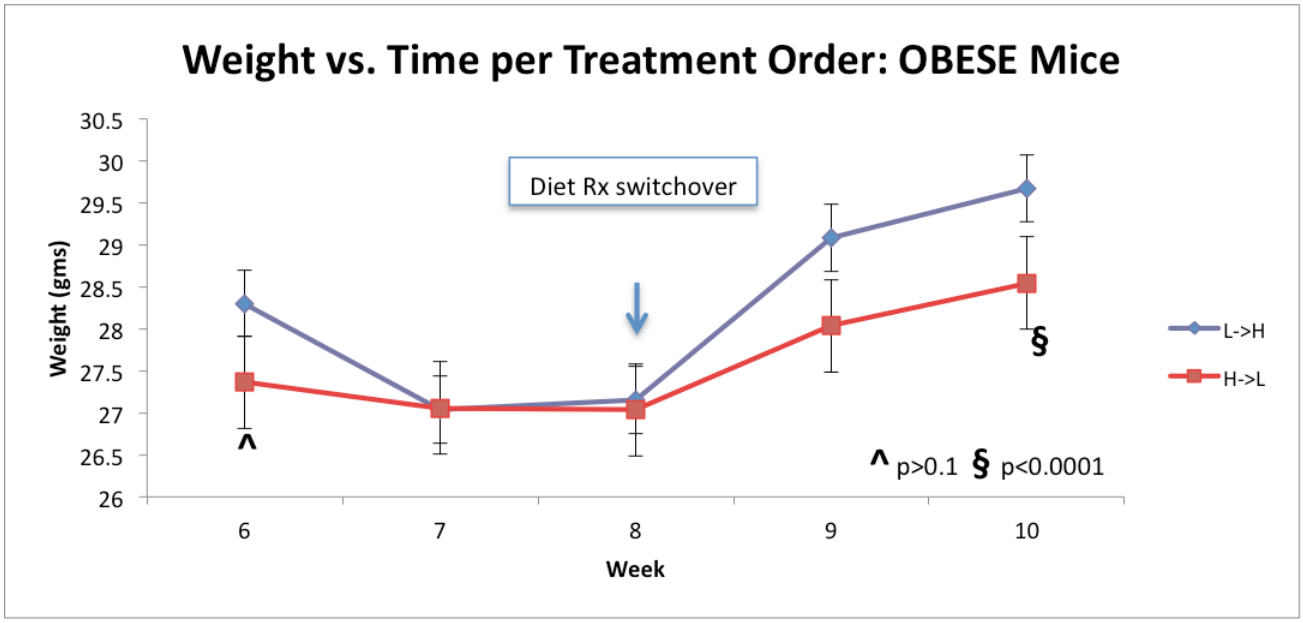


(a) LE mice **(b) OB mice**

Figure 3.9 Phase II: Weight vs. time per treatment order for **(a)** LE and **(b)** OB mice



(a) LE mice



(b) OB mice

NB: Experimental diet treatments switched over at Week 8

At the beginning of Phase II, there were no significant within group differences for weight. By the end of the 2 feeding periods, when weight over time was assessed, both groups had gained weight over Phase II (LE +1.6gm, +6%, OB: +2g, +7%; unadjusted p <0.0001) but when diet treatment order was added as a co-factor to the analysis, the weight changes only remained significant for the OB group.

For LE mice: Weight over time was significantly different but when diet treatment order was added as a co-factor to the analysis, differences were no longer significant (adjusted F (3, 0.9) = 0.3, p =0.8).

For OB mice: when week over the time was assessed, a significant difference was found which remained significant after diet treatment order was added as a co-factor: (adjusted F (1.96, 1.70) = 3.29, p =0.03 with the *Greenhouse-Geisser* correction).

3.4.5 Phase II: Experimental Diets and Secondary Outcomes

When considering secondary outcomes by the end of Phase II, the LE mice weighed less, were shorter and had higher levels of adiponectin than the OB mice.

Tables 3.7 and **3.8** show the secondary outcomes at the end of Phase II per phenotype and according to the order of the diet treatments respectively.

Table 3.7 End of Phase II: Secondary measures per phenotype

Group / Measures	**LEAN N=20**	**OBESE N=20**	**Analyses**
Length (mm)	81.9 ± 0.5	84.0 ± 0.6	*p = 0.02*
Weight (gms)	26.6 ± 0.3	28.3 ± 0.5	*p <0.01*
Total Abdominal Adiposity (gms)	0.7 ± 0.1	0.8 ± 0.1	*p* = 0.45
Glucose (mmol/L)	6.7 ± 0.3	6.9 ± 0.2	*p* = 0.53
Adiponectin (µg/mL)	59.0 ± 7.9	37.7 ± 4.4	*p <0.01*
Total Ghrelin (ng/mL)	9.3 ± 0.8	9.4 ± 0.8	*p* = 0.97
Insulin (ng/mL)	1.0 ± 0.1	1.3 ± 0.2	*p* = 0.22
Leptin (ng/mL)	6.7 ± 0.6	7.8 ± 0.6	*p* = 0.16

Table 3.8 End of Phase II: Secondary measures according to order of diet treatments

Group / Treatment order	**LEAN N=20**		**OBESE N=20**	
Measures	**LP –> HP N=10**	**HP –> LP N=10**	**LP –> HP N=10**	**HP –> LP N=10**
Length (mm)	80.8 ± 0.6	83.0 ± 0.8 *	84.9 ± 0.6	83.0 ±1.0
Weight (gms)	26.0 ± 0.4	27.1 ± 0.4	28.9 ± 0.7	27.7 ± 0.6
Abdominal Adiposity (gms)	0.6 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Glucose (mmol/L)	6.2 ± 0.3	7.1 ± 0.4	6.3 ± 0.4	7.4 ± 0.2 *
Adiponectin (µg/mL)	83.6 ± 8.5	65.0 ± 7.3	28.4 ± 3.3	46.9 ± 7.1 *
Total Ghrelin (ng/mL)	7.6 ± 0.9	11.0 ± 1.1 *	9.8 ± 1.4	8.9 ± 0.7
Insulin (ng/mL)	1.1 ± 0.2	0.9 ± 0.2	1.4 ± 0.3	1.1 ± 0.2
Leptin (ng/mL)	5.2 ± 0.5	8.1 ± 0.8 ¥	7.5 ± 1.0	8.1 ± 0.6

Statistical Differences: All p>0.05 except for: * $p < 0.05$ ¥ $p < 0.01$

3.4.5.1 Anthropometric Measures

In detail, when between phenotype comparisons were made of the anthropometric measures at the end of Phase II, all measures were greater for the OB mice but only for two reached significance: length (Δ LE vs. OB: -2.1 ± 0.8 cm, p=0.02) and weight (Δ: -1.7 ± 0.6 gm, p <0.01). All of the other anthropometric measures non-significantly favoured the OB mice (see **Table 3.7**).

Within group analyses were performed to determine if there were any effects from the order of the diet treatments (see **Table 3.8**).

For the LE mice significant differences were found for length (p=0.04) and gondal fat pad mass (p = 0.02), which favoured the HP -> LP fed group. There were also non-significant trends for weight (p = 0.07) and peri-renal fat pad mass (p = 0.07) which also were greater for the HP -> LP group. Total abdominal adiposity (p = 0.19) also favoured the HP -> LP group but was not significant.

For the OB mice when the anthropometric measures were analysed according to the order of the diet treatments, the differences were greater for the LP -> HP sub-group although all were non-significant.

Finally, when measures at the end of Phase II were compared to baseline (see **Tables 3.2** and **3.6**), for the LE mice the changes were greater for the Phase II mice but only body weight (p = 0.02) and gonadal fat pad mass (p = 0.02) were significant. For the

OB mice while all measures favoured the Phase II mice, no significant differences were found except a non-significant trend for gonadal fat pad mass (p = 0.07).

3.4.5.2 Glucose, adiponectin, ghrelin, insulin and leptin

When between phenotype comparisons were made of these measures at the end of Phase II, all measures, except adiponectin, were higher in the OB group but only the difference for adiponectin was significant (p = 0.001) (See **Table 3.7**).

Within-group analyses based on the order of the diet treatments (see **Table 3.8**) demonstrated for the LE mice significant differences for ghrelin (p = 0.04) and leptin (p <0.01), which favoured mice fed HP chow first. The non-significant differences for adiponectin and glucose also favoured HP -> LP feeding while the non-significant difference for insulin favoured LP -> HP feeding.

For the OB mice, BGL (p = 0.02) and adiponectin (p = 0.03) were significantly higher in the mice fed LP chow first with leptin also (non-significantly) favouring the HP -> LP group. The non-significant differences for ghrelin and insulin favoured the LP -> HP group.

Finally on comparisons of Phase II to baseline (see **Tables 3.2** and **3.6**), for the LE mice all differences favoured the Phase II mice but only leptin (p = 0.003) was significant. Trends towards difference were noted for adiponectin (p = 0.06) and ghrelin (p = 0.07).

For the OB mice all differences were greater in the end Phase II mice (except for insulin) but significant differences were found for ghrelin (p = 0.01) and leptin (p = 0.01) only with a non-significant trend towards difference also noted adiponectin (p = 0.07).

3.4.6 The "Top 10" mice: A re-analysis of the 10 most obese versus the 10 leanest mice

Data from the bottom and top quartile of mice for weight, which were the 10 leanest mice from the LE group and the 10 heaviest mice from the OB group (hence *"Top 10"* mice), was used in a sub-group analysis. The "*Top 10"* mice were re-analysed to reassess differences in food intake during Phase II and differences in body weight.

The weight and food intake data from the "*Top 10 mice"* re-analyses are presented in **Tables 3.9** and **3.10** respectively.

Selection of the *Top 10* lean and obese mice was based on their weight at the end of Phase I (week 6, end of the conditioning diet). For each group of *Top 10* mice the five leanest or most obese mice were selected from each the subgroups (5 from subgroup: LP -> HP and 5 from subgroup: HP -> LP).

The results from the re-analyses are congruent with the results from the main analysis; namely that for both phenotypes there was greater consumption of LP chow compared to HP chow although compared to the main analyses, there was an increased leveraging effect for the LE mice but a decreased effect in the OB mice.

Mean Food Intake per feeding period per *"Top 10"* mouse for LP chow vs. HP chow was:

LE mice: 53.4 vs. 44.0 gms / +16.2% LP chow/ 203.1 vs. 174.8 kcals; $p = 0.001$.

OB mice: 57.0 vs. 53.4 gms/ +7% LP chow/ 216.6 vs. 202.8 kcals; $p = 0.02$.

Table 3.9 Weight of the LE[10] and OB[10] mice

Weight (gms)	**LE[10]**	**OB[10]**	**Analyses**
Pre-conditioning diet (week 0)	22.1 ± 0.2	24.0 ± 0.3	p <0.0001
Post-conditioning diet (week 6)	24.8 ± 0.5	29.1 ± 0.3	p <0.0001

Table 3.10 Mean daily food intake per mouse for the LE[10] and OB[10] mice

Mean Daily Food Intake (gms)	**LE[10]**		**OB[10]**	
	LP - HP	**HP - LP**	**LP - HP**	**HP - LP**
Week 7	**LP** 3.3 ± 0.1	**HP** 3.2 ± 0.1 **¥**	**LP** 4.1 ± 0.1	**HP** 3.9 ± 0.1 **§**
Week 8	**LP** 3.3 ± 0.1	**HP** 3.5 ± 0.2 **¥**	**LP** 3.8 ± 0.1	**HP** 3.5 ± 0.2 **§**
Week 9	**HP** 3.2 ± 0.1	**LP** 4.3 ± 0.1 **¥**	**HP** 3.9 ± 0.1	**LP** 4.4 ± 0.2 **§**
Week 10	**HP** 3.2 ± 0.1	**LP** 4.1 ± 0.1 **¥**	**HP** 3.8 ± 0.1	**LP** 4.0 ± 0.2 **§**

Statistical Differences: Mean daily Food Intake over time: **¥** p<0.01 **§** p<0.0001

3.4.6.1 "Top 10": Body weight and adiposity

The mean weight of the LE and OB *Top 10* mice (LE^{10} and OB^{10}), taken at week 1 prior to the beginning of the conditioning diet, was compared and found to significantly differ by +9%, favouring the OB^{10} (LE^{10} vs. OB^{10}: 22.1 ± 0.2 vs. 24.0 ± 0.3 gms, Δwgt +1.9 gms; p <0.0001).

By the end of the Phase I the LE^{10} mice had increased their weight by 13% while the OB^{10} mice had increased by 21%. On a direct comparison of weight, the OB^{10} were 17% heavier than the LE^{10} mice (24.8 ± 0.5 vs. 29.1 ± 0.3 gms, Δwgt +4.3 gms; p <0.0001) and had greater adiposity (defined as total fat pad mass), which was just not significant (0.7 ± 0.1 vs. 1.1 ± 0.2 gms, Δtotal adiposity +66%, p = 0.05).

"Top 10" vs. Baseline weight and adiposity

Weight and total abdominal adiposity for the *Top 10* mice were compared to the baseline (obtained from the five LE and five OB mice culled at the end Phase I). For the LE^{10} there was a non-significant trend towards greater weight and a non-significant increased adiposity (LE^{10} Δwgt +1.8 gms, p = 0.06, Δtotal adiposity +0.05 gms, p = 0.2). For the OB^{10} there was a significant increase in weight and a non-significant increase in total adiposity (OB^{10} Δwgt +4.1 gms, p = 0.01, Δtotal adiposity +0.3 gms, p = 0.4). No other secondary outcomes were reassessed.

3.4.6.2 "Top 10": Experimental Diets and Food intake

The intake of the experimental diets over time were re-analysed for the *"Top 10"* mice. Like in the main analyses, for both groups mean daily intake over time differed as did total intake over both feeding periods, both of which favoured LP chow. The increase in LP chow intake remained more prominent in the LE group.

In detail, for the LE[10] group, both the total intake per chow (p = 0.001) and the mean daily food intake over time were significantly different. The latter remained so after diet treatment order was a co-factor in the analysis (F (1.4, 1.2) = 11.63, p <0.003). *Post-hoc* analyses confirmed that the main significant differences were between the intakes of week 7 vs. 9 (I – J = -0.5, p = 0.001), 7 vs. 10 (I – J = -0.41, p = 0.002).

For the OB[10] mice, both the total intake per chow (p=0.02) and mean daily intake over time were significantly different with the latter remaining so after diet treatment order was added as a co-factor to the analysis (F (3, 0.5) = 23.0, p <0.0001). *Post-hoc* analyses confirmed that main differences in the mean daily intakes were for weeks 7 vs. 8 (I – J = 0.3, p = 0.02), 7 vs. 9 (I – J = -0.3, p = 0.01), weeks 8 vs. 9 (I – J = -0.5, p <0.0001) and 8 vs. 10 (I – J = -0.3, p = 0.04).

3.5 DISCUSSION

The aim of this study was to determine whether obese mice, as lean mice do, prioritise their intake of food according to the P content. In a two-phased experiment, the first phase was the generation of two phenotypes of C57BL/ 6J mice, lean and obese, through 6 weeks of conditioning diets. It was demonstrated that under *ad libitum* feeding conditions obese mice, like their lean littermates, consumed significantly more low protein (LP) than high protein (HP) chow and as the experimental chows were isocaloric, this meant that while on the LP chow, both phenotypes significantly increased their energy intake.

These findings supports the main concept of the *Protein Leverage Hypothesis*[42], which is that the dietary P is prioritised, even at the expense of increasing caloric intake and that this occurs in obese as well as lean animals. The finding was emphasised by consideration of the results from mice at the extremes of weight, the leanest and most obese.

We also found that during the feeding of the experimental treatments, modest increases in body weight were reported for both phenotypes though the order of the experimental diets moderated the changes for the lean mice. Finally for both phenotypes greater bodyweight was associated with increased fasting levels of leptin and ghrelin and for OB mice decreased levels of adiponectin.

3.5.1 Protein Leveraging in obese mice

As this experiment is among the first to test the *Protein Leverage Hypothesis* in an obese phenotype prospectively we took care to follow the experimental protocols laid out in Sorensen *et al*[192]. This included using the same strain of mouse and the same protein-content modified bespoke chow manufactured according to the recipes laid out by a proposer of the *Hypothesis*.

Both phenotypes of mice ate more low P chow than high P chow, thus prioritising their intake of P at the expense of increasing their energy intake (see *Section 3.4.3*, **Table 3.3** and ***Figure 3.6***). These findings, which support the *Protein Leverage Hypothesis*, have not previously been reported in the literature.

The *PLH* also predicts that adverse consequences will arise from the long-term consumption of lower P diets. As the animal must sustain a trade-off of increased energy intake to meet their P targets, unless this is matched by a similarly sustained increase in the animal's energy expenditure, weight gain will result. In our study the weight changes over the period of the experimental diets were modest and only significant for the lean mice. However the period of feeding of the low P diets was of short duration and the cross-over design may have mitigated against larger changes in weight.

While overall obese mice consumed more of both chows than their lean littermates, when the differences in energy intake between LP to HP chow were considered, for the lean mice it was +14% while for the obese mice only +10%. Whilst acknowledging that

a 4% relative difference is relatively modest, this suggests that obesity may blunt *protein leveraging*.

Interestingly, the "*Top 10"* sub-analysis showed a larger *leveraging* effect (+16%) for the 10 leanest mice but a reduced effect (+7%) for the 10 fattest mice. This widened the relative differences in *protein leveraging* between the phenotypes to 9% and lends support to the idea that *protein leveraging* is blunted by obesity. This intriguing finding should be reassessed in longer-term studies of obesity and if borne out would have important implications in regards to dietary management in obesity.

3.5.2 Caveats: Diet Induced Obesity, puberty, the order of the diet treatments and variety in the diet

Diet Induced Obesity

Whilst at the end of the conditioning diet the OB mice were significantly heavier than the LE mice, the absolute difference in body weight (+1.3 gms/ +5.1%) was relatively modest.

Three studies[190,191,199] that validated the high fat diet/ diet induced obesity C57BL/ 6J mice model for diabetes and cardiovascular disease research tracked weight changes for mice fed HFC and SC for up to six months. Reporting on mice aged 5-8 weeks at the beginning of the studies, the studies showed that the changes in weight in the HFC groups consisted of three phases: the first was accelerating weight gain that peaked at 12 - 15 weeks of feeding, the second was decelerating but still above control group

weight gain until 20-24 weeks of feeding with the third being plateaued weight changes from 25 weeks onwards.

During the first 6 weeks of HFC feeding, all three studies reported modest but significantly increased weight, as in this study.

Another comparator against which the OB mice should be referenced are the weight tables published by the Jackson Laboratory, the originator of C57BL/ 6J strain of mice[211]. When referenced against the corresponding weight-for-age table for male C57BL/6J mice fed standard chow, our OB mice were on average +7% heavier.

Thirdly, the "*Top 10*" subgroup re-analyses of the heaviest and leanest 10 mice demonstrated not only the expected larger differences in weight by end of the conditioning diet (+4.3 gms / +17%) but more importantly that the direction of the findings for food intake on the experimental diets were the same as the main analyses.

Taken together, these additional findings increases confidence that the weight trajectory for the HFC fed mice in this study was as expected in the literature. It also follows that our conclusion of a reduction in protein prioritisation among the OB mice may indeed be due to the differences between the phenotypes, although this needs to be borne out in future studies.

Acclimatisation, puberty and the order of the diet treatments & variety

In this study both groups of mice clearly ate to a dietary target for P but the magnitude of leveraging in both phenotypes was less than the 15% difference in energy intake between the chows reported by Sorensen *et al*[192]. While obesity itself may impact on *protein leveraging*, there are three other explanations for this discrepancy.

Acclimatisation

Firstly, Sorensen *et al.* reported that the results from their first experiment (two choice diet of high and low P chow) revealed conflicting shifts in eating patterns that only became evident by day 20. In the groups offered 9% P vs. 31% P or 48% P the shift favoured an increased intake of the low P chow while for the groups offered 17% P vs. 48% P and 23% P vs. 48% P the shift favoured the higher P food. In the second (no-choice diets) experiment mice offered 9%P chow also increased their intake ½ way through the 32-day experiment. These changes in the eating pattern of the mice were thought to represent acclimatisation and increased familiarity with the chow which our shorter period of experimental diet feeding (14 days vs. 32 days) mitigated. The net result may have been a reduction of the differences in food intake.

Puberty

Secondly, male rodents are known to alter their preferences for macronutrients as they experience and then leave puberty[186]. To meet the increased metabolic demands of this period, there is a marked peak in the intake for P, increased F intake and a relative

decrease in the intake of CHO at puberty then for two weeks following puberty, this changes again with the intake of CHO increasing at the expense of F and to a lesser extent P. Afterwards, macronutrient preferences stabilise and thereafter remain relatively steady.

The C57BL/ 6J mice used in the *PLH* experiments reach puberty between the ages of 5 to 8 weeks with puberty lasting 1-2 weeks[212]. The current study protocol sought to avoid the known puberty associated changes in macronutrient preferences by starting the study with mice aged 8 weeks. By providing them with a week of acclimatisation and 6 weeks of conditioning diet the mice would have been aged ~15 weeks before the start of the experimental feeds, which was deemed to be old enough to be well beyond any lingering puberty related effects on appetite.

However due to logistical issues with the supplier the mice received were aged 5 to 6 weeks at the start of the study which resulted in the mice being 12 to 13 weeks of age at the start of the experimental feeding and therefore, within the window of post-puberty related appetite changes.

Mitigating this is that puberty related appetite changes favour increased CHO intake at the expense of F and P whilst in our study, a reduced leveraging effect was noted compared to Sorensen *et al*[192]. Indeed, and as acknowledged by the authors, puberty related changes in food preferences may have enhanced the degree of *protein leveraging* reported in the original study and our findings of 10 - 14% differences in energy intake may represent a truer measure of *protein leveraging* in the adult mouse.

The effects of diet order and variety

Thirdly a phenomenon not seen in the original study was observed; the total intake for either chows was lower when it was given in the 1st feeding period compared to when it given in the 2nd feeding period (see *Section 3.4.3:* **Table 3.5** and ***Figure 3.7***). This treatment ordering effect was only observed because we utilised a cross-over diet design in the experimental protocol.

The ordering effect was noted in both phenotypes of mice and had an unclear net result on *protein leveraging*. While the differences in energy intake were at least twice as great in the subgroups of mice given HP chow first compared to those given the chows in reverse order (hereby enhancing *protein leveraging*), this should be balanced against the groups given LP and then HP diets, which may have resulted in an increased intake of the HP chow thereby reducing the extent of dietary P prioritisation.

However as the overall direction of food intake demonstrated an increased intake of LP chow regardless of treatment order and as the statistical analyses took treatment order into consideration, we can remain confident in the conclusion that the prioritising of protein over total energy intake is a genuine phenomenon.

One explanation of the ordering effect is to consider the role that choice in food plays in regulating appetite and food intake. There is evidence that animals and humans prefer variety in their choice of food and respond to an increased number of food options by increasing total energy consumption. This in turn is associated with an increased risk of overweight and obesity[213].

In a major review of this topic, *Raynor and Epstein*[214] summarised the relevant animal and human studies that have examined the role of variety and increased palatable food choices and their effects on food intake, weight and body fat to postulate that *sensory-specific* satiety exists and influences food intake. They argued that the sensory characteristics of food are more powerful than the food's energy density or macronutrient content and that this influences the development of overweight and obesity. More recently *Remick et al*[215] reviewed the subsequent research and while acknowledging methodological issues clouded much of the research, concluded that the evidence supported the concept of *sensory-specific* satiety, albeit in a modified form.

While the current study was not designed to test this alternative hypothesis of a driver for food intake, it did switch food choices three times in four weeks and altered the palatability of the chow. This suggests we provided the mice with choice that influenced their food intake. Taken together, the effects of ordering / variety may have moderated or undermined the underlying drive towards *protein prioritising* and could explain the observation of reduction in food intake that was noted in the first week of experimental diet feeding regardless of the type of chow being consumed.

However the response to variety in food is more nuanced than that proposed by the above authors. The more qualified response to variety is illustrated through the results of three studies that examined food variety in rodents. Firstly, *Rolls et al*[216] showed that increased food consumption was seen only in the mice offered simultaneous highly palatable options and not in the mice offered sequential highly palatable food offerings. Secondly, *Armitage et al*[217] demonstrated that rats offered sequentially

differing food options decreased their food intake for several days after the withdrawal of a palatable food choice before returning to baseline. Thirdly, *Zylan et al*[218] reported that rats offered sequential single-choice palatable food versus multiple-choice palatable foods in a cross-over fashion did not, as expected, increase their food intake after the first switch over / feeding period but only after the second switch over.

In this study, while the experimental feeding was associated with increased weight in both groups, there were also plateaus in weight gain during the first week of the experimental diets for both groups and for the OB mice, diet treatment order rendered the increase in weight non-significant (see ***Figures 3.8*** and ***3.9***). This was likely to be effects from diet treatment order and variety; in particular a moderating of food intake associated with the switch-over from the conditioning chows (which were probably considered more palatable chow by the mice) and / or the temporary reduction in food intake in response to the first switch over of chows as noted by *Zylan et al*[218].

The final interesting aspect of the ordering effect was that it was up to four times greater in lean mice compared to the obese, raising the question of how obesity affects the response to variety in the diet.

An answer may be found in a study by *Ackroff et al*[219], who reported on a well-designed series of experiments in obese adult rats comparing different types of energy rich foods and different means of enhancing palatability. The strength of the study was that the authors used adult rats, which addressed issues related to food intake and puberty and took care to resolve methodological flaws noted by *Remick et al* such as standardising

pre-treatment diets, standardising palatability enhancement and testing different forms of palatability enhanced food.

However the authors concluded that contrary to the concept of *sensory-specific* satiety, the physical form of food and its macronutrient content was more important than increased numbers of food choices or flavouring. Drawing from their findings, it suggests that obese animals do have a differential response to variety and furthermore, in hierarchy of contributors to food intake, palatability and variety are important but macronutrient content is more so. It is worth noting that this moves the argument in a direction which is tantalisingly sympathetic to the *Protein Leverage Hypothesis*.

3.5.3 Anthropometric measures, biochemistry and hormones

Anthropometric measures

When the anthropometric measures at the end of Phase II for the LE and OB mice were compared, the OB mice were heavier, longer and had increased adiposity, but only the changes for weight and length were significant. Within group comparisons to assess the effects of the order of diet treatments showed a significant change for length in the LE mice only. Finally, within group comparisons of these measures for end of Phase II with baseline (end of Phase I) showed all older mice to be heavier and with increased adiposity but the change that met significance was weight in the LE mice only.

Although all of the measures on the between group comparisons favoured the OB mice, as the underlining weight difference was modest it is unsurprising that few changes met significance. In addition and perhaps as important, compared to other studies that used the DIO C57BL/6J model and found significant anthropometric differences[195,199,200], the duration of the conditioning and experimental diets was at the lower end of the range of feeding times. Both of these issues prevented the development of large differences in body composition.

However the *Top 10* sub-group analysis of the heaviest and leanest mice found a larger difference in body weight and a larger and almost significant difference in adiposity. Finally, the original *PLH* study[192] did report only one significant anthropometric measure difference, though it was only carried out in lean mice.

The current study's cross-over experimental diet design meant that the mice consumed each experimental chow for 1 feeding period (14 days) only but the anthropometric measures were collected at the end of 2 feeding periods. It is therefore unsurprising that few significant differences according to the order of the diet treatments were found. Taken with the original *PLH* study, it is concluded that for both LE and OB mice despite the animals consuming more energy on the LP diet treatments, treatments of 9% P and 23% P and less than 32 days duration are associated with few significant changes to body composition. Whether longer interventions are associated with an increase in the number of significant changes in body composition still remains unknown.

One final note is the intriguing finding that the changes to anthropometric measures when assessed according to the order of the experimental diets were in opposing directions for the LE and OB mice. That is for the LE mice the subgroup given HP followed by LP diets were heavier and had increased adiposity while the opposite held for the OB subgroup given LP then HP diets. This raises the possibility that for differing phenotypes of mice, dietary P effects their body composition differentially but before taking this notion further, it should be thoroughly tested in a revised and longer study of the *PLH* in mice.

Biochemistry and hormones

For both phenotypes of mice, fasting levels of leptin and ghrelin were higher at the completion of the study (end Phase II) compared to baseline (end Phase I). On the direct comparison between the phenotypes at end Phase II, levels of adiponectin were significantly higher in the lean mice, as might be expected. Although the changes were non-significant, the direction of the findings for glucose and insulin in both the within-group end Phase II vs. end Phase I analysis and between phenotypes at end Phase II analysis were again as might be expected with the heavier mice insulin resistant and having higher levels of glucose.

The overall direction of these findings reflects the known results from studies in DIO C57BL/6J mice that have examined the effects of increased body weight on the secretion of adipokines (adiponectin[195] and leptin[199,200]), glucose and insulin[189] and ghrelin[196,220]. The relatively small number of significant findings between the phenotypes is likely to be related to the relatively modest weight difference between

the groups, which reduced the statistical power to assess for secondary outcome differences and increased the likelihood of a Type II error.

There exists some controversy as to how obesity and diet affect ghrelin secretion. There is evidence that fasting levels of ghrelin are significantly lower in obese compared to lean rodents (DIO C57BL/ 6J mice[196] and DIO Zucker rats[204]) as is seen in humans. However, other studies have reported that levels of ghrelin are generally attenuated in the obese rodent, regardless of the status of fasting[197]. The role of P in ghrelin secretion is also unclear, some evidence points to elevated levels of ghrelin being associated with low P diets that are also high in CHO[221], whilst others (*Vallejo-Cremades et al[222])* found that rats fed a high P diet, compared to the rats fed high CHO, high F or standard diets, had the highest levels of fasting ghrelin. They also found that the high P diet was associated with the greatest prolonging of post-prandial ghrelin secretion and suggested that for rats, dietary P had potent ghrelin modifying properties. However it should be noted that compared to the other studies *Vallejo-Cremades et al* used a different species of rat (not Zucker rats) and dispensed the experimental feeds as fixed volumes rather than *ad libitum*.

As many elements of our protocol were relevant to examining the interactions between weight, dietary P and ghrelin, it was disappointing that the study found few differences for ghrelin, probably due to methodological limitations. The effects of dietary modifications on the secretion of adipokines and gut hormones remain an open and relevant area of research.

3.5.4 Additional comments: strengths and limitations and future directions

Given the primary outcome was difference in food intake, having a high degree of confidence and accurately measuring food consumption were key to the study. By adhering closely to the design of the original *PLH* study (using the same specialised feeding inserts to collect spilled and uneaten food, having a single investigator dispense and measure the experimental chow and measuring four times per week) this study maintained a high degree of accuracy for its food data.

The results and experiences from this study will inform on-going research in this still very open topic. Reflecting on the limitations in the current study that have already been acknowledged, the following suggestions for future mouse *PLH* studies are made. Firstly older mice should be used and they be offered a longer period of conditioning diets to remove any puberty and post-puberty related effects on food intake, to enhance the differences in body weight and to enhance phenotypic differentiation. Doing so would also help to confirm the extent that obesity moderates protein prioritisation.

Secondly, the experiment should involve at least 80 mice as larger numbers would allow for additional comparator groups of mice which would be offered only low and high P diets. This would provide the opportunity to determine the effects of differing P chows on the anthropometric measures accurately without the clouding of results that occurs in a cross-over diet design.

Finally the period of experimental diet feeding should be lengthened as this should minimise the changes in food intake and weight that resulted from the cross-over from conditioning to experimental diets and the second cross-over between the different experimental diets.

3.6 CONCLUSION

In the current experiment we compared the intake of experimental isocaloric diets differing in % P in obese and lean mice. It was shown that under *ad libitum* feeding conditions, both lean and obese mice over-consumed low P chow at the expense of increasing their energy intake, though the overconsumption was more marked in lean mice. The findings in the obese mouse are novel. Taken together, these findings support the *Protein Leverage Hypothesis* which states that the main driver for food intake is dietary P and that its intake is prioritised above total energy consumption though there is the intriguing suggestion that obesity blunts *protein leveraging*.

Because of the cross-order feeding design, an ordering effect was observed for the experimental diets, suggesting that food variety and palatability also influence food intake. However the inclusion of a cross-over comparator and the results of revised statistical analyses showed that this effect did not impact on the overall direction of the findings for food intake.

The weight related changes were significant but modest and associated with appropriate changes in levels of fasting leptin and adiponectin. The direction of these findings confirmed the known effects of obesity and body composition on the secretion of adipokines.

Finally the finding that obese and lean mice leverage their food intake as predicted by the *Protein Leverage Hypothesis* sheds light on one of the potential drivers of the increased rates of human overweight and obesity. Understanding the limitations of

protein leveraging and the mechanisms that underlie it should point to new directions for research into dietary and medical treatments that may help ameliorate overweight and obesity.

CHAPTER 4

Lessons from South Korea and its diet: Lifestyle-induced weight loss and the secretion of appetite and body weight influencing hormones

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4.1 INTRODUCTION

The global phenomenon of increasing overweight and obesity is a well-documented and serious health risk[1,4,223-225]. The traditional lifestyle approach to weight loss involves: recommending dietary changes, increasing levels of incidental activity and regular physical exercise, introducing behavioural (habit) changes and providing psychological support. These programs are popular with patients[226], are cost-effective[227] and are recommended by experts and national health agencies as the cornerstone in the clinical management of the overweight and obese[17,18,228,229].

However the challenges for the overweight and obese to maintain the weight lost by traditional methods are well known and illustrated by a fifth or fewer subjects able to maintain 10% weight loss for 12 months or more[77,230] and average weight loss at 5 years being little more than 3%[20].

These limitations stand in contrast to the literature published from studies into modern bariatric surgical procedures (laparoscopic adjustable gastric banding, vertical

sleeve gastrectomy, and gastric – duodenal bypass / switch procedures). These clearly demonstrate that modern bariatric surgery is associated with greater weight loss, greater long-term weight maintenance [24,27,231], the remission of obesity complications (especially hypertension and Type 2 Diabetes mellitus [T2DM][24,232,233]) and significantly improved 10 year survival (from reduced cardiovascular events and all-cause mortality)[234].

Yet several barriers do limit the wider use of bariatric surgery: The evidence is unclear as to which type of bariatric surgery is most appropriate for patient groups, with the type of surgery left to the individual preferences of the patient and the whim or expertise of the surgeon while surgery itself carries a risk for morbidity and mortality[28,234].

Furthermore in Australia and other Western countries bariatric surgery is predominately privately funded and not available equitably[30] and long-term questions about outcomes, complications and overall cost effectiveness remain unanswered[29].

Therefore the challenges in sustaining weight lost by traditional lifestyle interventions remains a highly relevant area of research[235,236]. Historically physical, psychosocial and physiological factors have been considered the major contributors to weight regain[237,238] but the counter-regulatory changes in the neuro-endocrine control of appetite and body weight after weight loss, and in particular to peripheral mediators, have increasingly been recognised as having a central role in both limiting further weight loss and promoting weight regain[239,240].

4.1.1 Neuro-endocrine adaptation to weight loss and the role of peripheral mediators

Another way to frame overweight and obesity is to consider it a physiological response to the chronic exposure to an environment and lifestyle that combines the ready availability of energy dense foods, favours the use of vehicular transportation, shifts paid labour away from tasks involving strenuous physical exertion and favours less active forms of entertainment. Taken together there is a powerful argument that the *post-industrial* combination of lifestyle and environment is *"obesogenic"*[48].

Given these powerful environmental forces collectively favour increased energy intake and reduced energy expenditure it is important to examine how these factors interact with the homeostatic control of body weight. In two recent reviews *Chaput et al*[50] and *MacLean et al*[240] summarised the last decade of research into weight loss and maintenance, focusing on the complex neuro-endocrine system that regulates appetite and body weight.

In its simplest form, a feedback loop connects the periphery to the brain; with peripherally secreted hormones signalling the long-term availability of energy stores (predominately adipose tissue) and the short-term availability of sources of macronutrients and energy. Numerous central and peripheral components are involved and in concert work to maintain body weight homeostasis. However, for much of human existence the feedback system operated in and adapted to dietary environments where nutrient sources were limited[48,49] and because of this, the homeostatic mechanisms are much more responsive to dietary states where nutrient

supply is scare rather than to the current *post-industrial* dietary state of general global energy excess[50].

As a result of these evolutionary influences, during periods of increasing weight, the homeostatic mechanisms react to these changes not by reversing or stopping or even slowing the weight gain but instead by allowing body weight to drift upwards unchecked and by continually re-tuning to the new levels of obesity[240].

In contrast significant weight loss does not occur generally without a sustained intentional effort to reduce food intake and increase levels of physical activity. However, any weight loss that is induced will lead to the development of counter-regulatory changes in these homeostatic controls that work to prevent further weight loss. This is illustrated by the well-known phenomenon of reaching a plateau in lifestyle induced weight loss known colloquially "hitting the wall".

To lose more weight the diet, exercise and behavioural strategies must be intensified and to maintain weight loss, strategies such continually altering exercise programs and monitoring and adjusting the diet must be applied long term[230,237,240].

The full details of the many mediators of the homeostatic response to weight loss is complex and beyond the scope of this review but in short, it involves mechanisms such as a disproportionate reduction in energy expenditure compared to the weight lost[241], alterations in adipose tissue signalling that are also disproportionate to the remaining fat mass, reduced central sensitivity to satiety signals, and the altered secretion of

appetite, satiety and body weight influencing peripheral hormones[240]. It is the latter that is the focus of the remainder of this chapter.

4.1.2 Key gut hormones & adipokines and the effects of body weight

The peripheral hormones of interest are the adipocyte-secreted leptin and the gut hormones GLP-1 and ghrelin; collectively these hormones have anorectic and orexigenic properties; influence the initiation, continuation and termination of food consumption and act as short and long term signals of energy intake and adiposity. The effect of reductions in body weight on the secretion of these hormones has been studied and the findings are reviewed below.

Ghrelin was reviewed in detail in **Chapter 2**, *Section 2.1.3.2* and GLP-1 in **Chapter 2**, *Section 2.1.3.3*. Leptin will be discussed in detail below.

4.1.2.1 Ghrelin and changes in body weight

Compared to the levels of ghrelin at stable body weight, in the overweight and obese weight loss by diet or by exercise alone has been shown to significantly increase the fasting, post-prandial and 24 hr levels of ghrelin by at least 10%[25,73,239,242-244]. In contrast when bariatric surgery is the method of inducing weight loss, compared to baseline levels of ghrelin, fasting and 24 hr profiles are either unchanged or reduced[25,245].

It is hypothesised that the increased levels of ghrelin seen after conventionally induced weight loss signal to the brain a state of energy deficit, with the subsequent result of an

increase in the urgency to consume food and a reduction in the interval between meals[246]. In comparison, the attenuated or improved levels of ghrelin following weight loss by bariatric surgery are thought to contribute to the reductions in subjective measures of hunger and the superior rates of weight maintenance that are reported with bariatric surgery[25,26,247,248].

4.1.2.2 Glucagon-Like Peptide-1 and changes in body weight

Unlike the other hormones of interest the effects of overweight and obesity on fasting concentrations of GLP-1 are still unclear. Several studies[67,249] have demonstrated that obesity is not associated with changes but at least one study reported that fasting levels were lower in the obese[249]. However the effects of obesity (and T2DM) on the normal post-prandial increase in GLP-1 have been more clearly demonstrated, with both conditions attenuating the duration and magnitude of the expected rise[250,251].

In a well-designed study[252] of post-prandial GLP-1 secretion in obesity and during weight loss, levels of GLP-1 were measured at baseline, after 6 weeks of weight loss by a very low calorie diet using liquid meal replacement products, and after 3 months of weight maintenance by an energy restricted diet. Subjects lost ~8% body weight during the active phase but regained ~1.5% during the period of weight maintenance.

Compared to the baseline levels of GLP-1, post-prandial secretion was significantly reduced at the end of weight loss and during the period of weight maintenance and it was proposed that these changes had the physiological effect of reducing satiety and

delaying gastric emptying, thereby shifting the regulation of appetite to favour increased meal consumption and weight regain.

In comparison, weight loss induced by bariatric surgery has been associated with short and medium term increases in the fasting and post-prandial secretion of GLP-1[26,253]; an enhancement of satiety and depending on the type of surgery, increased or decreased gastric emptying. The net result from these changes is to shift the balance in the homeostatic regulation of appetite and body weight to favour further weight loss. These differences in GLP-1 secretion have been proposed as contributing to the superior rates of obesity remission seen following bariatric surgery compared to conventional weight loss therapies[254,255].

4.1.2.3 Leptin

Leptin was discovered in 1994 as the result of studies into a unique strain of hyperphagic obese mice (known as the *ob/ob* mouse) which lost weight after their circulatory systems were attached to normal, phenotypic lean mice via parabiosis[256,257]. Leptin, originally known as the *ob* protein, is a 167 amino acid length molecule that belongs to the cytokine family of proteins.

Leptin was originally thought be only secreted by white adipose tissue but while it is now known to be secreted by brown adipose tissue, in the gut and in the placenta[258,259], the majority of leptin still arises from subcutaneous white adipose tissue[201,260,261].

There are five leptin receptors with the most widely distributed being the short form, which is the leptin receptor iso-form present in most tissues and the form that facilitates its transport across the blood-brain and into the intra-cerebral circulation, where leptin has its main effect[262].

Leptin acts within the hypothalamus to trigger a signalling cascade that inhibits the release of orexigenic peptides, stimulates the release of anorectic peptides and reduces food intake. There follows multiple effects including reduced hunger, enhanced post-meal satiety and reduced sense of food as a positive reward[201,263,264].

Experiments in normal weight mice made obese by feeding of high fat chow[199] then treated with exogenous leptin illustrate these actions. Initially all the mice received peripherally administered leptin and responded by reducing their food intake. However as the mice became obese the response became muted, but for a sub-group of mice receiving leptin via their cerebral ventricles the physiological effects were restored.

Another illustration of the biological effects of leptin is seen from studies in individuals and families with the rare condition of congenital leptin deficiency[265,266]. These groups have a phenotype marked by extreme hyperphagia and extreme early-onset obesity but both food intake and body weight were dramatically reduced following the administration of exogenous leptin. Importantly, these experiments help give rise to the understanding that common obesity is a state of hyper-leptinaemia but also one of apparent resistance to its effects[267].

The serum concentrations and mRNA levels of leptin rapidly decrease during states of caloric restriction and negative energy balance, pointing to the two broad functions of leptin: the first function is seen during periods of weight maintenance where increased levels of leptin acts as a long-term, peripherally secreted signal notifying the brain as to the levels of adipose tissue stores but without leading to changes in food consumption.

The second function can be seen during periods where energy balance is in flux, where reductions to the levels of leptin act as a short-term signal to the brain, indicating an imbalance in energy intake and leading to increased food consumption[268].

While the primary role of leptin is through its actions on the central nervous system, its cytokine-like structure suggests a secondary, immunogenic role. Leptin has been shown to increase inflammation through modulating the secretion of TNF-α and macrophage and platelet activation[269] and based on these findings, leptin has been proposed to contribute to the low-grade inflammatory state that marks obesity[269].

Leptin and body weight

In humans the concentrations of leptin in the serum and its production (as measured by mRNA in adipose tissue) are both highly correlated with total fat mass[261,270]. Given the known differences in fat distribution between the genders, concentrations of leptin are higher in women than in men.

While leptin deficiency is a well-recognised genetic cause of severe obesity, genetic surveys of common obesity have not demonstrated a high prevalence of mutations to

the leptin gene[271,272] and it is widely accepted that common obesity is not a state of leptin deficiency but rather a state marked by leptin excess[201,260].

Unlike the other hormones of interest, weight loss achieved through lifestyle changes (diet, exercise or combined diet and exercise programs[273,274]) and by bariatric surgery[245,247] (though possibly less so following bypass procedures[275]) have both been associated with reduced fasting and 24 hr secretion of leptin.

It has been proposed that the net result of these changes is that of a "reduced adiposity signal" which is interpreted centrally to shift appetite and weight homeostasis to favour weight regain[240].

Whether the changing levels of leptin play only a short term role in appetite and body weight homeostasis following weight loss is unclear: the levels fluctuate proportionally to changes in body weight and have been reported to increase after weight regain or maintenance[276,277] but more recent studies have brought this finding into question[239].

Interestingly, the administration of exogenous leptin after diet induced weight loss has been shown to limit the post-weight loss decline in metabolic rate[278], suggesting that elevating levels of leptin after weight loss may be some clinical use in weight maintenance.

4.1.3 Methods of weight loss on the secretion of peripheral hormones

Bariatric surgery

The effects of weight loss by bariatric surgery on the peripheral mediators of appetite and body weight regulation have been well established: there are immediate and persistent reductions to the fasting and post-prandial levels of ghrelin[25,245]; short- to medium- term increases in fasting and post-prandial GLP-1 secretion[245,253,275] and additional favourable changes to other gut hormones that influence appetite including Peptide YY[26,247]. There are also marked reductions to the fasting and post-prandial secretion of leptin[245,275,279] which unlike the gut hormones above, have been reported consistently regardless of the method of weight loss.

It has been proposed that bariatric surgery itself induces the changes in the secretion of the gut hormones (ghrelin, GLP-1, peptide YY) which militate against the usual homeostatic counter-regulatory response that develops after weight loss (which can still be seen following bariatric surgery in the marked reductions to levels of leptin) and which normally leads to weight regain. Arguably the combined effect from the changed secretion of gut hormones is sufficiently powerful to overcome the remaining counter-regulatory changes that favour weight regain and that this mechanism is one of the central reasons for the superior rates of long-term weight reduction observed after bariatric surgery[254,255,280].

Lifestyle interventions

By comparison, the number of studies that have set out to examine the effects of conventional methods of weight loss on the secretion of gut hormones (i.e. ghrelin and GLP-1) is sparse and the findings mixed.

Four studies have demonstrated that in the obese, fasting levels of total ghrelin and 24 hour ghrelin profiles following weight loss by a hypocaloric liquid diet and following weight stabilisation by low energy intake diets were significantly higher compared to levels before weight loss or to lean control populations[25,242-244]. Furthermore, a fifth 8-week study[281] of an energy restricted (but not liquid meal replacement) diet also demonstrated that following weight loss there was a significant increase to the fasting levels of ghrelin.

Studies on the fasting and post-prandial levels of GLP-1 following weight loss by very low calorie diets (by liquid meal replacement) found that, compared to lean controls, obese subjects had reduced fasting and post-prandial GLP-1 secretion at baseline with levels further reduced following weight loss[249,282]. An extension of one of these studies but published separately[252] found that after a period of weight stabilisation by hypocaloric diet, the changes to the fasting and post-prandial secretion of GLP-1 had increased but were still reduced compared to baseline. Against these findings are the results of a study of weight loss and maintenance which demonstrated that both were associated with increased secretion of GLP-1[250]. The sum of these studies is that the direction of the changes in the secretion of GLP-1 that develop following weight loss is still debatable.

In contrast to the number of weight loss studies examining very low calorie diets, there have been only two studies of the effects on peripheral mediators of appetite and body weight regulation by exercise-only weight loss.

The first was a 12-month study[283] of post-menopausal obese women, randomised to an exercise program or to stretching, which reported that fasting ghrelin increased proportionally to weight loss. The second was a 12 week study[284] of overweight and obese middle-aged adults who lost a mean of 5% of baseline weight by exercise only. While they reported no significant changes in fasting ghrelin or GLP-1, there were trends towards prolonged suppression of post-prandial ghrelin release and enhanced post-prandial GLP-1 secretion.

When these two studies are considered together, the first suggests that exercise-induced weight loss also leads to maladaptive changes in the fasting secretion of ghrelin that favour weight gain whilst the second suggests that exercise-only weight loss induces post-prandial changes in ghrelin and GLP-1 that moderate the expected counter-regulatory changes. While it is possible that the direction of fasting and post-prandial changes is in opposition, it is also reasonable to conclude that the results are contradictory. When the methodologies are compared, the frequency and intensity of the exercise programs were the same for both studies but the subject groups differed with those in the second study younger and overweight but not obese. The question of whether exercise-induced weight loss is associated with the same counter-regulatory adaptions that constrain further weight loss remains open.

Finally, only two studies have examined the effects of weight loss by combined diet and exercise on the secretion of the key peripheral mediators of appetite and body weight regulation with the results also mixed.

The first study[273] examined an overweight cohort and compared the effects of 6 months of hypocaloric diet vs. hypocaloric diet and regular exercising. They found that diet and exercise was associated with greater weight loss but for fasting leptin compared to baseline, there were with-in group reductions but no significant differences between groups. Furthermore, by 12 months subjects had regained weight but unfortunately, as leptin was not measured, there was no assessment of the persistence of any changes to leptin.

In the second study[285], obese Mexican-Americans who received a 12 month combination diet, exercise and adjuvant pharmacotherapy program lost and maintained ~5% weight loss, which was associated with increases to the fasting levels of ghrelin at 6 months, but at 12 months fasting levels had returned towards baseline.

A final point of comparison is to review the literature from studies of the management of adolescent obesity. Due to the restrictions on the use of very low calorie diets and pharmacotherapy with this population there have been more studies of weight loss by combined diet and exercise interventions on the release of peripheral hormonal mediators of appetite and weight loss regulation. In a recent review, *Roth et al*[286] examined the studies and while unable to apply conventional meta-analyses techniques, reported that on balance, there was good evidence to support the notion

that in adolescents, both during and after weight loss changes, the secretion of ghrelin and leptin are in similar direction to the counter-regulatory changes reported in adults.

In addition, the persistence of the maladaptive homeostatic changes has recently and uniquely been demonstrated in a 2011 study of obese subjects who achieved and maintained an impressive 8% weight loss by very low calorie diet therapy[239]. At 12 months, weight maintenance was associated with persistent changes in the fasting and post-prandial levels of leptin, insulin, ghrelin, CCK and peptide YY, which were all counter-regulatory and against further weight loss. Coupled with persistently elevated measures of subjective hunger, the overall direction of all these changes favoured weight regain.

To date, this study has demonstrated the long term nature of the maladaptive changes to peripheral hormonal mediators of appetite and body weight and strengthens the main argument, which is that following conventional weight loss, there are sustained and persistent counter-regulatory changes to the homeostatic mechanisms of body weight regulation that act in concert to regain weight. Understanding these changes could be of great importance to the development of more effective strategies to address overweight and obesity.

4.1.4 Body weight and inflammation: adiponectin, insulin and C-reactive protein

Obesity is not only a state of energy excess and increased adiposity, it is also a metabolically active state marked by low-grade subclinical inflammation that contributes to the development of obesity- related complications[269]. In comparison, weight loss is associated with lower levels of C-reactive protein (CRP), increased levels of adiponectin and improved insulin sensitivity[287,288] and as all are biological markers of subclinical inflammation, these improvements correspond to weight loss itself reducing subclinical inflammation. It has been clearly demonstrated that the magnitude of these changes is dependent on the degree of weight loss rather than the methods used to achieve it[279,287].

4.1.4.1 Adiponectin

Adiponectin (ADP) was fully identified in 1995[289] and is an adipocyte-derived 28-kDa 244 amino-acid protein that is abundantly found throughout human circulation. ADP's physiological functions include modulating the inflammatory response, influencing glucose and lipid metabolism and reducing circulating levels of free fatty acids. Levels of ADP are inversely associated with insulin resistance, T2DM and coronary artery disease and in contrast to other adipokines, increased levels of ADP are associated with decreased rates of overweight and obesity.

ADP is predominately synthesized and secreted from white adipose tissue at concentrations (5 - 30 micrograms per millilitre) that are significantly higher than those of all other adipokines[290]. Circulating ADP is found in the form of multimers

with the high molecular weight multimer considered the most clinically relevant[291]. ADP acts on one of two identified receptors that are expressed in varying affinities throughout tissues of the body, with the ADP $_1$ receptor found in skeletal muscle and vascular endothelial cells while the ADP $_2$ receptor is found in the liver[292,293].

Studies in rodents have demonstrated that low levels of ADP are associated with insulin resistance[294,295] while other animal and *in vitro* studies have shown that ADP levels are inversely associated with increased platelet and thrombus activity[296,297] and increased levels of the pro-inflammatory cytokine *Tumour Necrosis Factor* α [298].

In humans levels of ADP have been found to inversely correlate with insulin resistance, the metabolic syndrome and the risk for T2DM[299-301]. Levels of ADP are also inversely associated with levels of serum triglycerides and positively correlated with levels of high-density lipoprotein cholesterol. These are all risk (or for HDL protective) factors for cardiovascular disease (CVD) and unsurprisingly, a relationship exists between high levels of ADP and a reduced risk for CVD, although the relationship is weak and attenuated by the presence of traditional CVD risk factors[302,303].

Clinically, increased levels of ADP are found after the use of *thiazolidinediones* agents for T2DM, which act on the peroxisome proliferator-activated receptor in skeletal muscle, vascular endothelium, hepatocytes and adipose tissue to reduce insulin resistance and improve insulin sensitivity[304,305]. ADP also moderates hepatic glucose production by reducing the levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, two key enzymes involved in hepatic gluconeogenesis[306,307].

Physiologically, there is evidence that the beneficial effects of physical activity on insulin resistance are mediated by the ADP - ADP receptor pathway[308].

Adiponectin and body weight

The secretion of ADP is affected by changes in body weight with studies reporting significantly lower levels of ADP in obese populations compared to lean controls, in conditions of normal health and in the presence of metabolic co-morbidities[309-313]. Moreover, the results of a recent meta-analysis[314] clearly demonstrated that for overweight and obese subjects across a range of health conditions, clinically significant weight loss through exercise only, combined diet and behavioural changes or by bariatric surgical are all associated with significant increases to ADP levels. Furthermore, it has been proposed that the favourable metabolic changes that develop following weight loss are at least in part mediated by the increases to ADP[287,308,313,315].

Interestingly this finding appears to be proportional to the baseline weight and to the amount of weight loss. Studies have found the link weaker in lower grades of excess weight (overweight and Grade I obesity) while other studies where weight loss was less than 10% have failed to replicate the finding at all[287,316,317].

4.1.4.2 Insulin

Insulin was discussed in detail in **Chapter 2**, *Section 2.1.3.4*.

Insulin and body weight

Overweight and obesity is associated with the hyper-secretion and impaired clearance of insulin, leading to elevated levels both basally and in the post-prandial period[318]. Elevated levels of insulin and the development of resistance in peripheral (especially adipose) tissues to its actions is thought to be a major pathway mediating the metabolic and cardiovascular complications that associated with overweight and obesity[163].

Greater than 5% weight loss is associated with reduced concentrations of serum glucose and with decreased basal and post-prandial secretion of insulin with greater weight loss associated with greater improvements to both[239,287,313]. The greater than 20% weight loss typically reported after bariatric surgery has been shown to be associated with the medium-term remission of T2DM[232,287].

In contrast to the other hormones of interest in the current experiment, the changes following clinically significant weight loss in the secretion of ADP and insulin appear to contribute little to weight regain and the counter-regulatory changes that develop in the homeostatic regulation of appetite and body weight. Indeed, the net result of the changes to ADP and insulin are beneficial.

4.1.4.3 high-sensitivity C-reactive protein

C-reactive protein (CRP) is an acute phase reactant synthesised in the liver and released in response to cytokines secreted by immune-mediating cells including adipocytes. As the name suggests, CRP is released in response to inflammation and in the presence of severe inflammatory states such as sepsis, levels can increase up to 1000-fold [319].

However, persistently mild elevations of CRP (≤ 5 fold) have been noted in conditions of metabolic disorder such as obesity, the metabolic syndrome, insulin resistance and T2DM; leading to the conclusion that these are all conditions associated with chronic subclinical inflammation[320]. Furthermore, mildly elevated levels of CRP are weakly correlated with an increased risk for CVD but whether this represents an independent risk factor or is a proxy for conventional risk markers remains unclear[321,322].

As weight loss improves both subclinical inflammation and the metabolic profile, it is unsurprisingly that CRP levels fall after weight loss with the presence of pre-existing metabolic disturbances and the extent of weight loss influencing the extent of the reduction in levels of CRP. The greatest reductions have been reported following bariatric surgery in previously morbidly obese subjects with extensive metabolic complications[287], while more modest reductions have been reported following weight loss by lifestyle means in the overweight and obese with few metabolic complications[313].

(Finally it should be noted that high-sensitivity CRP (*hs*CRP) refers not to another form of CRP but to the laboratory technique of laser nephelometry, which allows for the measurement of very low levels of the protein.)

4.1.5 Rationale for the experiment and summary

National weight management guidelines advocate a comprehensive lifestyle approach that combines a hypocaloric diet, increasing physical activity and undertaking regular exercise and reassessing maladaptive food-related behaviours[17,19,229]. While compliance with these forms of therapy is associated with clinically significant weight loss, maintaining the lost weight remains a challenge.

Following weight loss, counter-regulatory changes develop in the homeostatic regulation of appetite and body weight that favour weight regain and studies have increasingly focused on the peripheral mediators of appetite and body weight regulation, especially ghrelin, GLP-1 and leptin. While the effects of diet-induced weight loss have been relatively well studied, there are fewer studies on the effects of weight loss by exercise and the impact of weight loss by an integrated lifestyle approach on these key hormones remains poorly understood.

The aim of the current study was to investigate whether in the overweight and obese, the changes in the peripheral hormonal mediators of appetite and body weight regulation that develop after weight loss by a single lifestyle modality are also seen in those who lose weight by a combined and comprehensive lifestyle program.

Working with data from a weight loss study, of main interest were changes in the fasting levels of ghrelin, GLP-1 and leptin, whilst of secondary interest was the change in the markers of obesity related to sub-clinical inflammation (adiponectin, insulin and *hs*CRP).

4.2 MATERIALS AND METHODS

4.2.1 Subject recruitment

Subjects were recruited by reference to an existing clinical trials database and by advertisements placed in local papers and on the intranets of the Sydney South West Area Health Service and the University of Sydney. Subjects were deemed eligible if they were aged 18 – 65 years, had a BMI between 25 – 45 kg/m^2, at least 3 months of stable weight and in good general health. Exclusion criteria included a diagnosis of diabetes mellitus or an glycosylated haemoglobin >6.5%, cardiovascular disease, renal or hepatic impairment, serious gastrointestinal disease or abnormal thyroid function; current pregnancy; or if within 6 months they had taken mediations known to alter body weight; or if they were vegetarians, vegans or had food intolerances; or if they consumed greater than 4 standard drinks of alcohol per day or 8 standard drinks per week.

Potential subjects attended for a screening review where they completed a standardised questionnaire and were assessed by a medical clinician. They had blood collected for screening tests of thyroid, renal and hepatic function and glycosylated haemoglobin. A urinary β human chorionic gonadotropin test was performed for women of child-bearing potential at the screening visit and at the completion of the study to assess for possible pregnancy. No positive results were recorded.

Seventy subjects were recruited and commenced the study but 10 withdrew due to time constraints or social circumstances that prevented compliance with the protocol. None

withdrew due to adverse events. Sixty subjects completed the study and there were no baseline differences for weight or BMI between completers and those who withdrew. A *per protocol* analyses was performed using data only from the 60 subjects who completed the study.

The Human Ethics Committee of the University of Sydney approved of the study (*Korean Diet Efficacy Clinical Trial.* Ref. No.: 11-2009/12124). Funding for the study was provided by the Department of Agriculture, Republic of (South) Korea. Subjects provided their written, informed, signed and personally dated consent prior to commencing any study related activities.

4.2.2 Lifestyle intervention: Diet, activity & exercise and behavioural changes

The 12-week combined lifestyle intervention included prescribing a hypocaloric diet (-500 calories [2092 kJs] per day from resting energy expenditure as estimated by Harris Benedict Equations[323]); 120 minutes per week of regular, moderate intensity exercising; increased levels of incidental activity (8kms or >10,000 steps per day was the goal) and tailored behavioural advice focused on revising their attitudes towards the intake of food and alcohol and prioritising incidental activities and exercise. Additionally, there was an education focus regarding the nutritional content of food and the merits and ease of home cooking. The primary outcome was weight loss at the end of the intervention.

At the screening visit, all subjects were also provided with a pedometer (calibrated with the assistance of study staff) and asked to record their daily step count to assist them to reach their targets for incidental activity.

The lifestyle intervention was designed to be consistent with the Australian "*Clinical Practice Guidelines for the Management of Overweight and Obesity in Adults*" put forward by the peak national health body, the NHMRC[17].

Following the screening visit, all subjects had a comprehensive 45-minute lifestyle review which was followed by weekly 20-minute lifestyle reviews until the completion of the study. Subject compliance was assessed during these visits with reference to the prescribed targets for diet and exercise. Food and exercise related behaviours were also reviewed.

Prior to their second (baseline) visit and week 4, 8 and 12 visits, subjects were asked to complete a diary of all their food intake and record their pedometer step-count for 3 days (2 week days and 1 weekend day). During these visits, they were also asked to complete the International Physical Activity Questionnaire (IPAQ) 2005 (short) Edition[3,24] (see **Appendix: Item E**)

Dietary Intervention

Before their baseline visit, subjects were randomised into groups for different dietary treatments: the Korean Diet (KD) group and the Conventional Diet group (CD). Subjects in the KD group were provided with a traditional Korean lunch and dinner, Monday to Saturday, for the 12-week duration of the study. The hypocaloric

traditional Korean meals were prepared according to standardised recipes at a specialist Korean cuisine restaurant (Open Korea, Camperdown, NSW). KD main meals were composed of small servings of rice, meats, soups, noodles, vegetable side dishes (*kim chi* / fermented cabbage and radish) and noodles, which were placed into separate compartments of a customised food tray. An example of the Korean main meals is seen in the **Appendix: Items B(1) and B(2).**

Subjects in CD group were provided with a recipe book (kindly provided by the Metabolism and Obesity Services, Royal Prince Alfred Hospital, NSW) that contained forty-two recipes for hypocaloric main meals, deserts and soups. CD subjects were also provided with a weekly food and grocery stipend to the value of 6 main Korean Diet meals.

The food diaries were analysed by the computer programs FoodWorks 2007 and CAN-pro 3.0 with mean daily energy intake (kJ) calculated for baseline and for study weeks 4, 8 and 12.

Exercise and incidental activities

Levels of exercise were calculated using the IPAQ short questionnaire[324]. This clinical research tool assesses a range of physical activities across domains including work, leisure time and transport time and grades physical activities into three tiers, generating a total score with reference to Metabolic Equivalent of Task or METs. 1 MET is defined as the energy output of the surface area of an average person seated at

rest (1 kcal·kg^{-1}·h^{-1}). To illustrate: walking is 3.3 METs, moderate physical activity is 4.0 METs while strenuous physical exercise is 8.0 METs[324].

Pedometer counts were measured by reference to the 3-day diary and validated against the stored values in the electronic memory of the devices. Mean daily step counts were calculated for baseline and for study weeks 4, 8 and 12.

Compliance

An individual subject's progress and compliance with their lifestyle program was assessed by review of completed 3-day food diaries and pedometer record diaries and by review of completed IPAQ questionnaires. Subjects not meeting their targets were counselled and provided with advice as to how to improve their performance.

As an additional measure of dietary compliance, the KD group was asked to return food containers and uneaten food while the CD group was asked to bring in their weekly shopping receipts.

All of the lifestyle interventions were conducted by the lead dietitian and exercise physiologist (NRF) or by one of three study clinicians supervised by NRF. Entry of data from the food and pedometer diaries and the computerised analyses of food intake were all kindly performed by students from the Master of Dietetics and Nutrition program, University of Sydney. A detailed description of the dietary interventions (including the background to the choice of Korean cuisine), methodology, results and conclusions was published in 2012[325].

4.2.3 Anthropometric measures

At the baseline visit, height was measured using a wall-mounted stadiometer (Wedderburn, Australia). At each visit, fasting early morning body weight (in light clothing) was measured on the same set of digital scales (Tanita BC-418, Wedderburn, Australia) as was waist and hip circumferences. Waist circumference was measured at the mid-point between the highest point of the iliac crest and lowest point of the costal margin in the mid-axillary line. Hip circumference was determined at the level of the greater trochanter. Body Mass Index was calculated as body weight divided by height squared (kg/m^2).

4.2.4 Phlebotomy, measuring hormones by RIA, analysis of hsCRP and glucose, and calculating insulin resistance

Blood samples were collected at the baseline visit and at the final (completion) visit at week 12. Subjects were asked to present in the early morning following an overnight fast. Blood was collected by antecubital venipuncture for (total) ghrelin, active GLP-1, leptin, insulin, adiponectin, glucose and *hs*CRP. Serum blood samples were collected in BD Vacutainers (SSTII advance with gel separator) while plasma samples were collected in K_2EDTA containing BD Vacutainers. Blood collected for GLP-1 was collected in BD Vacutainers (BD, NJ, USA) containing Dipeptidyl Peptidase-IV inhibitor (10 μL/mL blood; Millipore Corp, Billerica, MA, USA) to prevent GLP-1 degradation. These containers were stored at -20^{o}C until the morning of sampling.

Blood samples were immediately centrifuged for 15 min at 3000*g* then aliquotted and stored at -20°C until analysis.

Radioimmunoassays of adiponectin, GLP-1, total ghrelin, leptin

Adiponectin (ADP), total ghrelin (tGh) and leptin (Lep) were measured using human specific RIA kits manufactured by Millipore, USA (ADP: HADP-61HK; tGh: GHRT-89HK; Lep: HL-81HK). The RIAs for ADP and leptin (and the leptin assay's characteristics) have been described in ***Chapter 3***, *Section 3.2.6* while for total ghrelin in ***Chapter 2,*** *Section 2.2.6.2*. Though the ADP RIA kit described in ***Chapter 3,*** *Section 3.2.6* is mouse specific, the human kit follows the same assay technique but with the following assay characteristics: samples assayed in a 1:500 dilution; the lowest sensitivity is 1 ng/mL using a 100 mL diluted sample; intra-assay %CV was 1.2 – 6.2%; inter-assay %CV was 6.9 – 9.3%.

Concentrations of biologically active GLP-1 (7-36, 7-37) were measured using a human specific RIA kit (GLP-1A-35HK, Millipore, MA, USA) in a process that required the preliminary step of alcohol-based extraction of GLP-1 from the stored plasma samples. The RIA for GLP-1 and its assay characteristics have been described in detail in ***Chapter 2***, *Section 2.2.6.3*.

All RIAs were performed in the Endocrinology Laboratory of Royal Prince Alfred Hospital, NSW and all unknowns were assayed in duplicate. The author measured all of the hormones except for ADP. This latter RIA was kindly performed by Ms. Marilyn Brown (Senior Hospital Scientist at Royal Prince Alfred Hospital).

Biochemical analysis of insulin, glucose and hsCRP

Fasting insulin, serum glucose and *hs*CRP were all measured using the automated Architect ci16200 platform (Abbott Diagnostics, Il, USA) by immuno-chemiluminometric technique at the laboratories of Douglass Hanly Moir Pathology, Sydney.

Insulin resistance was quantified using the homeostatic model assessment (HOMA-IR) and calculated using the open access University of Oxford HOMA calculator[326].

4.3 DATA ENTRY AND STATISTICAL ANALYSES

Data was entered into a series of spreadsheets using Microsoft Excel 2008 for Mac (version 12.3.1 build 110725). Following data entry, a data cleaning cycle was undertaken that consisted of cross-referencing figures in the spreadsheets back to the original source material. All of the statistical analyses were calculated using the IBM SPSS Statistics Version 19 software package (IBM Corp, NY, USA) with the author performing all data entry and all of the statistical analyses.

The study's primary outcome was a clinically significant change in weight (≥5% off baseline). It was calculated that 30 subjects in each study arm would give the study 80% power (α= 0.05, two-tail). Incorporating a 20% drop out rate, the target subject recruitment was 72 subjects and while only 70 subjects were recruited, the dropout rate was lower than projected and hence the study maintained 80% power.

Data was analysed for normative distribution by the Kolmogorov–Smirnov test. Parametric data is reported as mean ± Standard Error Mean (SEM) while non-parametric data is reported as median with the inter-quartile range (IQR). All data is presented as mean ± SEM unless specified.

Parametric (normally) distributed data was analysed using the Two sample *t*-test to assess for between group differences at the completion of study and the Paired sample *t*-test used to assess for differences between baseline and completion for the entire cohort. Non-parametrically distributed data was analysed using the Mann-Whitney *U*

test for differences between the groups at completion and the Wilcoxon signed-rank test for the differences between baseline and completion for the entire cohort.

Spearman's rank correlation coefficients were calculated to test for relationships between changes in weight and changes in the levels of hormones.

Missing data for GLP-1 was corrected by multiple imputation data analyses using the Marcov Chain Monte Carlo method. Results of statistical testing were considered significant at $p<0.05$ unless noted.

4.4 RESULTS

4.4.1 Subjects, weight loss and lifestyle intervention

4.4.1.1 Analysis per dietary intervention: KD vs. CD

The disposition of the subjects in the study is presented in the **Appendix: Item C**. The results of the analyses according to dietary intervention are presented in the **Appendix: Item D** and were published in 2012[325].

There were no baseline differences between treatment groups (per dietary intervention) for any measure except adiponectin, which was higher in the KD group (+3.8 μg/mL, p = 0.02). For the primary outcome of weight loss, both treatment groups lost at least 5.7% from their baseline weight with no significant differences found for weight at completion between the diet treatment groups (p = 0.93).

At completion the KD group was found to have a higher daily energy intake (EI) (p<0.001) than the CD group but in comparison to baseline daily EI, both groups had significantly lowered their daily EIs at completion. There were no significant differences for any other completion measure (activity levels, hormones or biochemistry) except for levels of fasting adiponectin (p = 0.02), which again favoured the KD group.

As there were no between group differences for the primary outcome of weight loss or for any secondary measure (except fasting adiponectin) data from all subjects was

pooled to increase the statistical power of the analyses of the impact of weight loss on the fasting levels of GLP-1, tGh, leptin, *hs*CRP and insulin resistance.

This author was a co-investigator in the overall project with duties including designing and drafting the clinical trial protocol and assisting in the process of ethics submission, conducting subject screening, implementing research protocols, performing phlebotomy and sample processing and analysing samples by RIA. This author also co-authored the two research articles produced from the project thus far: "*A 12-week, randomised, controlled trial….of the Korean diet"*[325] and "*A 12-month, randomised, controlled trial…..of the Korean diet….follow up analysis"*[327].

4.4.1.2 Completion vs. baseline for the lifestyle intervention: Weight loss, energy intake and activity levels

The baseline demographic, anthropometric, energy intake and activity and exercise measures for the sixty subjects (58% female) who completed the study are presented in **Table 4.1**.

Table 4.1 Demographic, anthropometric, activity and exercise levels at baseline and completion

	Baseline	**Completion**	***P***
Gender (F/M)	35/25	32/25	-
Age (yrs)	42.4 ± 11.7	-	-
Weight (kg)	89.5 ± 16.5	84.4 ± 15.8	*<0.0001*
BMI (kg/m²)	31.2 ± 4.2	29.4 ± 4	*<0.0001*
Waist Circumference (cms)	98.5 ± 12.7	94.5 ± 12.6	<0.0001
Daily mean Energy Intake (kJ)	7705	6180	*<0.01*
Average Daily Step Count*	7950, 4336	11550, 3732	*<0.001*
MET minutes/week	2097 ± 1926	3416 ± 2186	*<0.001*

NB: Data are presented as mean ± SEM and analysed by Paired *t*-test unless otherwise noted

* non-parametrically distributed data as median, IQR and analysed by Wilcoxon signed-rank test.

The subjects at completion of the study achieved a mean -5.7% weight loss from baseline (Δ Wgt: -5.1 ± 4.2 kg/ Δ BMI: 1.3 ±1.4 kg/m²; p <0.0001). Overall 31 (50%) of subjects lost greater than 5% bodyweight and of those, 10 subjects lost more than 10%. Of the remainder 5% of subjects gained weight. Mean daily levels of physical activity (as measured by the IPAQ as MET equivalents) increased significantly during the intervention (+63%) as did mean daily step count (+45%) while mean daily EI decreased (-20%).

4.4.2 Baseline vs. completion: GLP-1, ghrelin, adiponectin and leptin

The results of the analyses of completion vs. baseline for fasting GLP-1, ghrelin, adiponectin, leptin, glucose, insulin and HOMA-IR are presented in **Table 4.2**.

Table 4.2 Fasting levels of hormones and glucose at baseline and at completion

	Baseline	**Completion**	***P***
total Ghrelin (pmol/L)*	304.9, 232.5	388, 260	*<0.0001*
Active GLP-1 (pg/mL)*	34.6, 22.9	28.8, 12.9	*0.03*
Leptin (ng/mL)	20.7 ± 1.9	12.6 ± 1.4	*<0.0001*
Adiponectin (µg/mL)	12.3 ± 0.9	11.8 ± 0.8	0.67
Insulin (mU/L)	9.7 ± 1.7	7.0 ± 0.6	*0.03*
Glucose (mmol/L)	4.3 ± 0.1	4.2 ± 0.1	0.11
HOMA-IR*	1.4, 1.3	1.1, 0.8	*0.002*
*hs*CRP (mg/L)	3.3 ± 0.4	3.5 ± 0.5	0.07

NB: Data are presented mean ± SEM and analysed by Paired sample *t*-test unless otherwise noted

* non-parametric distributed data as median, IQR and analysed by Wilcoxon signed-rank test.

When compared to levels at baseline, at completion there was a significant increase in the fasting levels of the orexigenic hormone ghrelin (+28%), a significant decrease in fasting levels of the anorectic hormone GLP-1 (-22%) and to the fasting levels of the adipokine leptin (-40%). However there was no change in the fasting levels of adiponectin.

4.4.3 Baseline vs. completion: insulin, glucose, HOMA-IR and hsCRP

At the completion of the study compared to baseline, there was a significant decrease in fasting levels of insulin (-38%) and a significant increase in insulin sensitivity as determined by HOMA-IR (Δ 30%). However, there was no significant change for fasting glucose and a non-significant trend for increased levels of *hs*CRP.

4.4.4 Correlation of weight loss with levels of GLP-1, ghrelin, leptin, adiponectin and insulin

The change in weight was significantly correlated with the changes for fasting GLP-1 (r_s = -0.3, p = 0.03), insulin (r_s = 0.4, p = 0.005) and leptin (r_s = 0.4, p = 0.001) and with a non-significant trend for fasting ADP (r_s = -0.2, p = 0.10) but not for fasting tGh (r_s = 0.08, p = 0.58).

4.5 DISCUSSION

The current study examined the impact of weight loss, achieved by 12-weeks of two different diets but the same exercise and behavioural intervention (these were combined for analysis and are henceforth called the combined lifestyle intervention) on the fasting levels of orexigenic and anorectic gut hormones and adipokines; and on other markers of inflammation in overweight and obese subjects. The lifestyle intervention was based on the obesity management recommendations of the NHMRC and consisted of several components: dietary education and the prescription of a hypocaloric diet (conventional or Korean), a focus on regular exercising and increasing incidental activity, and the promotion of appropriate behavioural changes.

While counter-regulatory changes in the homeostatic regulators of appetite and body weight, including to peripherally secreted hormones (e.g. GLP-1[252,282] and ghrelin[281]) have been demonstrated following weight loss by very low calorie and low calorie diets, there remains much uncertainty in this field and the results from the small number of studies of weight loss by exercise-only and combined diet and exercise programs have been mixed.

Overall the combined lifestyle intervention achieved clinically significant weight loss (mean -5.7% from baseline) by increasing levels of physical activity and by reducing energy intake (see **Table 4.1**). Following weight loss were falls in the fasting levels of GLP-1 and leptin, a rise in fasting levels of ghrelin, and an improvement in insulin sensitivity. However the overall direction from the changes in ghrelin, GLP-1 and leptin were such as to limit further weight loss and promote weight regain.

These findings show that weight loss from a combined lifestyle intervention leads to maladaptive counter-regulatory changes in multiple peripheral regulators of appetite and body weight and to our knowledge; this is the first time that these results have been demonstrated following such a program.

4.5.1 Lifestyle intervention and pooled analyses

At the end of the combined lifestyle intervention, subjects in this study had decreased their EI by 20% and increased their levels of physical activity: METs by 63% and mean daily step count by 45%. While the final component of the intervention, namely behavioural changes, were not formally quantified, the clinically significant weight loss (>5% from baseline) attests to the overall success of the intervention (see **Table 4.1**).

However while subjects were originally separated into two dietary treatment groups data from the entire cohort was used in the analyses of the effects of weight loss on the secretion of hormones and markers of inflammation. As such, comment should be made about the dietary interventions and on the decision to pool data.

In the study subjects were divided into a Korean Diet (KD) diet treatment group, which received 12 main meals per week (lunch and dinner Mon-Sat); and a Conventional Diet (CD) group; which received shopping vouchers to the value of the meals and a hypocaloric recipe book. While this component of the dietary intervention differed, the provision of commercially prepared low energy meals is utilised by many dietary programs and is recognised as a valid option in the treatment of obesity[17]. Furthermore, both groups received the same hypocaloric diet targets, the same

education regarding the nutritional composition of meals and the value of home cooking in addition to the same advice and targets for physical activity and behavioural changes.

At completion both groups reduced their EI compared to baseline EIs (which did not differ) but the KD group was found to have a significantly higher EI than the CD group (see **Appendix: Item D**).

One question is whether these results are genuine or whether they are due to errors in the subjective reporting of food intake in the 3-day food diaries. While under-reporting of food intake in food diaries is a known problem, the reporting of food intake remains consistent over time and the food diary has become a widely utilised and validated method of measuring food consumption in clinical trials[328,329]. In addition, both groups received the same education about calorie counting (intended to up-skill the subjects and aid their compliance with the prescribed diet).

Moreover, there were no baseline differences for EI (which suggests that randomisation of subjects did not lead to between group bias in the reporting of food intake). Interestingly the group with the higher EI was the KD group, where food intake was also assessed directly by study staff by weighing the leftover food from the returned food trays and there were no significant differences in EI between that reported in the KD food diaries and returned food trays (not shown).

Furthermore, the KD group was noted to have 20% lower levels of fasting ghrelin at completion than the CD group (see **Appendix: Item D**) consistent with the known

effects of food intake and levels of ghrelin see ***Chapter 2***, *Section 2.1.3.2* and *Section 4.1.2.1* above). Taking all these factors into consideration, while an error in results for EI cannot be excluded, the finding is less likely a reporting error than a true finding.

A possible contributory factor to the increased EI in the KD group was the nature of Korean cuisine. There is a known association between novelty and variety in food choices and the increased consumption of food[214,216] and while the KD group was instructed to eat *ad libitum*, the large number of different foods served per main Korean meal and the unique nature of the cuisine; coupled with the form in which the Korean meals were presented, may have influenced the subjects food related behaviours to favour over consumption (see *Section 4.2.2* and **Appendix: Items B**). Conversely, part of the focus of the behavioural intervention was on appropriate food selection and preparation and the CD group had greater ability to choose the form and portion size of their main meals, which should have made it easier to reduce food intake. However, whatever the balance between all these factors, it is worth restating that at completion both two groups lost an almost identical amount of weight.

To reiterate, despite these confounders, because there were no between-group differences for weight or any biochemical or hormone measure at completion (except adiponectin), the data from the KD and CD groups were pooled to increase statistical power for the baseline vs. completion analyses of the secondary outcomes.

4.5.2 Lifestyle induced weight loss and the peripheral mediators of appetite and body weight regulation

The findings of most interest, namely that weight loss was associated with increased levels of the fasting orexigenic hormone ghrelin[281] and decreased levels of the fasting anorectic hormones GLP-1[252,282] and leptin[65,66]; are consistent with the notion that weight loss induces counter-regulatory changes in the peripheral mediators of appetite and body weight regulation that work physiologically to limit further weight loss and promote weight regain. In the situation where weight loss is being attempted and weight maintenance desired, such changes may be considered "maladaptive". This is the first study to demonstrate these significant changes in fasting GLP-1, ghrelin and leptin following combined lifestyle-induced weight loss.

The changes in the hormone levels and in their overall direction are congruent to those reported in studies of weight loss with very low calorie and low calorie diets (see *Section 4.1.3*) and some of the results reported in the lesser number of studies into weight loss produced by combined diet and exercise, or an exercise only program[273,286]. However, given the paucity of the literature in this area, it is worth commenting that the results for ghrelin run contrary to the single study of weight loss by exercise only and ghrelin[284].

Ghrelin

There has been only one study of weight loss by combined diet and exercise and the effects on the secretion of ghrelin, by *Garcia et al*[285]. For obese Mexican-American women who achieved mean weight loss ~5% after 6 months of a combined diet, exercise and pharmacotherapy program, weight loss was associated with elevated fasting levels of ghrelin, which are consistent with the results for the current study (both for weight loss and the findings of elevated fasting ghrelin).

Interestingly, *Garcia et al* had an extension phase of 6 months weight-maintenance by low caloric diet and reported that by month 12, levels of ghrelin had returned to baseline despite the majority of subjects maintaining close to 5% weight loss. They concluded that the post-weight loss changes for ghrelin were not persistent and suggested that any role ghrelin played in weight-loss counter-regulation and weight regain was transient.

The findings at 12 months are partially in conflict with the main hypothesis but several factors may explain this discrepancy: firstly the improved levels of fasting ghrelin at 12 months may have been more influenced by the subjects who had regained weight than those who maintained weight loss; secondly the small sample size reduced statistical power and made *Garcia's* study more prone to Type II error; while finally there is a possibility (albeit remote) that the use of Orlistat as pharmacological adjuvant to the combined lifestyle weight loss led to an independent effect on the secretion of ghrelin from this drug.

Furthermore, the evidence from other studies does suggest that the 12 month ghrelin findings from *Garcia et al*'s are an aberration: *Sumithran et al*[239] demonstrated strongly that many peripheral regulators, including ghrelin, remain at counter-regulatory, weight regain promoting levels 12 months after weight loss and maintenance while *Foster-Schubert et al*[283] reported that obese post-menopausal women who achieved weight loss by exercise only had increased levels of fasting ghrelin in proportion with weight loss at 6 months and at 12 months after starting the intervention levels remained elevated. In addition, both studies had greater subject numbers than *Garcia et al* and therefore greater statistical power.

The current study found no correlation between changes in weight and changes in fasting levels of ghrelin which was contrary to other studies of diet induced weight loss and fasting ghrelin[243,244]. While this was a genuine finding, one mitigating issue has been already acknowledged; namely the differences at completion between the diet treatment groups for EI and (non-significantly) for fasting ghrelin (see **Appendix: Item D**).

Taken together these differences and the fact that the KD subjects made up ~45% of the entire cohort, the pooling of data for this particular analysis weakened the correlation between weight loss and changes in ghrelin. However these factors also raise an intriguing possibility; that different diets may be equally efficacious in achieving weight loss but have differential effects on appetite hormones. This idea was discussed at length in the main article arising from the original study[325] with further research is being planned into the Korean Diet and its effects on weight loss and on the secretion of gut hormones.

GLP-1

Compared to the other appetite regulation hormones, there is a paucity of studies of the effects of weight loss by any lifestyle intervention on the secretion of GLP-1, and none by combined lifestyle treatments. As such, the findings from the current study of reduced levels of fasting GLP-1 following lifestyle induced weight loss are entirely novel.

These findings are supported by the results of *Adams et al*[282] who reported that in obese subjects after weight loss with a very low calorie diet, there was a near significant trend towards reduced fasting GLP-1 and significantly attenuated post-prandial GLP-1 secretion compared to baseline (pre-weight loss) levels. Furthermore in an extension of the study that also had increased subjects[252], the effects of weight loss and maintenance were assessed with the same post-weight loss changes for fasting and post-prandial GLP-1 reported (the former now significant). Intriguingly, after 3 months of weight maintenance by a hypocaloric but not liquid meal replacement diet, levels of GLP-1 were reported to have recovered to close to pre-weight loss levels.

Counter to the results from this study and above, *Verdich et al*[250] reported that weight loss and maintenance with a very low calorie diet led to a non-significant increase to fasting levels of GLP-1 and improved post-prandial secretion. These findings are against the main hypothesis but mitigating them are the study's small sample size (the smallest of any of the studies discussed) and that the study was carried out prior to the adoption of a standardised technique for the RIA of active GLP-1.

While there have been no studies that utilise a combined lifestyle intervention for weight loss into GLP-1, our findings should also be compared against the one study of exercise-only induced weight loss and GLP-1.

Martins et al[284] reported that after weight loss by 12 weeks of regular and supervised exercising, overweight and obese subjects had enhanced post-prandial secretion of GLP-1 compared to baseline and concluded that exercise-induced weight loss may actually attenuate the expected counter-regulatory changes in GLP-1 secretion. However their subject numbers were small and the finding did not meet statistical significance.

Given the relatively small number of studies of non-surgical weight loss and GLP-1, the results of a study of weight loss with bariatric surgery compared to that with a very low calorie diet[253] should also be considered. In a well-designed protocol, which matched subjects according to % weight loss, diet-induced weight loss was found to be associated with reduced post-prandial GLP-1 secretion and surgical-induced weight loss with significantly increased post-prandial secretion. As noted in the introduction (*Sections 4.1.2.2* and *4.1.2.3*), the latter finding has been reported consistently in studies of bariatric surgery and GLP-1.

Taking the current results and evidence from the above studies, weight loss by diet and exercise induces the same counter-regulatory changes in GLP-1 as seen in diet-induced weight loss, while for post-prandial GLP-1 secretion it would appear that on-balance weight loss attenuates secretion. However as the post-prandial changes have been noted to recover after a period of weight maintenance, these changes in GLP-1

secretion are shorter lived than the changes in the other hormones. Fasting changes do appear to persist for longer as significantly reduced fasting (but not post-prandial) GLP-1 52-weeks after a weight loss intervention has been reported[239].

This study also found a correlation between change in weight and changes in fasting GLP-1, suggesting that for non-surgical weight loss, the reduction in fasting levels of GLP-1 are proportional to the degree of weight loss.

Leptin

Our findings for leptin are similar to that reported by *Volpe et al*[273], one of only two studies which have examined the combined effects of diet and exercise on peripheral regulators of appetite and body weight regulation. Like the current study, they reported that levels of fasting leptin fell and this was correlated with weight loss by both diet and exercise and diet only. Additionally, compared to *Volpe et al* the current study had greater statistical power (due to a larger numbers of total subjects and due to the pooling of data) and a study population that was heavier (mean weight in the range of obesity rather than overweight).

Furthermore such changes in leptin have been widely reported in studies of weight loss by bariatric surgery and in the small subset number of studies that directly compared bariatric surgery with very low calorie diets, levels of leptin have also been reported to decrease in proportion to weight loss[248,279].

All these findings show that levels of leptin fall following weight loss regardless of whether it achieved by hypocaloric diet, combined diet and exercise or bariatric surgery, with the reduction in leptin proportional to weight loss. The net result of these changes is to favour weight regain.

These findings also support the notion that leptin has dual physiological roles with levels chronically increased in obesity (where it acts as a long-term signal of adiposity) and acutely decreased following weight loss (where it acts as a short-term signal of energy deficit)[268]. Intriguingly, the recent finding that the changes for leptin remain present for 12 months after weight loss and weight maintenance[239] raises the possibility that the post-weight loss reductions in fasting leptin continue to exert physiological effects for longer than would be expected for a short-term signal of energy deficit.

4.5.3 Clinical implications: GLP-1 and leptin

Agents targeting the GLP-1 pathway, such as incretin mimetics which have a high affinity for the GLP-1 receptor, and DPP-IV inhibitors, which prolong the life of endogenously secreted GLP-1, have become accepted as therapeutic options in the management of T2DM[330,331]. In contrast to most other hypoglycaemic agents, these agents do not lead to weight gain and the incretin memetics (such as *Exenatide* and *Liraglutide)* are associated with weight reduction.

Two recent meta-analyses of *Exenatide* use in T2DM found that compared to standard treatment, *Exenatide* was associated with dose-dependent significant weight loss that

persisted for at least 2 years[332,333]. The unique weight loss properties of the incretin mimetics raise the possibility that these agents do have an adjuvantive role in the management of obesity. Supporting this notion a recent study found that *Exenatide,* compared to lifestyle management alone, enhanced weight loss in obese, non-diabetic subjects following 24 weeks of therapy[334] and *Liraglutide* is currently the subject of a Phase III study in overweight and obesity (ClinicalTrials.gov ID: NCT01272232).

The administration of leptin to congenitally leptin-deficient obese individuals results in dramatic improvements in body weight, insulin resistance and other metabolic parameters[265] but unfortunately trials of leptin therapy for weight loss in common obesity have so far been unsuccessful[335]. As found in the current study and others, it has been established that weight loss reduces levels of leptin and (in other studies) is associated with increased hunger[239,246].

Therefore it has been postulated that the administration of leptin post-weight loss might help weight maintenance by ameliorating one of the counter-regulatory changes seen after weight loss. This is supported by two studies, albeit with very small sample sizes. In the first study[336] 7 obese subjects who maintained clinically significant weight loss by liquid very low calorie diets were administered exogenous leptin. This treatment was found to be associated with increased resting metabolic rates and slight, but significant, increases to the circulating levels of thyroxine and triiodothyronine. In the second study[337] leptin or placebo was administered in a cross-over fashion to 6 subjects after they achieved 10% weight loss by liquid very low calorie diets. The leptin treated subjects reduced their food consumption which was found to be correlated with

increased neurological activity in the areas involved in the cognitive and emotional control of food intake (measured by functional magnetic resonance imaging).

While leptin therapy for weight maintenance remains very much in the pre-clinical stage and the use of leptin in common obesity yet to be established, these findings raise the possibility that modulating levels of leptin post weight loss may become a valid treatment option in the future.

4.5.4 Lifestyle induced weight loss and inflammation

Following weight loss, subjects had significant improvements to fasting insulin and insulin sensitivity. However for the other markers of obesity-related inflammation, i.e. adiponectin (ADP) and *hs*CRP, no significant changes were found. Finally, fasting glucose was also unchanged at completion.

Subjects in this study achieved 5.7% weight loss, generally considered a good weight loss result following lifestyle therapy[17]. Weight loss in this range is known to be associated with improvements in metabolic health, including in insulin secretion[17,229] and given the strong association between insulin resistance and overweight and obesity (and see *Section 4.1.4* above), the improvements in fasting insulin and insulin sensitivity were as expected. Likewise, the lack of change in glucose is unsurprising as individuals with T2DM were excluded from the study and as baseline glucose was well within the normal range (see **Table 4.2**).

Despite clinically significant weight loss, no significant changes were noted for ADP or *hs*CRP. While increased ADP has been reported after weight loss by all methods, the association is weaker when weight loss is under 10% and in lower grades of obesity (see *Section 4.1.4.1*). Our current results reflect both the weight loss outcomes and lower mean baseline BMI of subjects in our study.

Secondly, as the only hormone or biochemistry difference between the diet treatment groups was for ADP (both at baseline and completion favouring the KD group and see

Appendix: Item D) the pooling of data for ADP likely increased the risk for a Type II error and has thus been unsuitable for this analysis.

There was a non-significant trend of a small increase of *hs*CRP at completion. For this study population the finding was considered unlikely to be of any clinical relevance.

Generally reductions in *hs*CRP are expected after weight loss but the reduction is greater in those with greater weight loss and for those with a greater number of pre-existing metabolic co-morbidities (see *Section 4.1.4.3*). As the weight loss achieved in this study was modest and as the subjects had lower grade obesity without T2DM or active cardiovascular disease, these worked against a significant reduction in *hs*CRP.

4.5.5 Additional comments: strengths, limitations and other issues

This study successfully proved the main hypothesis, namely that counter-regulatory and maladaptive changes develop in the secretion of peripheral mediators of appetite and body weight regulation following weight loss. Furthermore in comparison to the available literature into this topic, several aspects of this study enhanced the findings; the choice of weight loss intervention was that of a combined diet, exercise and behavioural program, which has rarely been tested in clinical research despite being the cornerstone of obesity management. Clinically significant weight loss was achieved, measures of energy intake and of physical activity were recorded and there was a relatively large sample size with greater statistical power and a large number of hormonal and biochemical measures were assessed.

However, there were also several limitations to the current study. This included the absence of a standardised appetite survey, which prevented any investigation of the effects of these changes on subjective appetite and hunger. Secondly, the inclusion of a meal test at baseline and at completion would have provided an excellent opportunity to examine the effects of weight loss on the post-prandial secretion of the gut hormones and especially GLP-1, which is known to exert many of its physiological effects after food consumption. Post-prandial testing may have also clarified one area of uncertainty: whether lifestyle induced weight loss alters the secretion of these key hormones of appetite and body weight regulation in a similar fashion to diet-induced weight loss[20-24] or whether, as suggested by one exercise-only weight loss study[25], the act of exercising attenuates the changes for ghrelin and GLP-1 in the post-prandial period. Given that most clinical obesity management programs utilise diet and exercise, these remain important unanswered questions.

Finally, while the original study was extended to assess the effects of the different dietary interventions on weight maintenance[327], there was not the opportunity to re-measure the secretion of the gut hormones and adipokines in the extension phase. This was a lost opportunity to determine the persistence of the counter-regulatory changes which have been suggested to last for up to 12 months[239].

If the opportunity arises again to revisit this particular study protocol, a strong effort will be made to address the above limitations and if it proceeds, it would provide an occasion to confirm the findings while clarifying several of the outstanding issues raised in this discussion.

4.6 CONCLUSION

In conclusion, 12 weeks of a combined diet, exercise and behavioural program produced clinically significant weight loss associated with changes in the fasting levels of ghrelin, GLP-1 and leptin. The net result of these changes would be to mitigate against further weight loss and encourage weight regain.

These findings suggest that all methods of non-surgical weight loss are associated with counter-regulatory changes in the peripheral homeostatic regulators of appetite and body weight and as a combined lifestyle intervention remains the recommended first line of obesity management our findings have important "real world" implications.

These findings raise the possibility that treatments targeting these hormonal changes can affect clinical outcomes. The (to-date) limited use of GLP-1 receptor agonists for weight loss appears to be a promising first step in this new therapeutic direction.

Additional research could provide important therapeutic options in the non-surgical management of overweight and obesity and may give clinicians additional pharmacological tools to help individuals maintain long-term weight loss.

CHAPTER 5

Aspects of obesity: lessons learnt

5.1 INTRODUCTION

Overweight and obesity, the 5th leading risk for global death, is a well-recognised international health challenge[1]. In Australia over 25% of children[6] and over 63% of adults are overweight or obese, moreover the prevalence continues to increase[2]. The conditions are a significant drain on national budgets with recent local estimates of the annual direct health costs in the order of $8 billion. When indirect costs such as lost productivity are considered, this rises to a staggering $45 billion annually[13,14]

While the scale of the conditions, their costs and their associated morbidity and mortality have been the subject of much research and are all well understood, the mainstay of clinical treatment, the combined lifestyle intervention (which comprises a hypocaloric diet, increased physical activity, behavioural change and psychological support) has its antecedents in methods developed by physicians of antiquity[15,16]. Moreover combined lifestyle interventions are associated with only modest weight loss and a high rate of recidivism[20].

There have been advances made in the field of clinical treatments for obesity and this includes the use of pharmacological agents to induce weight loss. However while these

agents have generally had modest efficacy[21,22], they also have been troubled with significant side-effect profiles[338,339] and questionable cost-effectiveness[340].

The most successful modern advance has been contemporary bariatric surgery, which is associated with significant weight loss, long-term weight remission and improved mortality[24,27,234]. Nonetheless, unanswered questions remain about bariatric surgery's long-term safety and overall cost-effectiveness[27,29].

There remains much on-going research in the field of overweight and obesity and recently, novel insights regarding the aetiology of overweight and obesity have brought a renewed focus on two hitherto underexplored factors: the role of dietary protein in driving food intake and the physiological changes that develop after weight loss. Underlying both are the peripherally secreted mediators of appetite and body weight regulation.

5.2 THE EXPERIMENTS

The role of dietary protein in influencing food intake was examined in the first experiment and the *Protein Leverage Hypothesis* rigorously assessed in lean humans. The larger experiment covertly presented subjects with the same isocaloric food items but the diets differed in % protein. The results demonstrated that the subjects' intake of protein was prioritised above that of the other macronutrients, even at the expense of increasing their total energy intake.

In the associated experiment, 4 days of *ad libitum* diets differing in % protein followed by a meal test resulted in changes to the fasting and post-prandial secretion of key gut hormones in a direction favouring decreased hunger and increased satiety.

In the final experiment, the prioritisation of dietary protein according to the *Protein Leverage Hypothesis* was tested in an obese phenotype. In the first report of this kind, obese mice demonstrated the same priority for maintaining their intake of dietary protein, even at the expense of increasing their intake for total energy, as was found in their lean counterparts. However the degree of protein prioritisation and the effects from variety and novelty in the diet were less powerful than that seen in lean mice, suggesting that the state of obesity itself may modulate the usual regulators of food intake.

In the third experiment, which examined weight loss and remission in an obese cohort, for the first time clinically significant weight loss by a combined lifestyle program was shown to be associated with changes in the fasting secretion of several key gut

hormones and adipokines that regulate appetite and body weight. The overall direction from these changes would be to act to limit further weight loss and to promote weight regain.

5·3 IMPLICATIONS FOR FUTURE RESEARCH AND CLINICAL DIRECTIONS

Implications for research

There are studies currently underway involving humans and mice into the *Protein Leverage Hypothesis*. While this author is not directly involved in these studies, the new studies have acknowledged the limitations that arose from the current experiments and have addressed many of the limitations discussed in **Chapters 2** and **3**.

For testing the *Protein Leverage Hypothesis* in lean humans (**Chapter 2**), at the completion of the current study a revised protocol was developed that addressed practical limitations such as refining participant instructions for the Appetite Visual Analogue Scale and streamlining the collection of samples. A major protocol change was the incorporation of four diet treatment cycles, which give the new study an opportunity to explore the role of variety and the relative power of carbohydrates and fat in modifying *protein leveraging*. The revised study commenced recruitment in the middle of 2012.

The experience gained from testing the *Protein Leverage Hypothesis* in obese mice (**Chapter 3**) has helped inform the on-going mice studies. Protocol changes include increasing subject numbers, lengthening the duration of the conditioning diets and diet treatments and increasing the number of treatments. These changes allow for statistical power to be maintained despite the increase in diet treatments and provide

sufficient time for the development of more differentiated phenotypes. The larger number of comparator groups will provide a clearer assessment of the effects of low and high protein diets on body composition and on the secretion of orexigenic and anorexigenic hormones while maintaining the ability to directly compare food intake between the different protein diets.

There will be an extension of the study performed and described in **Chapter 4,** providing the opportunity to continue the investigation as to the effects of weight-loss on the secretion of peripheral mediators of appetite and body weight regulation. Firstly, a subjective measure of appetite and hunger will be added, to help determine whether changes in hormones are correlated with changes in subjective hunger. Secondly, the addition of a standardised meal test will allow for the measurement of the post-prandial secretion of hormones at baseline and at completion. Thirdly, measuring hormones during the period of weight-maintenance will allow for an assessment of the persistence of the counter-regulatory changes. Fourthly, an increased number of hormones will be measured (such as the gut hormone peptide YY). Finally, better control of the energy intake during the dietary intervention should prevent any differences in energy intake from clouding the results.

Clinical directions

The results of the three experiments point to novel insights into different aspects of obesity. While further and more detailed research is needed before this translates into changes to clinical practice, it is foreseeable that validation of the *Protein Leverage Hypothesis* could lead to changes in national dietary recommendations, which could assist in reducing the incidence of overweight and obesity. Moreover a deeper understanding of the effects of dietary protein on gut hormone secretion may also alter the dietary recommendations for weight loss. A more complete understanding of the changes which develop in peripheral mediators of appetite and body weight regulation, which could open up new areas for targeted pharmacotherapy with the goal of enhancing both weight loss and weight maintenance.

5.4 FINAL CONCLUSIONS

The first two experiments help to confirm the central role that dietary protein plays in the regulation of food intake and that *protein leveraging* occurs regardless of phenotype. When contrasted to the secular trends in the consumption of macronutrients and increased energy intake, the *Protein Leverage Hypothesis* offers a fresh perspective to the aetiology of the increase in global overweight and obesity while potentially offering a novel approach to its dietary management.

The third experiment demonstrated that following weight loss by conventional lifestyle strategies, the same physiological homeostatic counter-regulatory changes develop as has been reported following diet-induced weight loss. The cumulative direction of these changes is to limit further weight loss and promote weight regain. These novel findings help provide the physiological underpinning to the high rates of recidivism that follow weight loss by conventional combined lifestyle methods and as such, has *"real world"* implications.

When the three experiments are taken together, they highlight the important role of gut hormones and adipokines in mediating changes in appetite, changes in food intake and ultimately changes in body weight both during periods where body weight is stable and during periods when it is in flux. Further research exploring these concepts may assist in the development of an integrated obesity treatment paradigm, which involves prescribing both diet and pharmacotherapy to induce weight loss and to target changes in key hormones to both facilitate weight loss and to help maintain reduced body weight.

CHAPTER 6

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CHAPTER 7

Appendix

Item A The principle of the technique for Radioimmunoassays

The principle of the RIA technique follows:

A fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum so that the concentration of antigen binding sites on the antibody is limited (e.g. only 50% of total tracer concentration may be bound by antibody). Unlabelled antigen is added, which leads to competition between labelled tracer and unlabelled antigen for the limited number of antibody binding sites. The amount of tracer bound to antibody decreases as the concentration of unlabelled antigen increases and so a standard curve generated. When unknown samples are prepared with antibody and tracer and measured against the standard curve, this method allows for the value of the unknown sample to be calculated against the known values arising from the standard curve.

The following pieces of laboratory equipment were used for all RIAs:

- 10 - 1000 μL pipettes with disposable tips,
- 10 - 1000 μL repeating dispenser (Eppendorf Multi-pipette),
- a vortex mixer (Ratek Model MTV1),
- a Ratek rolling column mixer, a refrigerated swing bucket centrifuge (HD Scientific / Hettich Zentrifuger Rotixa 50RS),
- an automated gamma-counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma-counter; MultiCalc v 3.6),
- refrigerators (set at -80 ^{o}C, -20 ^{o}C and 3.5^{o}C) were used to store reagents and samples, to thaw frozen reagents and samples and to incubate samples during the RIA runs.

For the RIAs for total ghrelin and insulin, samples were placed into pointed polypropylene tubes (12 x 75 mm).

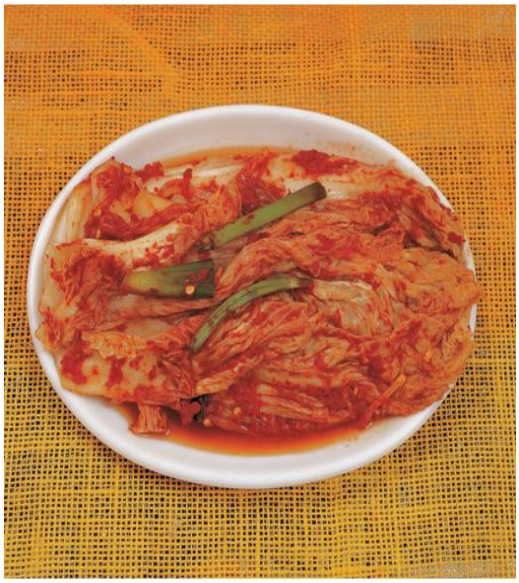
For the RIAS for CCK and GLP-1, the assay technique required a preliminary process of extraction of hormone from stored plasma. This involved the use of a 96% EtOH solution and a 36-well heating block with a 32-tube glass gas collector and distributor attached to a central source of nitrogen oxide. The extraction of GLP-1 from the frozen plasma samples also used Eppendorf microfuge tubes (1.5mL). Extracted and rehydrated samples were then placed into in borosilicate glass tubes (10 x 70 mm) for analysis.

Item B(1) An example of a main meal for the Korean Diet group

Rice	e.g. *Kimbap* (sushi rice), *Bibimbap* (combination rice dish) or *Kimchi bokkeumbap* (Kimchi fried rice)
Kimchi	e.g. *Tongbaechu-kimchi*, *Kkakdugi*
Soup	e.g. *Tteok Mandu-guk*, *Kongnamulguk*
Side dishes	e.g. *Beoseot Bokkeum* (mushroom), *Kongjaban* (black bean) or *Gyeran jjim* (egg roll)
Others	e.g. *Kimchi Jeon* (Kimchi pancake), *Haemul pajeon* (seafood pancake) or *tteokbokki* (rice cake)
Meats	e.g. *Bulgogi* (BBQ beef) or *Satae pyeon yuk* (beef)
Noodle	*Kongguksu* (noodle soup), *Memil-guksu* (buckwheat noodle) or *Japchae* (potato noodle)
Tea drinks	e.g. *Green tea* or *Sikhye* (rice beverage)

Item B(2) Images of traditional Korean meals

Traditional Korean vegetable sides



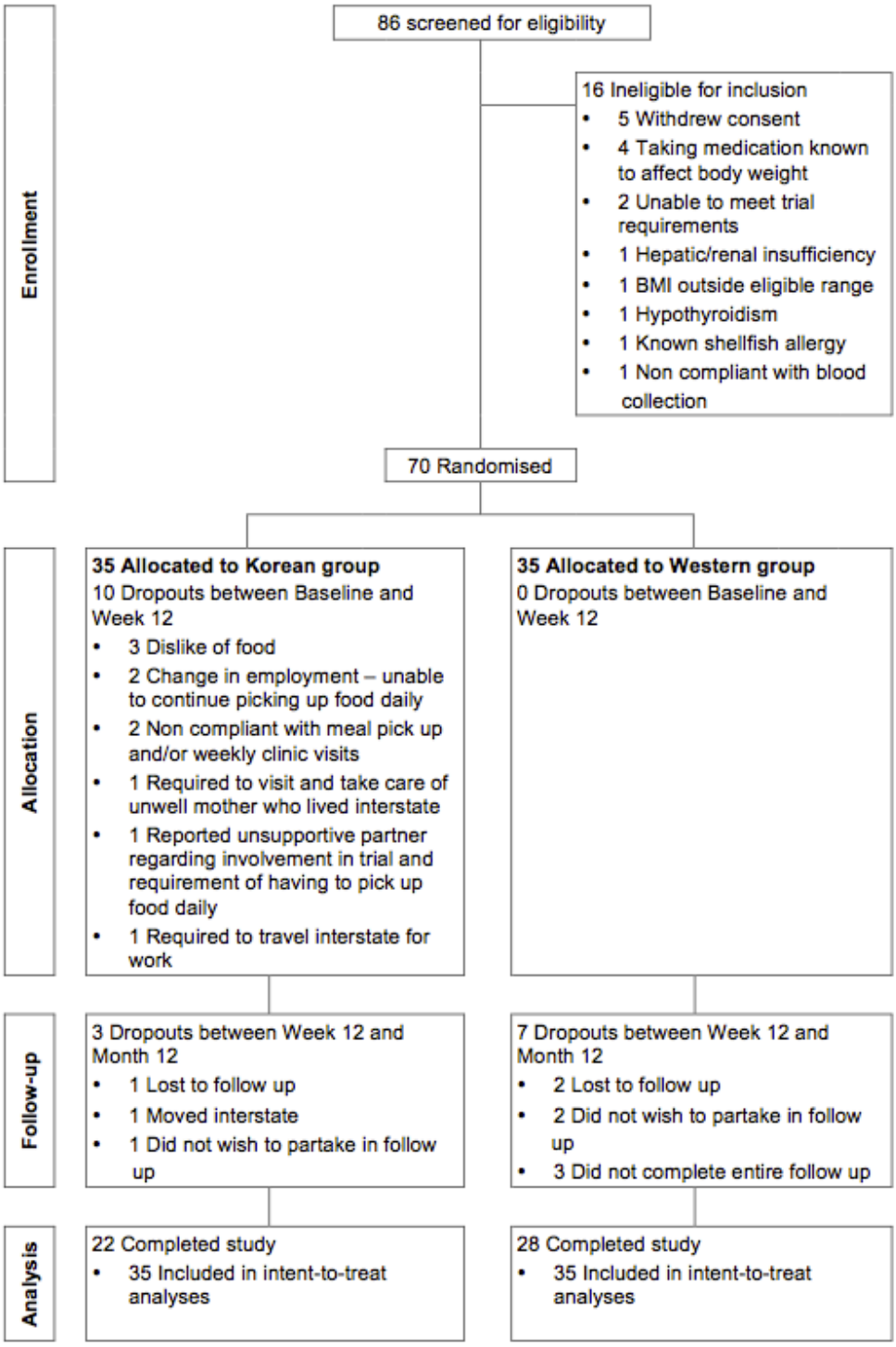
kimchi

Glass noodles with sautéed vegetables

Bokkeumbap stir fried *kimchi*, egg and rice

NB: Images obtained under the Creative Commons license from flickr – Korea.Net

Item C CONSORT diagram: Disposition of subjects for the Korean Diet Study



Item D Measures at completion (week 12) per dietary intervention

	Korean Diet	**Conventional Diet**	***P***
Number %Female	25 72%	35 54%	 0.14
Δ %Weight loss (% from baseline)	-5.8 ± 4.7	-5.7 ± 41	0.93
Δ BMI (kg/m²)	-1.7 ± 0.1	-1.7 ± 0.10	0.92
Δ Waist circumference (cms)	-5.1 ± 3.9	-3.4.0 ± 3.6	0.11
Step Count (steps/day)	11987±5130	12284±5214	0.42
Daily Energy Intake (kJ/day)	7407.9	5300.4	*<0.001*
Total Ghrelin (pmol/L)*	336.0, 179.2	420.7, 275.6	0.12
Active GLP-1 (pg/mL)*	25.5, 19.3	28.8, 10.3	0.26
Leptin (ng/mL)	13.3 ± 2.5	13.1 ± 1.9	0.86
Adiponectin (ug/mL)	13.5 ± 1.4	10.6 ± 0.8	*0.02*
Insulin (mU/L)*	5.5, 4.5	6, 5	0.33
Glucose (mmol/L)	4.0 ± 0.1	4.5 ± 0.1	0.07
HOMA-IR*	0.98, 0.95	1.15, 0.99	0.74
hsCRP (mg/L)	2.9 ± 0.6	4.4 ± 0.8	0.15

NB: Data are presented as mean ± SEM and analysed by Two samples *t*-test unless otherwise noted

* non-parametrically distributed data as median, IQR and analysed by Wilcoxon-Mann-Whitney *U* test.

Item E International Physical Activity Questionnaire (short form)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

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

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

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

   _____ **days per week**

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

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

   _____ **hours per day**
   _____ **minutes per day**

   ☐ Don't know/Not sure

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

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

_____ days per week

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

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

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

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

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

_____ days per week

☐ No walking → ***Skip to question 7***

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

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

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

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

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.