The impact of whey protein isolate on energy balance regulation

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

by

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Declaration

I hereby certify that the work presented here in this thesis, for which I now submit for assessment on the programme of study leading to the award of PhD, is my own original work and has not been previously submitted, in part or in whole, for any other degree, either to University College Cork or any other University.

Signed: _____ Liam McAllan

Date : _____

Author contributions

All the work herein was performed by myself, with the following exceptions:

CHAPTER 2

Dr. Deirdre Keane preformed the measurements of plasma insulin, glucose and GLP-1. Dr. Harriet Schellenkens preformed the insulin receptor immunoblot analysis.

CHAPTER 3

Paula O'Connor preformed the plasma amino acid analysis.

Peter Skuse preformed DNA isolation, sequencing, analysis and write up for the gut microbiota composition.

CHAPTER 5

Dr Eileen Murphy conducted the LFD vs. HFD mouse trial and provided the lean (+/?) and *ob/ob* mice epididymal adipose tissue samples.

Original Publications

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McAllan L, Skuse P, Cotter PD, O'Connor P, Cryan JF, Ross, RP, Fitzgerald G, Roche HM, Nilaweera KN (2013) Protein quality and the protein to carbohdate ratio within a high-fat diet influences energy balance energy and the gut microbiota in C57BL/6J mice. *Plos One* **9**: e88904

Abbreviations

ACC	Acetyl-CoA carboxylase
AgRP	Agouti-related protein
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
β3-AR	β3-adrenegenic receptor
BAT	Brown adipose tissue
BLAST	Basic local alignment search tool
BMR	Basal metabolic rate
BSA	Bovine serum albumin
CCK	Cholecystokinin
CCK-1	Cholecystokinin receptor 1
CCK-2	Cholecystokinin receptor 2
CD36	Cluster of differentiation 36
CD68	Cluster of differentiation 68
cDNA	Complementary DNA
CI	Cell index
CNS	Central nervous system
CO_2	Carbon dioxide
Cp	Crossing point of fluorescence
CPT1a	Carnitine palmitoyltransferase a
CPT1b	Carnitine palmitoyltransferase b
CPT1c	Carnitine palmitoyltransferase c
db/db	Leptin receptor gene deficiency
DEPC	Diethylpyrocarbonate
DGAT	Diglyceride acyltransferase
DIO	Diet-induced obesity
Diff	Differentiated
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DPPIV	Dipeptidyl peptidase-4
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EHA	European nucleotide archive
ELISA	Enzyme-linked immunosorbent assay
EWAT	Epididymal white adipose tissue
FABP1	Fatty acid binding protein 1
FASN	Fatty acid synthase
FATP1	Fatty acid transport protein 1
FATP4	Fatty acid transport protein 4
FATP5	Fatty acid transport protein 5
FBS	Foetal bovine serum
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCCR	Glucocorticoid receptor
GHS-R	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIT	Gastrointestinal tract
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GMP	Glycomacropeptide
GPR	G-protein coupled receptor
GPR93/92	G-protein coupled receptor 93 or 92(same as LPA_5)
HCL	Hydrochloric acid
HEPES	4(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HOMA-IR	Homeostasis model assessment of insulin resistance
HSL	Hormone sensitive lipase
ICV	Intraventricular

IGF-1	Insulin-like growth factor 1
IR (or INSR)	Insulin receptor
IRS-1	Insulin receptor substrate 1
IP	Intraperitoneal
JAK2	Janus kinase 2
Kcal	Kilocalorie
KING	Kinemage next generation
kJ	Kilojoule
LFD	Low fat diet
L-GS	Liver glycogen synthase
LH	Lateral hypothalamus
LPA	Lysophosphatidic acid
LPA_1	Lysophosphatidic acid receptor 1
LPA_5	Lysophosphatidic acid receptor 5 (same as GPR93/92)
LPL	Lipoprotein lipase
MC3R	Melanocortin-3 receptor
MC4R	Melanocortin-4 receptor
MEGAN	Metagenome analyser
M-GS	Muscle glycogen synthase
MJ	Megajoule
MOPS	3-(morpholino) propanesulfonic acid
mRNA	Messanger RNA
mTOR	Mammalian target of rapamycin
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaPPi	Sodium pyrophosphate
NCBI	National centre for biotechnology information
NEFA	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
NPY2-R	Neuropeptide Y2 receptor
NS	Non-significant

NTS	Nucleus of solitary tract
NZMP	New Zealand milk protein
ob	Leptin gene
ob/ob	Leptin gene deficiency
ObR	Leptin receptor
OTU's	Operational taxonomic units
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulphonyl fluoride
POMC	Pro-opiomelanocortin
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PTP1B	Protein-tyrosine phosphatase 1B
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
QIIME	Quantitative insights into microbial ecology
RDP	Ribosomal database project pyrosequencing
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGLT-1	Sodium-glucose transport protein 1
SNS	Sympathetic nervous system
SOCS-3	Suppressor of cytokine signalling 3
STC-1	Secretin tumour cell line 1
SWAT	subcutaneous white adipose tissue
TAG	Triacylglycerol (triglyceride)
TNF-α	Tumour necrosis factor alpha
Tyr	Tyrosine
UCP-2	Uncoupling protein 2
UCP-3	Uncoupling protein 3

Undiff	Undifferentiated
VCO_2	Carbon dioxide production
VLDL	Very low-density lipoprotein
VMH	Ventromedial hypothalamus
VO_2	Oxygen consumption
WAT	White adipose tissue
WNT10b	Wingless-type MMTV integration site family, member 10B
WPI	Whey protein isolate
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1

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ABSTRACT

Whey proteins are a by-product of cheese manufacture that have been shown to suppress high fat diet (HFD)-induced weight gain and adiposity independently of changes to energy intake. This project tested the hypothesis that whey protein isolate (WPI) directly impacts on adiposity by influencing tissue lipid metabolism. The hypothesis was tested using C57BL/6J mice fed WPI enriched high fat (HFD) or low-fat diets (LFD) and by assessing the functionality of the lysophosphatidic acid receptor 5, as a potential candidate mediating direct protein effects on adipose tissue.

WPI suppressed HFD-induced body fat at 8 weeks of dietary challenge and augmented lean mass. At a cellular level, WPI reduced HFD-associated hypothalamic leptin receptor, insulin receptor (IR), and carnitine palmitoyltransferase 1b (CPT1b) mRNA expression, and prevented HFD-associated reductions in adipose tissue expressions of IR and glucose transporter 4 (GLUT4). These changes occurred in a background of elevated plasma triacylglycerol (TAG), suggesting a reduced TAG storage in the tissues with WPI intake. While most of these effects were largely absent when the study was extended to 21 weeks, notably WPI specifically increased lean mass and caused a trend towards a decrease in fat mass. Analysis of faecal microbial populations revealed specific effects of WPI, namely increased proportions of *Lactobacillus* and decreased *Clostridiu*m. Increasing the (whey) protein to carbohydrate ratio within the HFD enhanced WPI specific effects on adiposity, and lean mass, similar to that observed in the short-term study and caused a shift in the composition of gut microbiota away from the HFD group.

Using mice fed a LFD enriched with WPI (LFD-WPI), the effect of the dietary protein on insulin and lipid-associated gene expression in the adipose tissue was further evaluated. The 7 weeks of LFD-WPI intake decreased insulin and lipid-related gene expression in the adipose tissue. In contrast, 15 weeks of LFD-WPI did not affect insulin-related gene expression but augmented lipid metabolism related gene expression. In both studies, WPI intake also reduced intestinal weight and length, suggesting a potential functional relationship between WPI, gastro-intestinal morphology and insulin and lipid metabolism related signalling in the adipose.

To obtain evidence of a direct route of action for WPI on adipose tissue, the functionality of LPA_5 in these cells were assessed. The gene expression of the receptor was increased in the adipose tissue by HFD feeding and *ob* gene mutation, as well as by the differentiation in 3T3-L1 cells. Over-expression of the receptor in 3T3-L1 pre-adipocytes provided a growth advantage to the cells and suppressed their cellular differentiation. These data suggest an important role for LPA_5 in pre-adipocyte growth and differentiation.

In conclusion, the data presented here provides support to the hypothesis that WPI can impact on adiposity by influencing lipid metabolism, by affecting lean mass, hypothalamic gene expression, changes to gut microbiota and alterations to gastrointestinal morphology. These effects were dependent on the macronutrient composition in the WPI enriched diet and its duration of feeding. Our data also showed the LPA_5 is a novel candidate in the regulation of pre-adipocyte growth and differentiation, and thus could potentially mediate direct effects of WPI on adipose cellular activity.

Chapter 1

The current understanding of how whey proteins influence the regulation of energy balance

Published works associated with this chapter are:

McAllan L, Cotter PD, Roche HM, Korpela R, Nilaweera KN (2012) Bioactivity in whey proteins influencing energy balance. *Journal of Metabolic Syndrome* **1**: 107.

McAllan L, Cotter PD, Roche HM, Korpela R, Nilaweera KN (2013) Impact of leucine on energy balance. *Journal of Physiology and Biochemistry* **69**: 155-163

1.1 ABSRACT

Body weight is determined by the balance between energy intake and energy expenditure. When energy intake exceeds energy expenditure, the surplus energy is stored as fat in the adipose tissue, which causes its expansion and eventually leads to the development of obesity. Regulation of energy balance is a complex physiological process that requires the interaction between multiple tissues, including the gastrointestinal tract, liver, pancreas, adipose tissue and the brain, and includes the regulation of lipid metabolism and glucose homeostasis. Nutrients that reduce the positive energy balance and thereby fat accumulation are thus being considered as therapies to combat obesity. In this regard, much interest has been focussed on the ability of whey proteins to suppress obesity development. Here, we review the literature related to the physiological, cellular and endocrine effects of intake of whole whey protein isolate/concentrate and individual whey proteins (α-lactalbumin, β-lactoglobulin, glycomacropeptide and lactoferrin). Moreover, we discuss how dietary composition and obesity may influence whey protein effects on the above parameters. Evidence suggests that intake of whey proteins causes a decrease in energy intake, increase in energy expenditure, influences insulin sensitivity and glucose homeostasis and alters lipid metabolism in the adipose, liver and muscle. These physiological changes are accompanied by alterations in the plasma levels of energy balance related hormones (cholecystokinin, ghrelin, insulin and glucagon-like peptide-1) and the expression of catabolic and anabolic genes in the above tissues in the direction of a negative energy balance. Evidence is also presented that whey proteins could reduce fat mass accumulation without substantial changes in energy intake or energy expenditure.

1.2 INTRODUCTION

Obesity has reached epidemic proportions globally (Caterson *et al*, 2004). The World Health Organisation is projecting 1.5 billion adults will be overweight and over 700 million will be obese by 2015. Due to its propensity to increase the risk of developing several clinical conditions including cardiovascular disease, stroke, hypertension, and type 2 diabetes, it is one of the major health problems associated with the world today (Schelbert, 2009). With the increased prevalence of obesity, economic costs are also projected to increase, with costs for a country such as the UK being predicted to increase by 1.9-2.0 billion £/year by 2030 in order to cope with a projected 11 million increase in obese individuals (Wang *et al*, 2011). Similarly, in Ireland, according to the National Task Force, obesity-related costs were up to €4 billion/year in 2005. This is expected to rise in the future, primarily because of the increasing prevalence of childhood obesity in Ireland, which at the time of this report was estimated at over 300,000 overweight and obese children, with this number projected to rise by 10,000 per annum. Importantly, the reversal of obesity is difficult to achieve as body fat is defended against acute perturbations, and the set point at which body fat is defended increases during obesity development (Ryan *et al*, 2012). Therefore, the growing opinion is that prevention of obesity from early on in life is the best solution to the future obesity epidemic. Thus, the discovery of nutritional factors which can reduce body weight is of critical importance in the prevention and/or reduction of obesity.

Epidemiological studies show that there is an inverse relationship between the consumption of diets high in dairy products and body mass index, as well as a decrease in the risk of developing type-2 diabetes and metabolic syndrome (Baxter

et al, 2006; Gao *et al*, 2013). Dairy proteins, particularly whey proteins, which are a by-product of cheese manufacture, have been linked to the weight regulating effects of dairy intake (Bendtsen *et al*, 2013). In this regard, whey protein isolate (WPI), which is the enriched protein form of whey, has received increasing research interest in relation to its impact on energy balance (Jakubowicz & Froy, 2013; Luhovyy *et al*, 2007). In fact, evidence suggests that whey proteins can impact on food intake (Luhovyy *et al*, 2007) and energy expenditure (Acheson *et al*, 2011; Hursel *et al*, 2010), and even on bodyweight in the absence of these latter changes (Pilvi *et al*, 2009; Royle *et al*, 2008). However, it is still not entirely understood how whey proteins mediate their action, although emerging evidence suggests that whey proteins may directly impact on the peripheral and central mechanisms regulating lipid metabolism.

Functional Foods are those that deliver health benefits beyond basic nutrition (Hasler, 2002). In this regard, WPI appears to be an attractive option for this use because of the energy balance-related effects described above and because this milk protein fraction is readily available and relatively inexpensive to isolate. Therefore, this offers the opportunity to assign considerable added value to WPI and to market it as a health promoting ingredient. In terms of the Irish Functional Food industry, such product development will allow job creation and expansion of the strong internationally recognised dairy industry in Ireland, and also contribute to the recovery of the Irish economy.

The main purpose of this thesis was to further investigate the potential of WPI to prevent the development of obesity, focusing on the impact of WPI on lipid metabolism and the mechanisms involved. The output of this research may help in the identification of bioactive components within whey proteins that could

specifically affect lipid metabolism. This introductory chapter will first briefly describe some of the key components and pathways involved in the regulation of energy balance and body weight, and then will provide an overview of the current understanding of how these systems are impacted upon by whey proteins.

1.3 REGULATION OF ENERGY BALANCE

Body weight is determined by a number of different components that fall under the three main categories of energy intake, energy expenditure and lipid metabolism (i.e. energy storage)(Hill *et al*, 2012). Mammals, including humans, obtain energy from food consumed daily, with this intake showing a rhythmic pattern, where food is ingested until fullness is achieved (satiation) and it will not resume for a period of time (satiety) to allow for the meal to be digested and its energy extracted. The energy consumed will replenish that spent on metabolic activity related to basal metabolic rate (BMR), physical activity and adaptive thermogenesis (Clarke & Henry, 2010). BMR is the amount of energy expended while at rest in the post-absorptive state to maintain the functions of vital organs, while adaptive thermogenesis is the regulated production of heat in response to environmental or dietary factors (Clarke & Henry, 2010; Lowell & Bachman, 2003). Positive energy balance occurs where energy consumed in excess of physical and metabolic requirements gets stored as triacylglycerol (TAG) in the adipose tissue. If the positive energy balance is sustained beyond the capacity of the adipose tissue to store it, then the progression of obesity development will occur, leading to the ectopic deposition of fat in other tissues (Fair & Montgomery, 2009).

Regulation of energy balance is a complex physiological process that requires the interaction between multiple tissues, including the gastrointestinal

tract (GIT), liver, pancreas, adipose tissue, skeletal muscle and the brain (Lenard & Berthoud, 2008). When there is a positive or negative energy balance, which is reflected by nutrient abundance or deficit respectively, the tissues are able to communicate this information, via the production of hormones (peripheral tissues) or neuropeptides (brain), to achieve energy homeostasis (Lenard & Berthoud, 2008). Thus, tissues not only metabolise nutrients, but also act as sensors of their abundance.

Lipid metabolism begins with the GI digestion of dietary proteins, fats and carbohydrates generating peptides, amino acids, fatty acids and glucose, which can then be absorbed through the intestine either passively or via active transport. Fatty acids that enter intestinal enterocytes are reconstituted as TAG and exported into the circulation within lipoproteins called chylomicrons (Mu & Hoy, 2004). The hydrolysis of circulating lipoprotein bound TAG, by lipoprotein lipase (LPL) at the outer cell surface, allows the uptake of fatty acids into tissues such as the skeletal muscle and adipose tissue, for the production of energy and/or storage as TAG, respectively (Voshol *et al*, 2009). Hydrolysis of adipose TAG stores generates free fatty acids (Robidoux *et al*, 2004), that when released into the circulation can be used as an energy source for other tissues (e.g. liver and muscle) (Frayn *et al*, 2006). Glucose is primarily utilised for the production of energy in the skeletal muscle, while in the liver and adipose tissue it is metabolised via the glycolytic and lipogenic pathways to generate fatty acids (Kersten, 2001), which can then be either stored as TAG (mainly by adipose), exported back into the circulatory system in lipoproteins (mainly by liver), or oxidised in the mitochondria to generate ATP (mainly by liver & skeletal muscle)(Frayn *et al*, 2006; Redinger, 2009).

The energy status in peripheral tissues is conveyed to the regions of the central nervous system (CNS) involved in the regulation of food intake and energy expenditure by hormones produced by the GIT, adipose tissue and pancreas. The main short-term satiety/satiation hormones are cholecystokinin (CCK), glucagon like protein 1 (GLP-1), peptide tyrosine-tyrosine (PYY) and ghrelin, and the long-term energy status-related signals are insulin and leptin (Woods & D'Alessio, 2008) (Table 1.1). The hindbrain and the hypothalamic nuclei, primarily the arcuate nucleus (ARC), are the principal brain sites for receiving and processing these peripheral signals (Berthoud *et al*, 2006; Lenard & Berthoud, 2008) (Figure 1.1). These hormonal signals are transmitted via vagal afferent neuronal fibres projecting into the nucleus of solitary tract (NTS) in the hindbrain, or via crossing the blood brain barrier (BBB) and acting directly on their target sites located in the CNS (Figure 1.1 & 1.2)(Berthoud *et al*, 2006; Sam *et al*, 2012). Neural projections between the hindbrain and hypothalamus transmit and allow integration of the hormonal signals via changes in neuronal activity and expression of several important neuropeptides, including pro-opiomelanocortin (POMC), Neuropeptide Y (NPY) and Agouti-related protein (AgRP) (Woods & D'Alessio, 2008) (Figure 1.2). The resulting behavioural, autonomic and endocrine responses are mediated through catabolic and anabolic output pathways originating from the activation of second order neurons. Indeed, the sympathetic nervous system (SNS) regulates peripheral lipid metabolism through its direct innervation of peripheral tissues, resulting from the balance between catabolic and anabolic CNS outputs (Figure 1.3) (Geerling *et al*, 2014). Thus, signals generated from multiples inputs are integrated centrally with the net result being changes to food intake, energy expenditure and/or lipid metabolism.

1.3.1 Short-term energy status-relaying signals

The underlying molecular mechanisms by which satiation and satiety arise, involves an interaction between the GIT and the CNS mediated through GI-produced hormones, namely ghrelin, CCK, GLP-1 and PYY. The hormone ghrelin, produced in the stomach, acts to initiate food intake (orexigenic effect) (Tschop *et al*, 2000; Wren *et al*, 2001a), and thus, its production increases prior to food ingestion. Once the food intake begins, ghrelin production decreases (Cummings *et al*, 2002). In contrast, CCK, GLP-1 and PYY are produced by the enteroendocrine cells in the intestinal epithelium in response to the availability of nutrients in the lumen (Nilaweera *et al*, 2010; Suominen *et al*, 1998) and inhibit food intake (anorexigenic) by inducing satiation and/or satiety (Neary & Batterham, 2009).

1.3.1.1 Cholecystokinin (CCK)

CCK is a peptide produced by mucosal enteroendocrine I cells in the proximal small intestine and is secreted in response to food ingestion (Figure 1.1). The principal stimulators of its release are dietary fats (fatty acids) and proteins (amino acids and peptides)(Woods & D'Alessio, 2008) (Table 1.1). Recent *in vitro* work using the enteroendocrine cell line STC-1 has demonstrated that protein hydrolysate stimulation of CCK gene expression and release is mediated through the activation of the G-protein coupled receptor, lysophosphatidic acid receptor 5 (LPA_5) (GPR92/93; see below) (Choi *et al*, 2007a; Choi *et al*, 2007b).

The ability of CCK to reduce food intake was first shown by Gibbs *et al.,* (1973), where a significant reduction in subsequent meal size was found in rats following the peripheral administration of CCK prior to food consumption. Since then, the ability of CCK to suppress short-term energy intake in both lean and

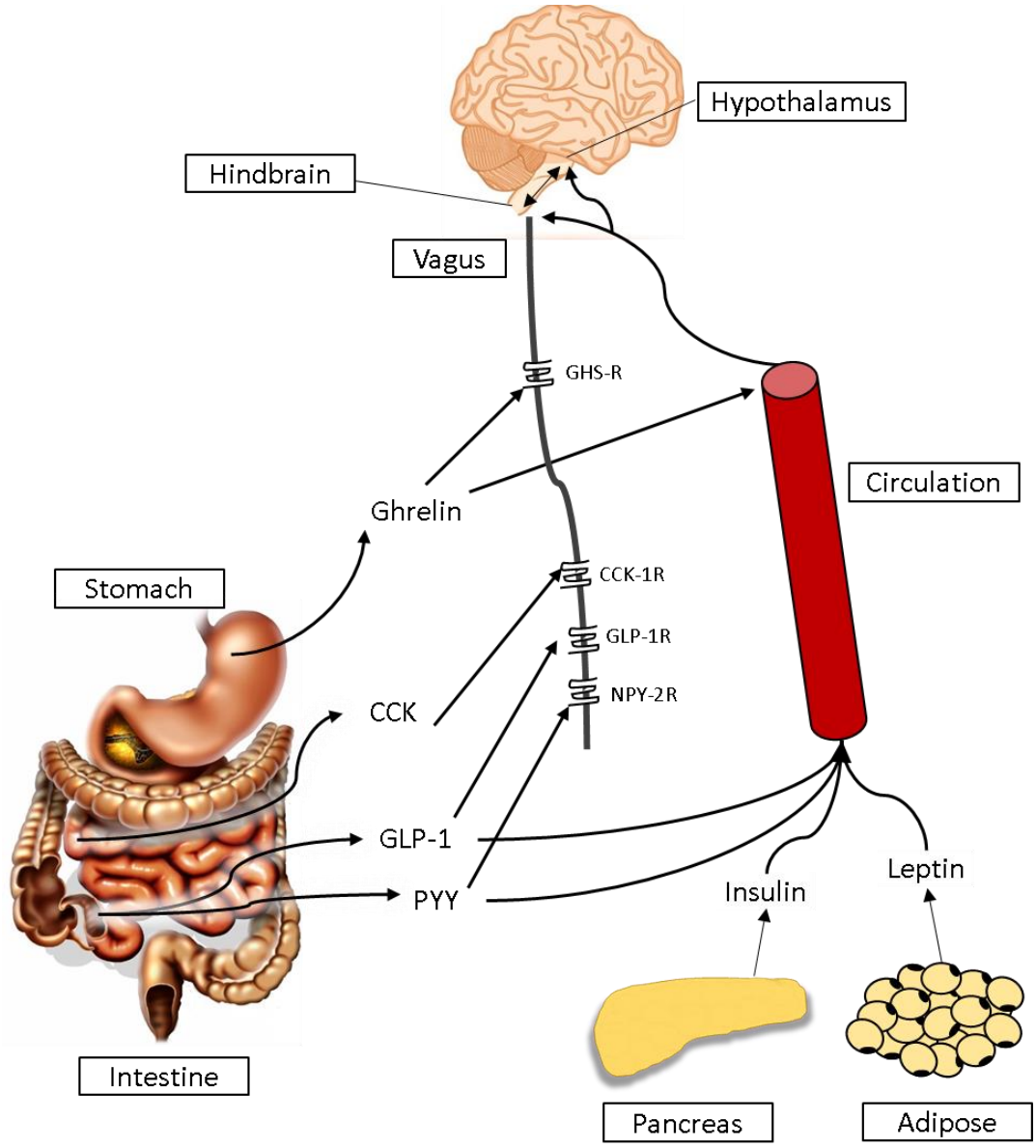

Figure 1.1. Overview of peripheral signals that influence energy balance regulation. During food ingestion short-term satiety/satiation signals, namely cholecystokinin (CCK), Glucagon-like peptide 1 (GLP-1) and Peptide YY (PYY) are released from small intestine. These anorexigenic signals are transmitted from gastrointestinal tract (GIT) to the hindbrain via vagal afferent neuronal fibres, or they can cross the blood brain barrier (BBB) and act directly upon their target sites located in the hindbrain and the hypothalamus. In contrast, the levels of ghrelin produced in the stomach rise in anticipation of food intake, and it induces its orexigenic effects via both vagal nerve activity and by direct action in the hypothalamus. Signals related to the body's long-term energy status, represented by the hormones leptin and insulin, are transported via the circulation to the brain, where they can cross the BBB and act upon hypothalamic (both) and hindbrain (leptin) areas involved in energy balance regulation. (Adapted from Duca & Covasa (2012)).

obese humans has been documented (Kissileff *et al*, 1981; Lieverse *et al*, 1995; Lieverse *et al*, 1994). Moreover, CCK has also been shown to suppress gastric emptying and transit, which would contribute to its satiating effect (Grider, 1994).

CCK mediates its anorexic effects by activation of its CCK-1 receptors, which are abundantly expressed on peripheral vagal afferents fibres, and in the hindbrain and the hypothalamus (Neary & Batterham, 2009) (Figure 1.1). Genetic deletion or pharmacological antagonism of these CCK-1 receptors abolishes the anorexic effect of CCK (Kopin *et al*, 1999; Moran *et al*, 1998). Also, given that complete ablation of the NTS (Edwards *et al*, 1986) or vagotomy (Smith *et al*, 1981) abolishes the anorexic effects of CCK, the primary route by which these effects are mediated appears to be through vagal afferents nerve fibres projecting to the hindbrain (Ritter *et al*, 1999). Indeed, only peripheral, and not central, CCK-1 receptor antagonism impairs the ability of peripheral CCK to suppress food intake (Brenner & Ritter, 1998; Reidelberger *et al*, 2003).

In summary, CCK influences food intake by reducing meal size (satiation) via signalling along the vagal afferent fibres from the GIT to the NTS in the hindbrain.

1.3.1.2 Glucagon-like peptide 1 (GLP-1)

GLP-1 is a peptide produced in L cells of the ileum and colon following enzymatic cleavage of proglucagon. It is secreted in response to nutrient ingestion and is rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPPIV) (Orskov *et al*, 1993) (Figure 1.1). Administration of GLP-1, either centrally or peripherally, reduces food intake in several species (Donahey *et al*, 1998; Tang-Christensen *et al*, 1996; Turton *et al*, 1996), including humans (Verdich *et al*, 2001). In addition,

GLP-1 has a variety of other functions, including reducing gut motility and glucagon synthesis, as well as enhancing insulin release and pancreatic β-cell growth (Donnelly, 2012).

GLP-1 receptors (GLP-1R) specifically mediate the anorexic effects of GLP-1, and are found in the hindbrain, hypothalamus, and on vagal afferents fibres projecting from the GIT to the NTS (Bullock *et al*, 1996) (Figure 1.1). Indeed, GLP-1's ability to suppress food intake is absent in rodents lacking GLP-1R (*glp1r-/-*) and in those peripherally treated with the GLP-1R antagonist, exendin 9-39 (Baggio *et al*, 2004; Williams *et al*, 2009a). Furthermore, peripheral GLP-1R agonist, exendin-4, administration, both reduces the subsequent meal size (satiation) and increases the inter-meal interval (satiety) (Williams *et al*, 2009a). Evidence has shown that the anorexic response to gut-derived GLP-1 is abolished following either vagotomy or disruption of the hindbrain-hypothalamus neural projections (Abbott *et al*, 2005).

In terms of energy expenditure, there are conflicting results for GLP-1. Firstly, infusion of GLP-1 in humans has both been reported to increase resting energy expenditure (Shalev *et al*, 1997) and contrastingly decrease diet-induced thermogenesis (Flint *et al*, 2000). Meanwhile, in rodents, central or peripheral GLP-1 injection dose-dependently increased oxygen consumption, heart rate and core body temperature (Osaka *et al*, 2005). In contrast, another study demonstrated a reduction in oxygen consumption (VO_2) and fat oxidation in mice receiving peripheral or central administration of exendin-4 (Baggio *et al.,* 2004). Thus, further studies are needed to determine the true nature of GLP-1's impact on energy expenditure.

In summary, GLP-1 can reduce food intake (satiety and/or satiation), via signalling along the vagus nerve to the hindbrain and/or by crossing the BBB and directly acting on GLP-1R's in the CNS.

1.3.1.3 Peptide tyrosine-tyrosine (PYY)

PYY is a peptide of the pancreatic polypeptide family which, like GLP-1, is synthesised and secreted from L-cells in the ileum and colon (Table 1.1). PYY is produced in proportion to the caloric intake, and is initially secreted as $PYY_{1-36,}$ before being readily cleaved into its main active form PYY_{3-36} by the enzyme DPPIV (Batterham *et al*, 2006). Peripheral PYY_{3-36} administration decreases food intake in several species (Adams *et al*, 2004; Moran *et al*, 2005; Scott *et al*, 2005) including humans (Batterham *et al*, 2003; Degen *et al*, 2005), and daily injections are accompanied by decreases in adiposity (Chelikani *et al*, 2006).

PYY_{3-36} is a selective agonist for the NPY2-R receptor of the NPY-receptor family (NPY1-R – NPY5-R), and these NPY2-R are expressed on vagal afferent nerves and central locations, including the hypothalamus and hindbrain (Karra & Batterham, 2010)(Figure 1.1). The evidence that the NPY2-R, which is an auto-inhibitory pre-synaptic receptor commonly found on NPY neurons, specifically mediates the anorexic effects of PYY has been shown by both antagonism and gene knockout of this receptor abolishing the anorexic effects of PYY (Batterham & Bloom, 2003; Scott *et al*, 2005). Moreover, direct ARC administration of PYY dose-dependently suppresses energy intake, indicating PYY has the ability to directly influence central energy balance regulation (Batterham *et al*, 2002). Furthermore, peripheral injection of PYY decreases hypothalamic NPY expression and release, while increasing the expression of POMC (Batterham *et al*, 2002; Challis *et al*,

2003). Yet, despite evidence of direct central action, both vagotomy and disruption of the hindbrain-hypothalamus neural pathway have demonstrated the importance of the hindbrain to PYY's anorexic effects (Abbott *et al*, 2005; Koda *et al*, 2005).

In recent years, it has emerged that PYY also influences energy expenditure, with elevated circulating PYY levels being associated with increased post-prandial energy expenditure (Doucet *et al*, 2008). Contrastingly, another study found fasting PYY levels to be negatively correlated with resting metabolic rate (Guo *et al*, 2006). However, transgenic mice over-expressing PYY have increased basal body temperatures, indicative of increased adaptive thermogenesis (Boey *et al*, 2008). Similarly, in humans peripheral injections of PYY stimulates lipolysis, enhancing thermogenesis and fat oxidation (Sloth *et al*, 2007). Furthermore, chronic PYY administration leads to a preference for fat as an energy substrate (Adams *et al*, 2006; van den Hoek *et al*, 2007). Overall, the evidence suggests PYY can increase energy expenditure, but the mechanism involved remains to be elucidated.

In summary, PYY reduces food intake (satiety and/or satiation), and potentially increases energy expenditure and fat oxidation, via signalling along the vagus nerve to the hindbrain and/or by directly acting on NPY2-R in the hypothalamus to inhibit NPY/AgRP neuronal activity.

1.3.1.4 Ghrelin

Ghrelin is a peptide formed from the cleavage of pre-proghrelin, and is released principally from gastric X/A-like cells in the stomach (Kojima *et al*, 1999) (Figure 1.1). In rodents, peripheral injections of ghrelin cause a dose-dependent rise in food intake (Bagnasco *et al*, 2003; Chen *et al*, 2004; Toshinai *et al*, 2003; Wren *et al*,

2000), with chronic administration increasing weight gain and adiposity (Tschop *et al*, 2000; Wren *et al*, 2001b). This effect has been similarly demonstrated in both lean and obese humans (Druce *et al*, 2005; Wren *et al*, 2001a). Central ghrelin action resulting from intracerebroventricular (ICV) administration also increases food intake and suppresses energy expenditure, leading to an increase in adiposity when chronically administered (Kamegai *et al*, 2001; Tschop *et al*, 2000).

Ghrelin acts via the growth hormone secretagogue receptor (GHS-R), which is expressed in the hypothalamus, hindbrain, and vagus afferent neurons (Faulconbridge *et al*, 2008; Shuto *et al*, 2001; Wren *et al*, 2001b) (Figure 1.1). In fact, the ability of ghrelin to induce food intake is absent in rodents lacking GHS-R (*ghsr-/-*) and in those treated with GHS-R antagonists (Asakawa *et al*, 2003; Zigman *et al*, 2005). Ghrelin signalling via the vagus nerve to the NTS seems to be important for ghrelin action (Date *et al*, 2002; Date *et al*, 2005), with both vagus nerve blockade or vagatomy abolishing ghrelin-induced feeding and NPY activity (Asakawa *et al*, 2001; Date *et al*, 2002; Faulconbridge *et al*, 2008). The central activation of GHS-R in the ARC stimulates the activity of NPY/AgRP neurons, promoting the release of orexigenic neuropeptides NPY and AgRP (Kamegai *et al*, 2001; Wang *et al*, 2002). The importance of this interaction to ghrelin's orexigenic ability can be seen following the treatment with NPY receptors antagonists (Shintani *et al*, 2001).

Increasing evidence suggests ghrelin also impacts on energy expenditure. Firstly, transgenic down-regulation of GHS-R in the ARC increases energy expenditure related parameters in mice, including VO_2, locomotor activity and BAT uncoupling protein 1 (UCP-1) expression (Mano-Otagiri *et al*, 2010). In fact, central ghrelin administration inhibits SNS activity in BAT, thereby suppressing

noradrenalin release and BAT thermogenesis (Mano-Otagiri *et al*, 2009; Yasuda *et al*, 2003). These finding support an inhibitory role for ghrelin in energy expenditure, and in particular adaptive thermogenesis.

In summary, ghrelin stimulates food intake and weight/fat mass gains and suppresses thermogenesis via its signalling along the vagus nerve to the hindbrain and by directly acting on its receptors in the hypothalamus, leading to a stimulation of NPY/AgRP neuronal activity and neuropeptide release.

1.3.2 Long-term energy status relaying signals

These signals include hormones that are found in circulation in proportion to body fat content, and as such, provide a signal of the body's energy stores to the CNS (Woods *et al*, 2004). These long-term acting signals regulate body weight by providing a negative feedback signal in response to changes in body fat mass, in order to facilitate appropriate changes in energy intake and energy expenditure (Morton *et al*, 2006). The two hormones that meet these criteria are leptin and insulin. Leptin is produced mainly by adipocytes and secreted into circulation in direct proportion to the levels of body fat, while insulin, which is secreted from pancreatic β-cells in response to elevated blood glucose levels, has fasting levels that are proportional to that of body fat (Woods & D'Alessio, 2008).

1.3.2.1 Leptin

Leptin is the product of the *ob* gene and is secreted from adipose tissue in proportion to the levels of body fat, with plasma levels rising and falling with weight gain and weight loss, respectively (Maffei *et al*, 1995) (Table 1.1). The administration of leptin, either centrally or peripherally, suppresses energy intake

and increases energy expenditure, and chronic administration leads to a reduction in body weight and to improvements in glucose tolerance (Halaas *et al*, 1997; Keung *et al*, 2011; Sivitz *et al*, 1997; Tang-Christensen *et al*, 1999). Genetic deficiency of the leptin gene (*ob/ob)* or its receptor (*db/db*) causes hyperphagia and decreases energy expenditure, resulting in severe obesity (Friedman & Halaas, 1998). Exogenous administration of leptin reverses this phenotype in *ob/ob* mice (Mercer *et al*, 1997). In contrast, HFD-induced obesity leads to increased resistance to leptin action both centrally and peripherally (Lin *et al*, 2000).

The leptin receptor has at least six isoforms, yet the expression of the long form Ob-R_b has been shown to be necessary and sufficient to mediate the actions of leptin on energy balance (Dam & Jockers, 2013). These receptors are highly expressed in the hypothalamus, including the ARC (Elmquist *et al*, 1998; Leshan *et al*, 2006), the hindbrain (Leshan *et al*, 2006; Williams *et al*, 2009b) and on vagal afferent fibres (Dockray, 2013). Leptin mediates its effects on energy balance by direct action on these receptors within the hypothalamus (Faouzi *et al*, 2007) and the hindbrain (Kanoski *et al*, 2012; Scott *et al*, 2011) (Figure 1.1).

Leptin action also impacts on short-term meal-related energy intake through interactions with satiety/satiation signals (Morton *et al*, 2005; Williams *et al*, 2006; Williams *et al*, 2009b; Williams *et al*, 2008). Leptin administration to the hindbrain at either the 4th ventricle or directly into the dorsal vagal complex, at doses which do not themselves influence food intake, significantly augments the anorexic response to IP injection of CCK (Williams *et al*, 2009b). Notably, the absence of functional leptin receptors in either the hypothalamus (Morton *et al*, 2005) or hindbrain (Hayes *et al*, 2010) blunts the response to CCK, while peripheral CCK-1 receptor antagonism abolishes the anorexic response to

hindbrain delivery of leptin (Williams *et al*, 2009b). Leptin similarly influences the anorexic response to GLP-1, with leptin pre-treatment significantly enhancing the anorexic effect of both GLP-1 and exendin-4, while leptin receptor deficiency in the NTS strongly attenuated this response (Williams *et al*, 2006).

In summary, leptin suppresses food intake and increases energy expenditure by directly acting upon its receptors within the ARC of the hypothalamus. Leptin action in the hindbrain also impacts on short-term energy intake by influencing the actions of satiety/satiation signals, CCK and GLP-1.

1.3.2.2 Insulin

Insulin is synthesised in β-cells of the pancreas in response to increases in blood glucose, although, as indicated above, fasting plasma levels are directly proportional to the adipose tissue mass (Bagdade *et al*, 1967) (Table 1.1). In addition, the incretin hormones GLP-1 and glucose-dependent insulinotropic peptide (GIP) both enhance glucose-dependent insulin release, as well pancreatic β-cell function (Garber, 2011). The central effects of insulin are also anorexigenic like leptin, with dose-dependent decreases in food intake and body weight following ICV administration of insulin (Chavez *et al*, 1995; McGowan *et al*, 1990; Sipols *et al*, 1995; Woods *et al*, 1979). Furthermore, the antagonism of insulin signalling via central administration of antibodies towards insulin, has been found to increase food intake and body weight (McGowan *et al*, 1992).

The central effects of insulin are mediated via direct activation of the insulin receptor (IR) and these are found to be extensively expressed in the CNS and particularly co-localised with POMC neurons within the ARC (Benoit *et al*, 2002; Havrankova *et al*, 1978) (Figure 1.1). Notably, like leptin, DIO induces resistance to

insulin action, thereby blunting the anorexic response to central insulin infusion (Clegg *et al*, 2005; Posey *et al*, 2009). In summary, insulin suppresses food intake by directly acting on its receptors within the hypothalamus.

1.3.3 Central regulation of energy balance

The ARC located in the mediobasal hypothalamus serves as the primary site for processing energy balance-related signals (Berthoud *et al*, 2006; Lenard & Berthoud, 2008). As discussed above, hormonal signals can either cross the relatively leaky BBB at the ARC (hypothalamus) or area postrema (hindbrain), and act directly upon their target sites, or they can relay their signal to the ARC via the hindbrain, following transduction along the vagal nerve from the GIT to the NTS (Berthoud *et al*, 2006; Cummings & Overduin, 2007). These signals are then integrated in the ARC and other hypothalamic nuclei to influence neuronal activity of two main neuronal populations, POMC and NPY/AgRP, that have opposing effects on energy balance regulation; POMC neurons propagate the catabolic response from peripheral signal inputs, while NPY/AgRP neurons facilitate the anabolic response (Woods & D'Alessio, 2008).

Table 1.1 Overview of peripheral hormones known to influence energy balance (adapted from Neary & Batterham (2009)).

Hormone	Site of production	Factors influencing hormone release (arrows denote effect on hormone release)	Hormone receptor	Location of action	Effects on energy balance
Cholecystokinin (CCK)	I cells (proximal intestine)	↑ Fat & protein intake ↑ Bariatric surgery	Cholecystokinin 1 receptor (CCK-1R)	Vagus nerve Hindbrain	↑ Satiation
Glucagon-like peptide 1 (GLP-1)	L cells (ileum/colon)	↑ Food intake ↑ Bariatric surgery ↓ Calorie restriction	Glucagon-like peptide 1 receptor (GLP-1R)	Vagus nerve Hindbrain Hypothalamus	↑ Satiety and/or satiation ↑ Insulin release ↓ Tissue lipid storage
Peptide YY (PYY)	L cells (ileum/colon)	↑ Food intake ↑ Bariatric surgery ↑ Exercise	Neuropeptide Y2 receptor (NPY2-R)	Vagus nerve Hindbrain Hypothalamus	↑ Satiety and/or Satiation ↑ Energy expenditure Alters fuel partitioning
Ghrelin	X/A- like cells (stomach)	↓ Food intake ↓ Bariatric surgery ↑ Calorie restriction	Growth hormone secretagogue receptor (GHS-R)	Vagus nerve Hindbrain Hypothalamus	↑ Food intake ↓ Energy expenditure ↑ Tissue lipid storage Alters fuel partitioning
Leptin	Adipocytes (adipose tissue)	Levels of body fat ↑ Body fat gain ↓ Body fat loss	Leptin Receptor (ObR)	Hindbrain Hypothalamus	↓ Food intake ↑ Energy expenditure ↓ Tissue lipid storage
Insulin	β-cells (pancreas)	Levels of blood glucose ↑ Glucose increase ↓ Glucose decrease	Insulin receptor (IR)	Hypothalamus	↓ Food intake ↑ Energy expenditure ↑ Tissue lipid storage

1.3.3.1 Melanocortin pathway

The POMC protein serves as the precursor to all melanocortin peptides, including the main mediator of the catabolic response, α-melanocortin stimulating hormone (α-MSH) (Pritchard *et al*, 2002). A deficiency in POMC gene expression induces hyperphagia and suppresses energy expenditure, resulting in severe obesity (Yaswen *et al*, 1999). In contrast, ICV administration of α-MSH to rodents suppresses food intake (McMinn *et al*, 2000; Petervari *et al*, 2009; Vergoni *et al*, 1998), and increases physical activity, oxygen consumption and BAT thermogenesis (Semjonous *et al*, 2009; Yasuda *et al*, 2004).

α-MSH acts via G-protein coupled melanocortin receptors 3 and 4 (MC3/4R), which are highly expressed within the hypothalamus (Shimizu *et al*, 2007), as well as being expressed within the NTS of the hindbrain (Li *et al*, 2007; Mountjoy *et al*, 1994; Voss-Andreae *et al*, 2007). Central administration of the non-selective melanocortin receptor agonist, melanotan II (MTII), suppresses food intake and increases oxygen consumption in rats (Skibicka & Grill, 2008; Skibicka & Grill, 2009), while central treatment with non-selective antagonists to melanocortin receptors, such as AgRP or SHU-9119, has the opposite effect (Fan *et al*, 1997). Notably, the effect of MTII is abolished in MC4R (*mc4r-/-*), but not MC3R (*mc3r-/-*), deficient mice (Chen *et al*, 2000a; Chen *et al*, 2000b; Marsh *et al*, 1999), thus suggesting MC4R is the primary regulator of the effects of α-MSH on food intake and energy expenditure.

1.3.3.2 Neuropeptide Y/ Agouti-related protein pathway

In the ARC, two orexigenic neuropeptides NPY and AgRP are expressed and released from a population of neurons distinct from those expressing POMC (Elias

et al, 1999). Central infusion of NPY increases food intake and reduces energy expenditure, with chronic administration leading to the development of obesity via sustained hyperphagia (Clark *et al*, 1984; Hwa *et al*, 1999; Stanley *et al*, 1986; Stanley & Leibowitz, 1985; Zarjevski *et al*, 1993). Notably, the release of the γ-aminobutyric acid (GABA) from NPY/AgRP neurons following their activation also inhibits POMC neuronal activity (Cowley *et al*, 2001).

Six NPY receptors (NPY1-6-R) have been identified, of which NPY1-R and NPY5-R principally mediate its influence on energy balance. Administering rats with selective agonists for NPY1-R or NPY5-R receptors has been shown to induce hyperphagia (Della-Zuana *et al*, 2004; Fekete *et al*, 2002; Jewett *et al*, 1993; Kalra *et al*, 1991; Mullins *et al*, 2001), with chronic administration in mice significantly increasing bodyweight and adiposity (MacNeil, 2007; Mashiko *et al*, 2003). However, in rodent models with either NPY1-R or NPY5-R receptor gene knockout, there is no significant reduction in food intake (Lin *et al*, 2004), suggesting a level of redundancy in the system of energy balance regulation.

Similar orexigenic effects as that described for NPY are also seen with acute and chronic central administration of AgRP (Hagan *et al*, 2000; Small *et al*, 2001; Small *et al*, 2003). However, the orexigenic effects of AgRP are mediated by inhibition of melanocortin signalling via an antagonism of MC3/4R (Ollmann *et al*, 1997). Indeed, co-administration of AgRP with MTII completely abolished the anorexic effects of this non-selective MC3/4R agonist (Hagan *et al*, 2000). Interestingly, a recent study showed by triple knockout of genes for NPY, AgRP and the associated neurotransmitter GABA, which is released by NPY/AgRP neurons and induces a potent orexigenic response, that the orexigenic effect of NPY/AgRP neuronal activation was completely abolished, while this response was unaffected

by any single gene knockout (Krashes *et al*, 2013). This suggests these three components are responsible for the orexigenic effects of NPY/AgRP neuronal activation.

1.3.3.3 Central integration of peripheral signals

In the ARC, leptin and insulin receptors are expressed within NPY/AgRP and POMC neurons (Benoit *et al*, 2002; Cheung *et al*, 1997; Mercer *et al*, 1996; Morton *et al*, 2006; Wilson *et al*, 1999). Notably evidence does suggest a degree of segregation of these two receptors, and thus their actions, into distinct POMC subpopulations (Williams *et al*, 2010). Activation of hypothalamic ARC leptin receptors inhibits NPY/AgRP neuronal activity, significantly decreasing the mRNA expression and release of orexigenic neuropeptides NPY and AgRP (Broberger *et al*, 1998; Elias *et al*, 1999; Morrison *et al*, 2005). Conversely, leptin activates POMC neurons, increasing the expression of POMC, and thereby the production of α-MSH (Elias *et al*, 1999; Elias *et al*, 1998; Vrang *et al*, 2002).

In a similar way to leptin, insulin administration into the 3rd ventricle has been shown to significantly increase ARC expression of POMC (Benoit *et al*, 2002; Fekete *et al*, 2006), while decreasing NPY expression (Fekete *et al*, 2006; Sato *et al*, 2005; Schwartz *et al*, 1991), thereby causing the associated reduction in energy intake. Evidence demonstrates that suppressing melanocortin signalling via MC3/4R antagonism, blocks the catabolic actions of both insulin and leptin (Benoit *et al*, 2002; Choi *et al*, 2003). As with central leptin receptor deficiency (i.e. *db/db* mice), complete insulin receptor deficiency in the CNS leads to hyperphagia and the development of obesity (Bruning *et al*, 2000). Interestingly however, specific

insulin receptor ablation in both POMC and AgRP neurons does not result in pronounced hyperphagia or obesity (Konner *et al*, 2007).

In addition to long-term energy status-relaying signals, short-term satiety/satiation signals can also act on hypothalamic neuronal populations important in energy balance regulation. Indeed, direct activation of central GLP-1R in fasting mice was shown to increase hypothalamic POMC expression, while suppressing fasting induced-increases in AgRP and NPY expression (Seo *et al*, 2008). In contrast to GLP-1, peripheral PYY administration has been shown to act centrally to suppress food intake by binding to NPY2-R's and suppressing hypothalamic NPY/AgRP neural activity and neuropeptide expression(Batterham *et al*, 2002). In fact, the anorexic effect of PYY does not appear to involve the melanocortin signalling pathway as it is still evident in both POMC and MC3/4R knock-out mice. In contrast, ghrelin activation of central GHS-R has been shown to stimulate NPY/AgRP neural activity and NPY and AgRP neuropeptide expression (Kamegai *et al*, 2001). This is associated with a simultaneous inhibition of POMC neuronal activity, which is a product of the latter effect on NPY/AgRP neural activity rather than a direct inhibitory effect (Cowley *et al*, 2003). Finally, the food intake suppressive effects of CCK have been shown to be influenced by melanocortin signalling in the NTS, with 4th, but not 3rd ventricle SHU-9119 treatment preventing the effects of CCK action (Blevins *et al*, 2009).

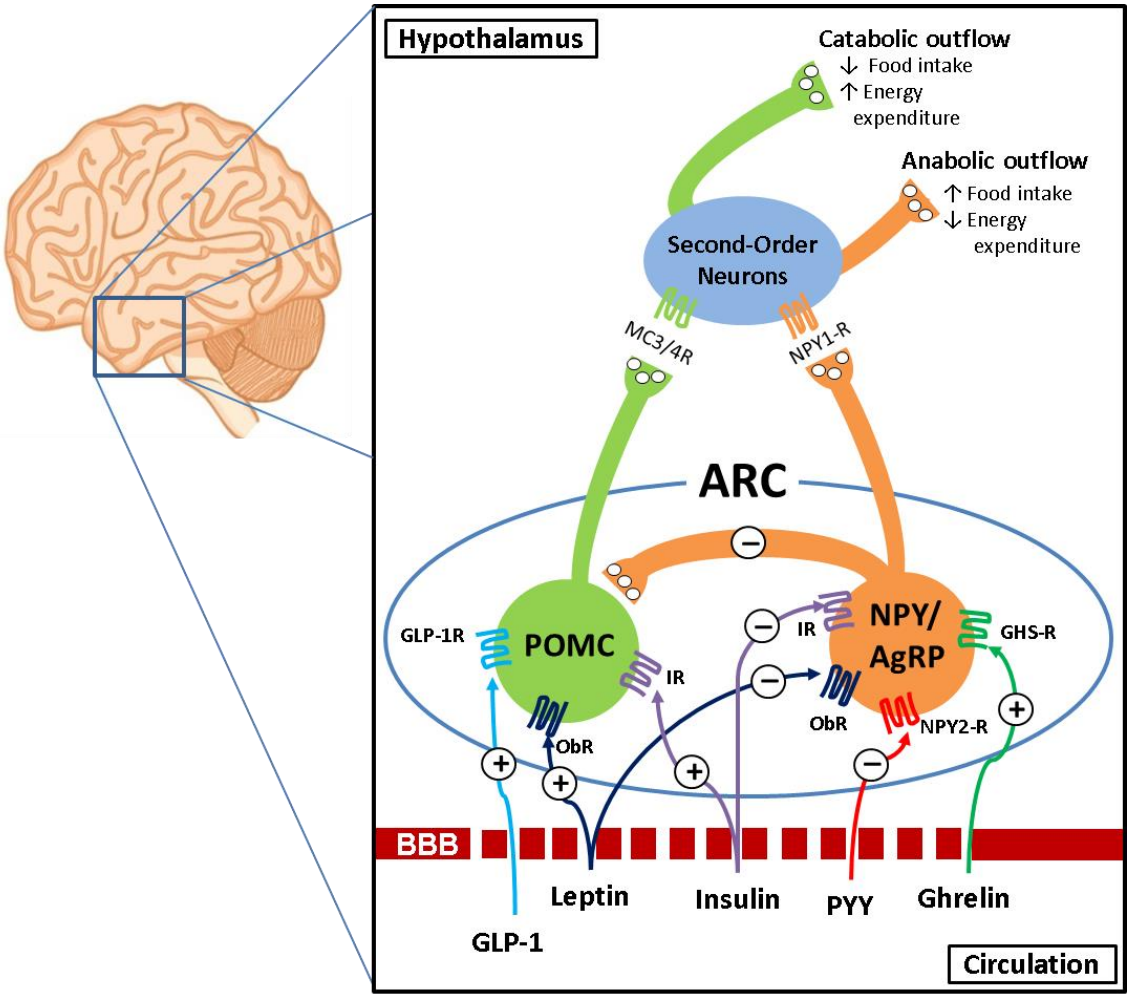

Figure 1.2. Central effects of peripheral hormones on neuronal activity in the arcuate nucleus (ARC) of the hypothalamus. Leptin, insulin and GLP-1 stimulate POMC neuronal activity, while leptin, insulin and PYY inhibit NPY/AgRP neuronal action, which promotes the activation of melanocortin signalling via MC3/4R. This initiates a catabolic response, which leads to a reduction in food intake and an increase in energy expenditure. In contrast, stimulation of NPY/AgRP neurons by ghrelin promotes an anabolic response via direct NPY effects, as well as indirect effects via AgRP inhibition of MC3/4R signalling, resulting in an increase in food intake and a reduction in energy expenditure.

AgRP, agouti-related protein; GHS-R, growth hormone secretagogue receptor; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; IR, insulin receptor; MC3/4R, melanocortin 3/4 receptors; ObR, leptin receptor; NPY, neuropeptide Y; NPY2-R, NPY 2 receptor; POMC, pro-opiomelanocortin; PYY, Peptide YY; BBB, Blood brain barrier.

1.4 REGULATION OF LIPID AND GLUCOSE METABOLISM

1.4.1 Introduction

This section provides background information on lipid metabolism in the intestine, liver and adipose tissue, with the view of providing an overview of the regulators of this physiological process. Firstly, due to the intimate involvement of insulin signalling in glucose homeostasis, a brief overview of this interaction and its influence on tissue lipid metabolism is provided, before providing a more detailed background in subsequent sections.

1.4.2 Insulin signalling and glucose metabolism

The classically defined function of insulin is to regulate glucose homeostasis by acting on key energy balance-related tissues, such as the skeletal muscle, liver, and adipose tissue. This is achieved by both direct action on these tissues and indirect action via the CNS (Marino *et al*, 2011; Saltiel & Kahn, 2001). In the former case, insulin binds to its receptor (IR), located at the cell membrane, phosphorylating nearby insulin receptor substrate (IRS) proteins, which in turn recruit and activate the phosphatidylinositol 3-Kinase (PI3K) pathway, ultimately resulting in a stimulation of its downstream targets, including protein kinase B (PKB/Akt) and protein kinase C (PKC) (Watson *et al*, 2004). The activation of the PI3K pathway facilitates the uptake of glucose into adipose and skeletal muscle cells by increasing the translocation of the glucose transporter 4 (GLUT4) to the cell membrane (Clarke *et al*, 1994). Upon entry into adipocytes, glucose can be converted by glycolysis to a substrate for insulin-induced *de novo* lipogenesis (Large *et al*, 2004). In the skeletal muscle, insulin promotes the disposal of excess

glucose by promoting its conversion into glycogen, which can then be utilized in times of energy deficit (Jensen & Lai, 2009). In contrast, in the liver, insulin does not facilitate glucose uptake, but instead regulates production and release of glucose by the inhibition of hepatic gluconeogenesis (Michael *et al*, 2000). Central insulin signaling, acting through the SNS, also suppresses hepatic glucose production (Obici *et al*, 2002), as well as stimulating muscle glycogen synthesis (Perrin *et al*, 2004). Lastly, as in adipose tissue, insulin also stimulates the conversion of glucose to TAG in the liver by prompting glycolysis and lipogenesis (Saltiel & Kahn, 2001).

1.4.3 Intestinal lipid metabolism

Dietary lipids in the intestinal lumen are digested by pancreatic lipases in order to release free fatty acids for subsequent uptake (Mu & Hoy, 2004). The uptake of these fatty acids into enterocytes occurs via both passive diffusion and transport protein mediated mechanisms (Mansbach & Gorelick, 2007). The main transport proteins involved are cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4) (Drover *et al*, 2005; Nassir *et al*, 2007; Stahl *et al*, 1999). Once inside the enterocytes, the fatty acids are used to re-synthesis TAG for inclusion into lipoproteins called chylomicrons. This process begins in the endoplasmic reticulum (ER), where fatty acids are used by monoacylglycerol acyltranferase (MGAT) and diacylglycerol acyltransferase (DGAT) sequentially to re-synthesise TAG for inclusion into pre-chylomicron particles (Mansbach & Gorelick, 2007). These particles are further modified in the Golgi apparatus before being released into the lymph and eventually finding their way into the circulation via the thoracic duct (Black, 2007; Pan & Hussain, 2012). The chylomicrons are then transported

via the circulatory system and hydrolysed by tissue specific lipoprotein lipase (LPL) at the cell surface to release free fatty acids, which can then be utilised by tissues such as the skeletal muscle and adipose tissue, for energy production and storage, respectively (Voshol *et al*, 2009).

1.4.4 Hepatic lipid metabolism

The liver is involved in lipid production (lipogenesis & TAG synthesis), lipid export (lipoprotein production), and lipid-derived energy production (β-oxidation) (Nguyen *et al*, 2008). Fatty acids taken up into the liver from the circulation are derived from chylomicron remnants and the non-esterified fatty acid (NEFA) pool. The latter is the primary source of fatty acids for the liver and it is predominately made up of fatty acids released from the adipose tissue following TAG storage hydrolysis (Tamura & Shimomura, 2005). Like in the intestine, the process of fatty acid uptake occurs in the liver by both membrane protein mediated transport (FATP's and CD36) and by passive mechanisms (Glatz *et al*, 2010). In addition to external sources, hepatic fatty acids can also be derived via endogenous production which is termed *de novo* lipogenesis. This process involves the synthesis of fatty acids from precursor substrates by the sequential conversion of acetyl-CoA to malonyl-CoA and then fatty acids via lipogenic enzymes acetyl CoA carboxylase (ACC) and fatty acid synthase (FASN), respectively (Large *et al*, 2004; Nguyen *et al*, 2008).

Insulin and glucose are key regulators of hepatic lipogenesis by activation of sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate responsive element-binding protein (ChREBP) transcription factors, respectively (Xu *et al*, 2013b). These, along with the liver X receptor (LXR), induce the

expression of genes involved in glucose uptake, glycolysis and lipogenesis within the liver, including glucose transporter 2 (GLUT2), pyruvate kinase, ACC, FASN, stearoyl-CoA desaturase (SCD-1) and glycerol phosphate acyltransferase (GPAT) (Iizuka & Horikawa, 2008; Jump, 2011). Fatty acids derived from direct plasma uptake and/or endogenous production can then be utilised either to synthesise TAG for storage or for export within very low density lipoprotein (VLDL), or to produce energy via β-oxidation (Nguyen *et al*, 2008). β-oxidation of fatty acids generates energy in the form of ATP for crucial cellular processes, and this process is under the influence of the transcription factor peroxisome proliferator-activated receptor (PPAR)-α, given its ability to regulate the expression of key β-oxidation associated enzymes (Reddy *et al*, 1986; Reddy & Hashimoto, 2001), namely carnitine palmitoyltransferase 1a (CPT1a), which is the rate limiting enzyme in the process and regulates long-chain fatty acid entry into mitochondria (Kerner & Hoppel, 2000; Reddy & Hashimoto, 2001). Notably, CPT1 activity is inhibited by the product of ACC enzyme activity, malonyl-CoA, thus highlighting the reciprocal regulation of lipogenesis and β-oxidation (Kerner & Hoppel, 2000).

1.4.5 Adipocyte lipid metabolism

The adipose tissue sources fatty acids primarily from TAG-rich lipoproteins (i.e. VLDL and chylomicrons), and albumin-bound fatty acids in the plasma (Voshol *et al*, 2009). The actions of cell surface LPL on circulating lipoproteins liberates TAG and further hydrolyses it to release fatty acids, which are then available for subsequent uptake into the cell (Weinstock *et al*, 1997). Insulin is a primary regulator of LPL function, up-regulating both its expression and activity (Goldberg *et al*, 2009; Semenkovich *et al*, 1989). The uptake of fatty acids into adipocytes is

primarily mediated by specific membrane transporters, namely FATP1, FATP4 and CD36 (Goldberg *et al*, 2009; Lobo *et al*, 2007). In addition to facilitating fatty acid uptake, insulin also regulates the uptake of glucose into adipocytes by increasing the expression and translocation of GLUT4 to the cell surface (Saltiel & Kahn, 2001). Upon transport into the cell, glucose can be converted by glycolysis to acetyl-CoA and then serve as a substrate for *de novo* lipogenesis (Large *et al*, 2004). Moreover, as in the liver, insulin-induced SREBP-1c actively regulates lipogenesis in adipocytes by promoting lipogenic enzymes, such as ACC and FASN (Carobbio *et al*, 2013). Fatty acids, both from extracellular sources and *de novo* lipogenesis, are either utilised for the production and storage of TAG via successive esterification steps (Large *et al*, 2004) or are oxidised via β–oxidation to generate energy.

In times of energy shortage, such as during periods of sustained physical activity, hydrolysis of adipose TAG stores, termed lipolysis, releases fatty acids into circulation for utilisation as an energy substrate in other tissues (Horowitz & Klein, 2000). This is initiated by the activation of the SNS innervating the adipose tissue (Thompson *et al*, 2010). In this instance the release of catecholamine's at the nerve axon terminals activates β-adrenergic receptors located at the cell surface (Frayn *et al*, 2003; Lafontan & Berlan, 1993). Three subtypes of β-adrenergic receptor (β_1-AR, β_2-AR and β_3-AR) are found in white adipose tissue (WAT); β_3AR is the major lipolysis mediator in rodents, while in humans its β_1-AR and β_2-AR (Lafontan & Berlan, 1993). Specifically, binding of catecholamine's to β-AR receptors in adipocytes leads to the phosphorylation of both perilipin and hormone sensitive lipase (HSL)(Belfrage *et al*, 1980; Clifford *et al*, 2000). Upon phosphorylation, perilipin dissociates from the surface of TAG-rich lipid droplets (Clifford *et al*, 2000), allowing HSL and adipose triglyceride lipase (ATGL) access, in order to

facilitate their hydrolysis (Lass *et al*, 2006; Miyoshi *et al*, 2007; Miyoshi *et al*, 2006). The main physiological inhibitor of adipose tissue lipolysis is insulin and it inhibits lipolysis by both reducing cAMP levels and dephosphorylating HSL (Carmen & Victor, 2006; Langin, 2006; Nilsson *et al*, 1980).

In addition to the effects noted above, insulin is also a potent stimulator of adipogenesis (Fajas *et al*, 1999), the process by which pre-adipocytes are differentiated into their mature fat cells. The key transcription factors controlling differentiation and maturation of adipocytes include PPAR-γ, CCAAT/enhancer-binding proteins (C/EBPs α, β & δ) and SREBP-1c (White & Stephens, 2010). PPARγ is considered the master regulator of adipogenesis, as its expression is not only sufficient but necessary to induce adipocyte differentiation (Rosen & MacDougald, 2006). Indeed, insulin's ability to promote adipocyte differentiation occurs by up-regulation of PPARγ and SREBP-1 activity, with the latter further augmenting PPARγ expression (Fajas *et al*, 1999).

1.4.6 Lysophosphatidic acid

LPA is small bioactive glycerophospholipid that functions as an extracellular signalling molecule (Choi *et al*, 2010). Through binding to G-protein coupled receptors (GPCRs), it regulates a wide range of biological responses including mitogenesis (Fang *et al*, 2002), cell survival (Weiner & Chun, 1999), cell migration (Panetti *et al*, 2001), platelet aggregation (Rother *et al*, 2003) and hormone secretion (Choi *et al*, 2007b). There are currently six LPA receptors ($LPA_{1\text{-}6}$), with more awaiting validation (Choi *et al*, 2010).

LPA is produced by the hydrolysis of phospholipids by the enzyme autotaxin (ATX) (Ferry *et al*, 2003) and is found in serum and in other biological

fluids, including that associated with the adipose tissue (Valet *et al*, 1998). Indeed, LPA is endogenously produced in the extracellular fluid of adipose tissue (Ferry *et al*, 2003; Valet *et al*, 1998) and has been shown to increase the proliferation of pre-adipocytes (Pages *et al*, 2001; Valet *et al*, 1998). Furthermore, a study by Simon *et al,* (2005) evaluated the effects of LPA treatment on differentiating 3T3F442A adipocytes and found that it significantly down-regulates the expression of PPARγ. The authors suggested the involvement of $LPA_{1,}$ with knockout mice for LPA_1 being found to have 24% increased lipid content in their adipocytes, while pre-adipocytes isolated from these transgenic mice did not show an anti-adipogenic response to LPA stimulation (Simon *et al*, 2005). Thus, the data demonstrates that LPA action, possibly though LPA_1, can both induce pre-adipocyte proliferation while suppressing their differentiation into mature lipid laden adipocytes.

Another LPA receptor, LPA_5 (GPR93 or 92) is widely expressed in murine tissues (Choi *et al*, 2010), and as mentioned previously, its activation by protein hydrolysate has been shown to stimulate CCK gene expression and peptide release in the STC-1 enteroendocrine cell line (Choi *et al*, 2007b). This raises the possibility that this receptor may mediate dietary proteins effects on other tissues, including adipose tissue. Interestingly, the whey associated protein, bovine serum albumin (BSA) has been shown to activate LPA_5 in enterocytes (Choi *et al*, 2007a) and suppress adipogenesis in 3T3-L1 adipocytes (Schlesinger *et al*, 2006). It remains to be determined whether whey proteins could directly activate LPA_5 in the adipose tissue and thereby affect lipid metabolism in the cell.

1.4.7 Central control of peripheral lipid metabolism

The SNS outflow from the CNS has been shown to play an important role in the regulation of peripheral lipid metabolism, as evidenced by direct SNS innervation of both the liver and WAT causing a change to their lipid metabolism (Geerling *et al*, 2014). This SNS control of lipid metabolism appears to be regulated by the balance between catabolic and anabolic outputs from hypothalamic POMC and NPY neuronal signalling; therefore, central effects of ghrelin, leptin, insulin and GLP-1 on peripheral lipid metabolism are thought to be mediated through their actions on these two neuronal populations (Geerling *et al*, 2014; Nogueiras *et al*, 2010).

The melanocortin system in the hypothalamus is a key component of CNS regulation of peripheral lipid metabolism and nutrient partitioning (Nogueiras *et al*, 2009; Nogueiras *et al*, 2007; Shrestha *et al*, 2010) (Figure 1.3). Indeed, melanocortin-mediated SNS outflow to WAT directly influences adipocyte metabolism independently of energy intake, with central administration of melanocortin receptor agonist, MTII, reducing body fat levels and increasing adipose tissue lipolytic gene expression, while blockade of this signalling pathway, using SHU9119, increases fat accumulation in association with augmented expression of lipogenic enzymes (Nogueiras *et al*, 2007; Raposinho *et al*, 2003; Shrestha *et al*, 2010). Furthermore, these effects on adipocyte metabolism have been shown to be absent in mice lacking all three forms of β-adrenergic receptors in the adipose tissue (Nogueiras *et al*, 2007). In addition, data also suggests melanocortin signalling regulates hepatic lipid metabolism, with central MTII administration reducing both liver TAG and hepatic lipogenic gene expression in both rats and diabetic mice (Leckstrom *et al*, 2011; Wiedmer *et al*, 2012).

Central infusions of NPY into rats pair fed to saline treated controls, results in significant increases in adiposity, that are thus independent of its effect on food

intake (Zarjevski *et al*, 1993; Zarjevski *et al*, 1994) (Figure 1.3). Accompanying these effects, the gene expressions of ACC, LPL and GLUT4 were up-regulated in the adipose tissue, while ACC expression was also increased in the liver. Moreover, the administration of NPY has also been shown to inhibit melanocortin induced lipolysis (Bradley *et al*, 2005; Martinez *et al*, 2000). Correspondingly, antagonism of the NPY signalling or knockdown of its expression in the dorsomedial nucleus, increases lipolysis and lipolytic gene expression, as well reduces body fat (Chao *et al*, 2011; Margareto *et al*, 2000). ICV administration of NPY has also been shown to enhance TAG synthesis in the liver, augmenting VLDL production and release even under hyperinsulinemic conditions (Stafford *et al*, 2008; van den Hoek *et al*, 2004). Denervation of the sympathetic nerve to the liver has been shown to block most of the effects of NPY, supporting SNS involvement in NPY action on hepatic lipid metabolism (Bruinstroop *et al*, 2012).

Central leptin and insulin show contrasting effects on peripheral lipid metabolism. Central leptin action suppresses lipogenesis-related gene expression and TAG content in both the adipose and liver, whilst also increasing lipolytic gene expression in the adipose (Buettner *et al*, 2008; Gallardo *et al*, 2007; Shen *et al*, 2007; Tajima *et al*, 2005) (Figure 1.3). In contrast, insulin causes an anabolic effect on peripheral lipid metabolism by specifically suppressing SNS outflow to the adipose tissue, suppressing lipolysis and increasing lipogenesis (Koch *et al*, 2008; Scherer & Buettner, 2011; Scherer *et al*, 2011). The involvement of the melanocortin signalling in leptin action is supported by evidence that pre-treatment with the melanocortin receptor antagonist SHU119 completely abolishes leptin induced suppression of adipose tissue lipogenesis (Choi *et al*, 2003; Lin *et al*, 2003) (Figure 1.3). In addition to its effect on adipose tissue

metabolism, hypothalamic leptin action has also been shown to suppress hepatic lipogenesis and TAG accumulation via a SNS route (Warne *et al*, 2011).

Given the ability of ghrelin to stimulate NPY/AgRP neuronal activity it is no surprise that central ghrelin administration promotes the storage of lipid in the adipose tissue independently of its effects on food intake, by up-regulating the expression of lipogenic genes (Andrews *et al*, 2010; Sangiao-Alvarellos *et al*, 2009; Theander-Carrillo *et al*, 2006) (Figure 1.3). These studies also showed a corresponding suppression of adipose tissue β-oxidation via a down-regulation of CPT1b expression. The ability of ghrelin to induce body weight gain and alter lipid metabolism-related gene expression is lost in mice lacking β-adrenergic receptors in the adipose tissue, thus supporting an SNS pathway involvement (Theander-Carrillo *et al*, 2006). Similarly, this study also showed that ghrelin gene knockout (*Ghrl-/-)* in mice results in an opposite effect on lipogenic gene expression to that induced by central ghrelin treatment.

Centrally administrated GLP-1 significantly decreases adipocyte lipogenesis and lipid storage independently of its effect on food intake (Nogueiras *et al*, 2009) (Figure 1.3). This corresponded to the down-regulation of key lipogenic enzymes, FASN, ACC and SCD-1 in association with increased SNS activity at the adipose tissue. Furthermore, mice lacking β-adrenergic receptors did not display these responses to centrally administered GLP-1. In addition, central GLP-1 action has also been shown to influence hepatic lipid metabolism, resulting in a reduction in liver TAG accumulation and a suppression of hepatic lipogenic enzyme expression (Nogueiras *et al*, 2009; Panjwani *et al*, 2013).

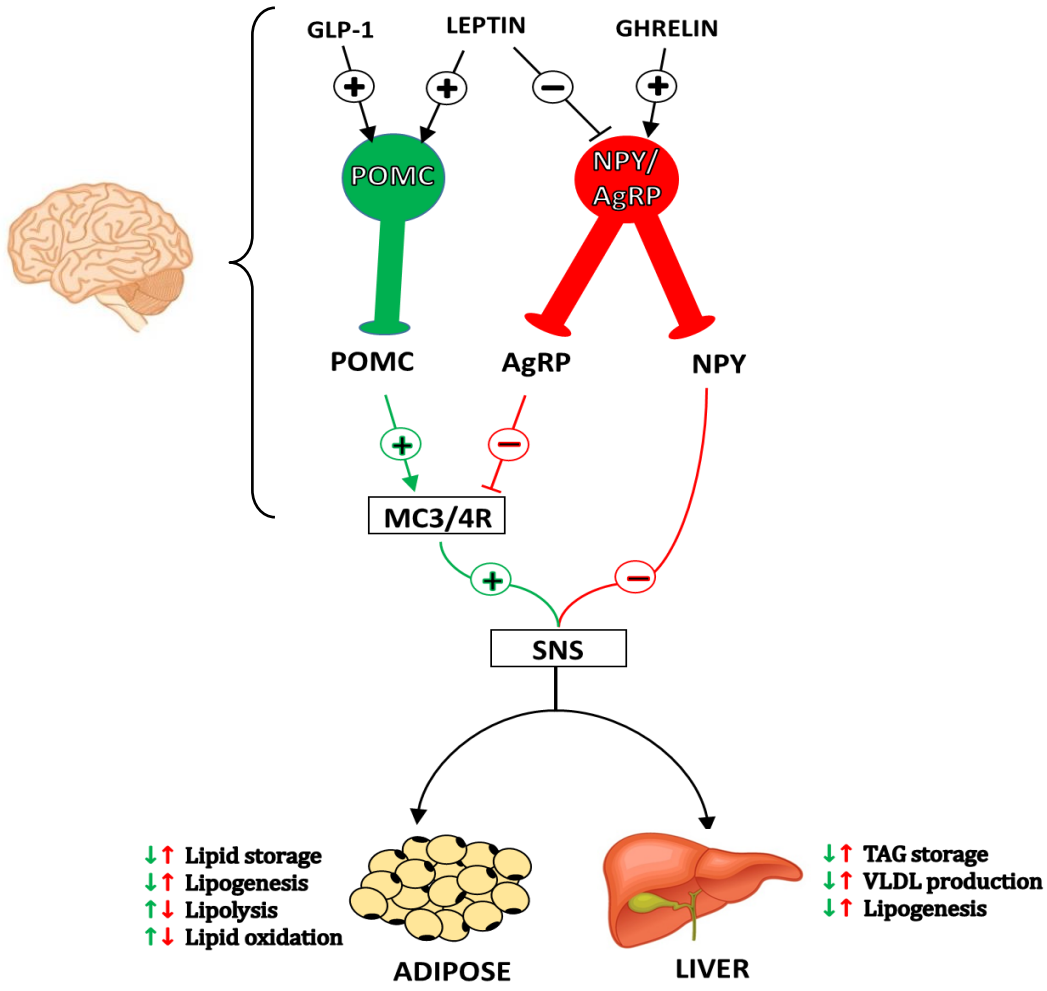

Figure 1.3. Model of central nervous system (CNS) effects on peripheral tissue lipid metabolism. Leptin and GLP-1 activate POMC neurons, while leptin inhibits NPY/AgRP neuronal activity, promoting melanocortin signalling via MC3/4R activation. This initiates a catabolic response on peripheral lipid metabolism via the sympathetic nervous system (SNS), which results in a reduction in lipid storage. In contrast, stimulation of NPY/AgRP neurons by ghrelin, promotes an anabolic response on peripheral lipid metabolism via the direct effects of NPY on SNS outflow, as well as the indirect effects of AgRP inhibition of MC3/4R, which result in increased lipid storage. Green arrows (↓, ↑) denote the effects on tissue lipid metabolism in response to central melanocortin signalling via the SNS, whereas red arrows (↑, ↓) denote the tissue lipid metabolism effects of central NPY/AgRP neuronal activation. (Adapted from (Nogueiras *et al*, 2010).

AgRP, agouti-related protein; GLP-1, glucagon-like peptide 1; MC3/4R, melanocortin 3/4 receptors; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; VLDL, very low density lipoprotein; TAG, triacylglycerol.

1.5 HIGH FAT DIET DYSREGUALTION OF ENERGY BALANCE

1.5.1 Lipotoxicity

Under normal physiological conditions the adipose tissue is able to store excess energy as TAG, while high fat consumption increases the amount of energy that needs to be stored in this tissue (Slawik & Vidal-Puig, 2007). To accompany this increased availability of TAG, adipocytes first undergo hypertrophy (enlargement) to store the excess lipid, followed by hyperplasia (increase in cell number) (Cusi, 2012). If the latter changes are not able to reduce plasma TAG levels, there is then an ectopic deposition of the excess energy as fat in other tissues such as the skeletal muscle and liver (Despres *et al*, 2008). In fact, HFD-induced increases in plasma NEFA results in increased liver and skeletal muscle lipid accumulation, and this has been linked to a dramatic reduction in the sensitivity to insulin action in both tissues (Bachmann *et al*, 2001; Longato *et al*, 2012). Storage of excess lipid increases both cellular stress and the accompanying inflammatory response, further exacerbating the dysregulation of cellular signalling pathways including that of leptin and insulin, thereby increasing the risk of developing associated co-morbidities such as type 2 diabetes (Donath *et al*, 2013). Notably, the gut microbiota has also been implicated in the development of lipotoxicity and the inflammatory response associated with HFD-induced dysregulation of energy balance (Cani *et al*, 2007; Cani *et al*, 2008).

1.5.2 Insulin resistance

The metabolic stress resulting from the continued expansion of the adipose tissue during obesity development eventually leads to an increased inflammatory

response in these cells, which is characterised by increased endogenous inflammatory cytokine release from adipocytes and the infiltration of macrophages (Maury & Brichard, 2010). Accompanying the inflammation is a reduction in the sensitivity to insulin action in the adipose tissue (Gustafson *et al*, 2009; Ruan & Lodish, 2003). This leads to adipose TAG breakdown and release as NEFA into the circulation (hyperlipidemia), along with increasing plasma glucose levels (hyperglycemia)(Groop *et al*, 1989; Karpe *et al*, 2011). Notably, circulating levels of NEFA are a major contributor to ectopic fat accumulation and associated tissue lipotoxicity in tissues such as the skeletal muscle and liver (Karpe *et al*, 2011; McQuaid *et al*, 2011).

Given the major contribution of the skeletal muscle to whole body glucose clearance, increased IMCL accumulation is of great consequence to whole body glucose homeostasis (DeFronzo & Tripathy, 2009). Notably, it is not necessarily total lipid content *per se* that is detrimental to insulin signalling and glucose transport, but rather the increased storage of the lipid derived toxic metabolites such as ceramides, diacylglycerols (DAG) and acyl-CoA's (Bosma *et al*, 2012; Corcoran *et al*, 2007). Increased synthesis and accumulation of these toxic lipid-intermediates, particularly DAG and ceramide, leads to activation of inflammatory pathways which then phosphorylate IRS-1, thereby reducing GLUT4 translocation to the plasma membrane (Bosma *et al*, 2012; Eckardt *et al*, 2011; Itani *et al*, 2002).

Hepatic lipid accumulation results in the reduced ability of insulin to stimulate glycogen synthesis and suppress hepatic glucose production in the liver (Samuel *et al*, 2004). Moreover, the hyperinsulinemia present in obesity not only increases fatty acid uptake into the liver, but also increases hepatic *de novo* lipogenesis and TAG production (Diraison *et al*, 2003; Schwarz *et al*, 2003;

Shimomura *et al*, 1999), thus increasing both lipid storage and export in VLDL (Adiels *et al*, 2008; Donnelly *et al*, 2005). The progression of NAFLD to steatohepatitis (NASH) occurs by the promotion of a chronic inflammatory response in the liver via increased storage of toxic lipid derivatives (Reddy & Rao, 2006).

In addition to the development of peripheral tissue insulin resistance, HFD-induced obesity also induces central (hypothalamic) insulin resistance, and this leads to an attenuation of insulin's ability to suppress food intake (Yue & Lam, 2012). This diminished central response occurs via both reduced transport of insulin across the BBB (Kaiyala *et al*, 2000), as well as impaired insulin signalling in target neurons in the hypothalamus (Belgardt & Bruning, 2010). This impairment of insulin action involves an increase in hypothalamic pro-inflammatory lipid accumulation (Posey *et al*, 2009). Indeed, the induction of a hypothalamic inflammatory response attenuates the anorexic effects of central insulin action (Kleinridders *et al*, 2009; Milanski *et al*, 2009; Zhang *et al*, 2008). In addition, high fat feeding also impairs the ability of central insulin action to suppress hepatic glucose production (Ono *et al*, 2008), which further contributes to the development of peripheral tissue insulin resistance and will eventually lead to type-2 diabetes.

1.5.3 Leptin resistance

HFD-induced obesity is associated with the progressive reduction in peripheral and central sensitivity to leptin action despite the fact that leptin levels continue to be released into circulation in proportion to body fat levels, and as such are elevated in obese individuals/animals (Kolaczynski *et al*, 1996; Lin *et al*, 2000).

This resistance to leptin action leads to further hyperphagia and obesity, which in turn further exacerbates leptin resistance (Enriori *et al*, 2007). Leptin resistance, like insulin resistance, has been proposed to result from both reduced transport of leptin into the brain and impaired leptin signalling in hypothalamic target neurons (Morris & Rui, 2009). Evidence suggests the latter to be more important, given that even the response to direct central leptin administration is attenuated in obesity; this outcome manifests itself as dramatic reductions in the sensitivity of both POMC and NPY/AgRP neurons to leptin action (Enriori *et al*, 2007).

1.5.4 Gut microbiota

There is a growing body of evidence suggesting that the gut microbiota plays a crucial role in host energy metabolism (Tremaroli & Backhed, 2012). Their importance in energy balance regulation has been demonstrated by germ free (GF) mice (an absence of gut microbiota) being both protected against HFD-induced obesity (Backhed *et al*, 2007) and considerably leaner than conventionally raised (CON-R) mice, despite consuming more energy (Backhed *et al*, 2004). Moreover, colonisation of GF mice with cecum microbiota from CON-R mice, leads to a dramatic increase in adiposity. Interestingly, this suggests that gut microbiota composition is an important determinant of adiposity via food intake independent mechanism(s). Moreover, by analysis of the gut microbial composition, clear links between the gut microbiota profile and the obese state have been found (Ley *et al*, 2005; Murphy *et al*, 2010; Turnbaugh *et al*, 2008), whereby certain microbes display a greater capacity to promote energy harvest (Turnbaugh *et al*, 2006).

The underlying mechanisms of microbiota induced adiposity appear to involve changes in the activity and morphology of cells associated with the

intestine, liver and adipose tissue (Backhed *et al*, 2004; Cani *et al*, 2008; Cani *et al*, 2009). Indeed, evidence has demonstrated gut microbiota can contribute to the pathophysiology of HFD-induced obesity by causing metabolic endotoxemia as a result of increased gut permeability and associated absorption of bacterially-derived lipopolysaccharide (LPS) (Cani *et al*, 2007; Cani *et al*, 2008). This involves an impairment of intestinal tight-junction protein expression, namely zona occludens 1 (ZO-1) and occludin, and an increased incidence of LPS producing microbiota; thereby allowing increased LPS translocation across the gut wall into the circulation (Cani *et al*, 2008). The observed endotoxemia is thought to be responsible for the progression of inflammation and associated metabolic disorders, such as insulin resistance. Correspondingly, it has been shown that subcutaneous infusion of LPS induces obesity and associated metabolic disorders, to the same extent as that caused by a HFD feeding (Cani *et al*, 2007). In contrast, antibiotic treatment recovers intestinal integrity, significantly decreasing the incidence of metabolic endotoxemia and as such the development of HFD-induced obesity and inflammation (Cani *et al*, 2008).

Thus, the data suggests that HFD-induced changes in gut microbiota not only influence the production of LPS but also facilitate its passage through the gut wall by affecting gut wall integrity, thereby increasing the effect of metabolic endotoxemia on tissues such as the liver and the adipose tissue. In addition, in the adipose tissue LPS also inhibits adipocyte differentiation (Muccioli *et al*, 2010), which would no doubt cause hypertrophy by increased storage of TAG in the existing mature adipocytes, and thereby exacerbating the inflammatory response by augmenting cytokine release, macrophage infiltration and cell apoptosis.

1.6 WHEY PROTEINS INFLUENCE ON THE REGULATION OF ENERGY BALANCE

1.6.1 Introduction

Whey is the milk serum that remains after precipitation of casein during cheese production, and it contains proteins, vitamins, minerals and trace amounts of fat. In this regard, its potential as a nutritional and health promoting product is of great research interest and economic value to the dairy industry, especially to those involved in cheese manufacture. Whey associated proteins include α-lactalbumin, β-lactoglobulin, glycomacropeptide (GMP), BSA and lactoferrin (Krissansen, 2007). There is accumulating evidence suggesting that whey protein intake influences the balance between energy (food) intake and energy expenditure, insulin sensitivity and glucose homeostasis, as well as tissue lipid metabolism (Jakubowicz & Froy, 2013). Data from recent studies also suggests the potential for whey proteins to influence the gut microbiota composition, which may likewise impact on energy balance regulation. Here, data are reviewed from *in vivo* (human and rodent) and *in vitro* studies related to the above effects of whole WPI/whey protein concentrate (WPC) and individual whey proteins. Furthermore, how dietary composition and obesity could influence the actions of these dietary proteins is discussed, exposing several unanswered questions which we aim to further investigate in this study.

1.6.2 Energy intake

Studies conducted in humans have shown that 20 to 50g of whey protein intake reduces short-term *ad libitum* food intake. Akhavan *et al,* showed that WPC (20-

40g) dose-dependently reduced subsequent (30 min) *ad libitum* pizza meal energy intake in lean subjects, with the 40g dose having the greatest impact compared to the water control (Akhavan *et al*, 2010). This effect extends up to 2h in lean subjects (Anderson *et al*, 2004). In comparison to casein, whey protein (48g) preloads were found to suppress subsequent *ad libitum* lunch energy intake and reduce the desire to eat for up to 180 mins in lean subjects (Hall *et al*, 2003), suggesting that whey proteins induce satiety in comparison to casein intake. Differences in the kinetics of digestion of these two protein sources have been proposed as being involved in the differential effects on satiety, with whey being described as a "fast" digesting protein and casein a "slow" digesting protein (Boirie, 2013; Boirie *et al*, 1997; He & Giuseppin, 2014). Indeed, compared to casein, whey proteins rapidly increase post-prandial plasma amino acids levels, which correlate with increases in satiety (Hall *et al*, 2003). The results of rodent studies with regard to the effects of whey proteins on food (energy) intake are largely in agreement with that observed in humans. Indeed, the suggestion that whey proteins induce satiety has been further confirmed in mice by showing that WPI providing 30% of total dietary energy increases the intermeal interval (satiety) compared to soy protein during a 7 day study period (Yu *et al*, 2009). Similar findings have been reported from a long term study lasting 10 weeks (Zhou *et al*, 2011). These data are consistent with the findings from human studies that whey proteins reduce food intake by inducing satiety in the lean state. However, a functional relationship between whey proteins and food intake has not been consistently reported. For instance, although Hall *et al,* showed that whey proteins reduce food intake compared to casein in humans (Hall *et al*, 2003), this same response was not observed in rats given a WPI diet for 7 weeks in comparison to

casein (300g/kg diet), despite the fact that the rats on the WPI diet showed a significantly reduced weight gain compared to the those on the casein diet (Royle *et al*, 2008). It is possible that physiological and neuroendocrine differences that exist between the species (humans vs. rats) may have given rise to the inconsistent effects of whey protein on food intake.

There is evidence that macronutrient composition in the diet could also impact upon the effect of whey proteins on food intake. Veldhorst *et al,* showed that a diet containing proteins, carbohydrates and fat providing 10, 55 and 35% energy, respectively, reduces hunger in humans if whey proteins were included in comparison to either soy and casein, but when the macronutrient composition was altered to 25, 55 and 20 % energy from proteins, carbohydrates and fat, this satiety effect was no longer observed (Veldhorst *et al*, 2009a). Similar results have been obtained from a study conducted in rats, which showed that WPC with an energy content of 55% reduced cumulative energy intake compared to whole milk protein if the former protein was supplied in a diet with 35% and 10% energy from carbohydrates and lipids, respectively, or in a diet with 45% energy solely derived from lipids (Pichon *et al*, 2008). No differences in cumulative energy intake were observed if the proteins were provided in a diet with 15% and 30% energy from carbohydrates and fats, respectively. These data highlights the importance of the macronutrient composition in the diet to whey protein effects on food intake. In addition to dietary composition, weight gain also seems to influence the effect of whey proteins on short-term food intake. This is revealed by the finding that intake of WPI (50g) reduced subsequent pizza intake in normal weight but not in obese subjects (Bellissimo *et al*, 2008). Similar findings have been reported by other human studies (Bowen *et al*, 2006a; Bowen *et al*, 2007; Bowen *et al*, 2006b) and in

HFD-induced obese mice (Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2011b). In fact, studies have shown that the reductions in weight gain with whey protein intake in HFD fed mice can occur independently of an impact on energy intake (Pilvi *et al*, 2007; Shertzer *et al*, 2011). In the case of Shertzer *et al,* (2011), HFD-fed mice that drank water supplemented with WPI at 100g/L for 11 weeks showed a similar energy intake to those on the un-supplemented water, despite the former showing a significant decrease in body weight. Thus, in addition to protein source, quality and time of consumption mentioned by Anderson *et al,* (2004), both macronutrient composition in the diet and state of energy balance (lean vs. obese), should be considered as important factors influencing the whey protein effects on food intake.

A number of studies have attempted to identify the individual whey proteins that might be causing the reduction in food intake in humans and rodents. Because GMP has been shown to stimulate the production of the satiation hormone CCK (Pedersen *et al*, 2000), a role for this whey protein in the regulation of food intake has been suggested. The findings of Veldhorst *et al,* support the latter suggestion, as ingestion of a breakfast diet with whey protein providing 10% energy reduced lunchtime energy intake in lean subjects more than the intake of a whey protein without GMP associated breakfast (Veldhorst *et al*, 2009c). This finding is however not in agreement with data from other studies performed on lean subjects with diets containing whey protein without GMP (providing 44% energy)(Burton-Freeman, 2008), and whey protein diets with added GMP (21% w/w) (Lam *et al*, 2009). Taking into account the data from another study involving rats, which revealed that GMP does not impact on food intake (Royle *et al*, 2008), it could be argued that this whey protein does not have a significant effect on this

physiological process. However, a more recent study has shown that rats fed a GMP supplementation with a HFD show an improvement in their plasma and tissue lipid profiles in association with significant reductions in their food intake compared to HFD fed controls (Xu *et al*, 2013a). Utilising a similar approach as in their previous studies, Veldhorst *et al,* showed that breakfast diets with α-lactalbumin providing 10% or 25% energy reduces lunch time energy intake in lean subjects compared to a breakfast diet with casein, soy or whey (Veldhorst *et al*, 2009b). This data is in agreement with the results of another study (Hursel *et al*, 2010), which together suggest an important role for α-lactalbumin in the suppression of food intake in lean humans. In addition to this, Pichon *et al,* (2008) also found β-lactoglobulin consumption in rats to reduce energy intake in comparison to whole milk proteins.

In summary, consumption of whey proteins as an isolate or concentrate appears to reduce food (energy) intake in humans and rodents by inducing satiety compared to casein, soy or carbohydrates. This effect appears to be influenced by the macronutrient composition of the whey protein diet and by the development of obesity. Of the whey proteins that have been studied, data suggests an important role(s) for α-lactalbumin and β-lactoglobulin in the regulation of food (energy) intake, but a regulatory role for GMP in this physiological process is still unclear. Moreover, despite progress in the understanding of the effects of whey proteins on energy intake, there is still insufficient information on the mechanisms by which these proteins influence satiety and alter energy intake, and how variations to dietary composition and energy status (lean vs obese) can influence their action. Currently, to our knowledge, data is lacking on how WPI changes energy intake

during the 24 hr period, or how the intake of whey proteins can influence daily meal pattern (i.e. meal size and meal number).

1.6.3 Satiety hormones

It is well established that the hormones CCK, PYY, ghrelin, insulin, leptin and GLP-1 play important roles in energy balance by inhibiting (CCK, insulin, leptin and GLP-1) and stimulating (ghrelin) food (energy) intake and/or by inducing catabolic (GLP-1 and leptin) and anabolic (ghrelin and insulin) responses on lipid metabolism in tissues (Cummings & Overduin, 2007; Nogueiras *et al*, 2010). Given the effects of whey proteins on food intake and body weight, it is no surprise that whey proteins modulate these hormones. A study by Bowen *et al,* showed that whey protein decreases in energy intake in humans are accompanied by increased plasma levels of CCK and GLP-1, and reduced levels of ghrelin (Bowen *et al*, 2006a). These changes were noted as early as 15 mins and continued up to 180 mins after the dietary challenge, suggesting potential acute and chronic effects of whey proteins on hormonal levels. Hall *et al,* (2003) also demonstrated that increases in satiety with whey protein intake were associated with elevations in post-prandial CCK and GLP-1 levels up to 180mins compared to casein. Others have confirmed these post-prandial increases in GLP-1, as well as showing enhanced plasma PYY levels with whey protein intake (Akhavan *et al*, 2014; Veldhorst *et al*, 2009a). Furthermore, as the hydrolysis of WPI releases peptides that can suppress DPPIV activity (Lacroix & Li-Chan, 2012), whey protein intake may improve the maintenance of incretin hormone activity as well as their release. Prolonged whey protein intake has also been associated with reduced fasting ghrelin hormone levels compared to carbohydrate or soy protein supplementation

in humans, although no corresponding effects on energy intake were seen (Baer *et al*, 2011). In the case of insulin, whey proteins appear to acutely increase its release (Power *et al*, 2009), but with prolonged intake there appears to be a reduction in fasting insulin levels (Baer *et al*, 2011). The fact that similar effects on hormone levels have been observed in studies conducted using only lean (Hall *et al*, 2003) or only obese subjects (Bowen *et al*, 2007), suggests that whey proteins can modulate hormone levels independent of weight gain and that the changes are consistent with an attempt to increase catabolism.

Comparable data has been obtained from rodent studies. Zhou *et al,* showed that the reduction in food intake in lean rats on a diet supplemented with 24% (wt/wt) WPC, is accompanied by an increased GLP-1 level in the plasma (Zhou *et al*, 2011). Rodent studies have also highlighted whey protein effects on long-term signals involved in energy balance regulation. Similar to the effects in humans, whey proteins acutely promote an insulinotropic response in rodents, while also reducing fasting plasma insulin levels in the long-term (Belobrajdic *et al*, 2004; Gaudel *et al*, 2013). Acute insulin production may be, in part, related to stimulatory effect of whey proteins on the release of incretin hormones GLP-1 and GIP (Drucker, 2006), given that both these hormones enhance glucose-dependent insulin secretion from pancreatic β-cells (Garber, 2011; Hansotia *et al*, 2007). Additionally, a detailed microarray analysis of gene expression in the adipocytes of obese mice that had ingested whey proteins revealed an increased expression of leptin and several genes involved in the insulin signalling pathway in the whey protein group compared to the casein control (Pilvi *et al*, 2008b). Since the mass of the adipose tissue and the gene expression of leptin have previously been shown to be closely linked (Galic *et al*, 2010), the significance of this increased expression of

leptin in adipocytes with reduced adipocyte cross sectional area remains to be determined.

As mentioned above, GMP has been demonstrated to enhance plasma CCK levels (Pedersen *et al*, 2000), yet this is not consistently shown (Keogh *et al*, 2010). However, data from Royle *et al,* (2008) does suggest a role for GMP in mediating the reduction in fasting insulin levels observed with prolonged whey protein intake; the data show that rats fed a GMP-free WPI diet have elevated fasting insulin levels compared to casein and BBQ beef diets, while the same WPI diet with added GMP at either 100 or 200 g/kg resulted in fasting insulin levels that were significantly lower than all other diets groups. So far, only one study, using healthy lean subjects, failed to find the consumption of a α-lactalbumin breakfast meal to influence postprandial levels of GLP-1, ghrelin and insulin compared to a comparable breakfast meal with gelatin or gelatin plus tryptophan, despite having significantly reduced hunger scores (Nieuwenhuizen *et al*, 2009).

In summary, whey proteins appear to drive endocrine and cellular changes consistent with a catabolic effect in comparison to casein and carbohydrates (glucose and fructose). This is achieved by up-regulation of the production of catabolic hormones (CCK, GLP-1, PYY, leptin and insulin), and by the reduction in the production of the anabolic hormone ghrelin. The decrease in insulin levels observed over time with whey protein intake might be a mechanism to reduce the anabolic effects of this hormone upon adipocytes (Coomans *et al*, 2011). Here we reviewed how whey proteins impact on peripheral signals in energy balance regulation, but to our knowledge no investigations to date have been conducted to assess the impact of whey proteins on gene expression in centres of the brain important for regulation of energy balance.

1.6.4 Energy expenditure and muscle protein synthesis

Whey protein (18g) intake prior to a bout of heavy resistance training increases post-training resting energy expenditure in humans compared to carbohydrate intake (Hackney *et al*, 2010). Others have shown similar augmentations in carbohydrate oxidation and lean body mass in response to combined whey protein intake and resistance exercise (Betts *et al*, 2007; Volek *et al*, 2013), which may be related to the observed effect on energy expenditure. Interestingly, the effect on energy expenditure diminishes if the whey protein meal is ingested after the resistance exercise, even if the protein content in the diet is at 30g (Benton & Swan, 2007). The data suggest that the timing of the dietary challenge is crucial for detecting whey protein induced changes in resting energy expenditure. Intake of whey proteins also appears to increase thermogenesis, possibly because of its higher thermic effect compared to soy or casein (Acheson *et al*, 2011). Data from a recent rodent study clearly suggests such an effect, with diet-induced obese mice drinking water supplemented with whey proteins (100g/L) increasing their O_2 consumption compared to obese mice drinking un-supplemented water (Shertzer *et al*, 2011).

These effects on energy expenditure may possibly be due to the whey protein induced increased protein metabolism in tissues (Boirie *et al*, 1997), given that the bulk of resting energy expenditure is linked to lean body mass and that whey proteins both enhance the development of lean muscle mass and spare its loss during energy restriction (Frestedt *et al*, 2008; Volek *et al*, 2013). Indeed, a number of other reports have shown that whey intake increases muscle protein synthesis in comparison to casein (Pennings *et al*, 2011; Tang *et al*, 2009), and

evidence suggests that this response may be related to its ability to stimulate skeletal muscle mTOR activity (Farnfield *et al*, 2012; Kakigi *et al*, 2014). Moreover, whey has also been shown to be effective at stimulating muscle protein synthesis in elderly humans and animals, thus reducing their susceptibility to age related muscle loss (sarcopenia) (Pennings *et al*, 2011; Rieu *et al*, 2007; Walrand *et al*, 2011). However, findings that whey specifically promotes muscle protein synthesis compared to other proteins sources, are not consistently reported (Dideriksen *et al*, 2011; Reitelseder *et al*, 2011). A recent study comparing post-prandial muscle protein synthesis, in response to different dietary proteins (egg, soy, wheat, whey) showed that the WPI was the most effective protein source for stimulating protein synthesis (Norton *et al*, 2012). The leucine content was a crucial factor in this effect, as supplementing the wheat protein diet with leucine to match the content in the whey diet led to similar levels of protein synthesis. This influence of leucine is not surprising as in addition to being a key protein synthesis substrate, leucine is also a regulator of the signalling pathway involved in protein synthesis via its effect on mTOR (Lynch *et al*, 2003; Lynch *et al*, 2002.).

To our knowledge, of the whey proteins associated with regulation of energy balance, only α-lactalbumin has been shown to influence energy expenditure. The study by Hursel *et al,* which reported an effect of α-lactalbumin (41% energy from the protein) ingestion on lunchtime meal intake in the healthy humans, also found that this whey protein significantly increases diet-induced thermogenesis compared to intake of a whole milk protein rich diet (Hursel *et al*, 2010). Whether α-lactalbumin, or any other individual whey protein, could influence energy expenditure in obese humans remains to be determined.

In summary, compared to carbohydrates, soy and casein, intake of whey proteins appears to increase energy expenditure in lean and obese states by influencing thermogenesis and resting energy expenditure. In the latter case, the timing of the dietary challenge might be important, in particular when associated with exercise, for obtaining an effective change in this parameter linked to energy expenditure. Enhanced protein synthesis associated with whey protein intake is no doubt a contributing factor in this effect, and most likely influenced by the high leucine content in whey proteins. It is also tempting to suggest that the effect of whey proteins on energy expenditure might be due to the actions of α-lactalbumin present in the WPI or WPC, however, this may be premature given that there are no data showing whether other whey proteins could influence energy expenditure to the same extent. Finally, a key unanswered question to date is, do alterations to energy expenditure and/or muscle protein synthesis play a role in the reductions in HFD-induced adiposity associated with whey protein intake?

1.6.5 Adiposity and Lipid metabolism

Over the last decade, an increasing number of reports have demonstrated that inclusion of WPI in the diet suppresses body weight gain in comparison to other dietary proteins, including casein (Pichon *et al*, 2008; Pilvi *et al*, 2007; Royle *et al*, 2008; Shi *et al*, 2012a; Shi *et al*, 2011b; Zhou *et al*, 2011). The reduction in body weight appears to be primarily driven by the pronounced decease in body fat gain in these animals (Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2011b). This effect is particularly visible during energy restriction, where whey protein intake enhances fat loss, while preserving lean mass content (Coker *et al*, 2012; Frestedt *et al*, 2008). As described earlier, whey protein induced reductions in body fat can

occur independently of an effect on energy intake (Pilvi *et al*, 2007; Shertzer *et al*, 2011), suggesting the involvement of alternative mechanisms in these outcomes. Given the importance of lipid metabolism for the storage and accumulation of fat, whey proteins may directly affect mechanisms regulating lipid metabolism in the tissues and this may underlie their ability to suppress adiposity.

In over-weight and/or obese humans and rodents, whey proteins have been shown to improve lipid metabolism, by reducing plasma, adipose and hepatic TAG levels. Recently Pal *et al,* demonstrated that intake of WPI (27g) twice daily for 12 weeks causes a reduction in fasting TAG levels in over-weight and obese subjects compared to casein intake (Pal *et al*, 2010). A similar effect has also been observed in obese rats with intake of WPC at 32% (wt/wt) (Belobrajdic *et al*, 2004). Additionally, even at a modest level of WPI (24% wt/wt providing 18% energy), these dietary proteins can reduce HFD (providing 60% energy)-induced weight gain and body fat accumulation in mice compared to those fed a HFD with casein (Pilvi *et al*, 2007). As the reduction in body weight and fat content was not a result of differences in energy intake or intestinal fat absorption between the two groups, the authors then investigated whether the WPI intake may have affected adipocyte lipid metabolism (Pilvi *et al*, 2008b) and demonstrated a reduction in adipocyte cross sectional area in the mice fed the HFD with WPI. This suggests that whey proteins influence lipid metabolism in adipocytes much more than casein, although the extent of the reduction in adipocyte size may have been influenced by the fact the diets had a high calcium content, which in itself is known to influence adipocyte lipid metabolism (Zemel, 2004). Furthermore, recent data has shown whey proteins to influence lipid metabolism-related gene expression in adipocytes, hepatocytes and skeletal muscle cells (Freudenberg *et al*, 2012; Morifuji *et al*,

2005; Pilvi *et al*, 2008b; Tauriainen *et al*, 2011). The study by Pilvi *et al,* (2008b) mentioned above performed a detailed microarray analysis of adipose tissue gene expression in the HFD fed mice that had ingested whey proteins. The microarray data revealed an increased expression of β3-AR, suggesting a potential functional relationship between the reduction in the adipose tissue and increased β3-AR expression, given that activation of this receptor is known to increase HSL-mediated hydrolysis of fat, increase fat oxidation and induce uncoupling protein-mediated thermogenesis (Holm, 2003; Weyer *et al*, 1999), all of which are likely to reduce the mass of the adipose tissue.

Given that the liver is also important for regulation of lipid metabolism, Pilvi *et al,* also assessed in a separate study how the same dietary challenge may impact upon the lipid metabolism in the liver of HFD-induced obese mice subjected to a 7 week calorie restriction (Pilvi *et al*, 2008a). Indeed, compared to obese mice on the calorie restricted casein diet, hepatic TAG levels in obese mice on the calorie restricted WPI diet were reduced to a far greater extent. The specificity with which WPI reduces hepatic lipid content has been further demonstrated in the absence of calorie restriction (Hamad *et al*, 2011; Shertzer *et al*, 2011). In the studies mentioned above, except for the study by Shertzer *et al,* (2011), the observed effects of whey protein on lipid metabolism were shown relative to casein. Interestingly, rats fed a LFD containing soy proteins at 24% (wt/wt) had a body weight and an abdominal fat mass that was comparable to rats fed a similar diet with WPC (Zhou *et al*, 2011). This data could be interpreted to suggest that whey protein effects on lipid metabolism are detectable only when casein is used as the control protein or that the lack of an effect on adiposity in comparison to soy might be due to macronutrient composition, in particular the fat content in the diet, with

body fat content in HFD fed animals being more likely to be counteracted by whey protein effects on lipid metabolism. At the molecular level, whey protein intake in rats, in comparison to casein intake, reduces the activity of several lipogenic enzymes including FASN in the liver, while contrastingly increasing FASN expression and activity in the muscle (Morifuji *et al*, 2005), possibly to reduce hepatic production of lipids and to promote lipid oxidation in the muscle cells to generate energy in the form of ATP.

A number of studies have attempted to determine how individual whey proteins affect lipid metabolism. By providing overweight human subjects with enteric coated lactoferrin (300mg/d) or placebo tablets for a period of 8 weeks, it has been found that lactoferrin reduces body weight, visceral fat mass, as well as hip circumference (Ono *et al*, 2010). In rats ingesting a WPI diet supplemented with GMP at 100 and 200g/kg a reduced carcass fat was found compared to rats on either a WPI or casein diet (Royle *et al*, 2008). Because there were no differences in food intake between the groups, the data suggests a food intake-independent effect of GMP on lipid metabolism (fat content) in these animals. Likewise, in another recent rat study, similar reductions in body fat and improved plasma and hepatic lipid profiles were seen following daily intragastric GMP supplementation to HFD fed obese rats (Xu *et al*, 2013a).

With the view to assessing how whey proteins, namely α-lactalbumin, β-lactoglobulin and lactoferrin influence body weight and body fat content in rodents, Pilvi *et al,* subjected HFD-induced obese mice to a 70% energy restricted diet containing WPI, α-lactalbumin, β-lactoglobulin or lactoferrin at 18% of dietary energy content for a period of 7 weeks to induce weight loss, and then subsequently allowed *ad libitum* access to these diets for a further 7 weeks to

allow weight gain (Pilvi *et al*, 2009). Results suggest that α-lactalbumin is the most beneficial whey protein in terms of causing fat loss when provided as part of an energy restricted diet or fed *ad libitum,* and similar findings to these were subsequently demonstrated in Shi *et al*, (2011a) by this same research group. In contrast to Pilvi *et al,* (2009), who showed that β-lactoglobulin was the least effective whey protein for inducing weight loss and for preventing weight gain, another study by Pichon *et al,* (2008) found that β-lactoglobulin (55% energy) causes the least weight gain when supplemented in a carbohydrate free diet containing 45% energy as fat, in comparison to a diet containing 35% and 10% energy from carbohydrates and fat or in a diet with 15% and 30% energy from the same macronutrients, respectively. The data again highlights the importance of macronutrient composition on whey proteins effects on energy balance. With regard to the above mentioned changes in the cellular activity observed in the adipose, muscle and liver, it is important to further define how they arise by investigating the impact of these whey proteins implicated in lipid metabolism, namely α-lactalbumin, β-lactoglobulin, lactoferrin and GMP.

In summary, WPI appears to reduce body weight and lipid metabolism in both obese humans and rodents compared to casein intake, with specific effects observed in the adipocytes and in the liver; although these effects appear to be influenced by macronutrient composition in the diet. Additionally whey proteins alter the expression or activity of lipogenic, lipolytic and fat oxidation related genes in the liver, adipose and in the muscle. Available data suggest an important role(s) for α-lactalbumin, β-lactoglobulin, lactoferrin and GMP in the regulation of lipid metabolism in humans and/or in rodents, but this requires further investigation to clarify the roles and to understand the mechanisms involved. It is

important to note that the above mentioned changes in adiposity and lipid metabolism occurred, for the most part, independently of changes to energy intake and/or expenditure, but the underlying mechanisms involved remain to be determined.

1.6.6 Insulin sensitivity and glucose homeostasis

In both humans and rodents, whey protein intake has been shown to cause an acute insulinotropic response (Gaudel *et al*, 2013; Power *et al*, 2009). This has been demonstrated by supplementation of whey proteins into glucose drinks or test meals, which augments postprandial insulin release, resulting in an enhancement of glucose disposal in both healthy (Acheson *et al*, 2011; Nilsson *et al*, 2007) and type-2 diabetic subjects (Frid *et al*, 2005). Similar effects have been observed in anesthetised mice, where gastric gavage of whey protein (75mg) and glucose (75mg) together augmented the insulin response 3-fold and increased the glucose disposal by 31% in comparison to glucose alone (Gunnarsson *et al*, 2006). *In vitro* work using the pancreatic BRIN-BD11 β-cell line has also demonstrated that whey protein hydrolysate can directly stimulate insulin release in a dose dependent manner (Gaudel *et al*, 2013). In addition to these acute responses, prolonged whey protein intake also improves insulin sensitivity in the obese state of both rodents (Belobrajdic *et al*, 2004; Huang *et al*, 2008; Pilvi *et al*, 2008a; Shertzer *et al*, 2011; Shi *et al*, 2011b) and humans (Pal *et al*, 2010). In fact, a recent study has demonstrated significant improvements to glycaemic control in obese *ob/ob* mice consuming a whey protein hydrolysate diet (Gaudel *et al*, 2013). At the molecular level, the mRNA expression of GLUT4 was increased by augmenting the WPI content in a HFD fed to mice (Freudenberg *et al*, 2012). Moreover, Morato *et*

al, (2013) examined the acute effects of WPI and its hydrolysate on GLUT4 translocation to the cell surface in Wistar rats and showed enhanced GLUT4 translocation to the plasma membrane without any alterations to fasting insulin levels in comparison to casein fed animals. The authors proposed that this could indicate a whey protein insulin-independent effect on GLUT4. This notion was also proposed by Akhavan *et al,* (2014), as they found comparable post-meal glucose levels in young lean males given whey protein and glucose preloads, despite reduced levels of post-meal insulin levels in the whey preload group. As discussed before, the ability of whey protein to stimulate the release of the incretin hormones GIP and GLP-1, and also to increase their activity by the suppression of DPPIV, may have also contributed to the insulinotropic effect associated with whey protein intake.

Of the whey proteins that have been tested, both α-lactalbumin and GMP have been found to increase the postprandial insulin release (in comparison to casein) with GMP having a greater effect (Veldhorst *et al*, 2009b). Long-term GMP intake has also been shown to improve fasting blood insulin levels in both humans (Keogh & Clifton, 2008) and rats (Royle *et al*, 2008). Similarly a high protein diet (55% kcal) with β-lactoglobulin as it source of protein was shown to reduce insulin resistance and improve fasting blood insulin levels in rats to a greater extent than the comparable high WPC diet (Pichon *et al*, 2008). Lactoferrin circulating levels also correlate negatively with hyperglycemia and positively with insulin sensitivity (Moreno-Navarrete *et al*, 2009), and inclusion of lactoferrin (Shi *et al*, 2012b) or a lactoferrin-rich WPI (Shi *et al*, 2011b) into a HFD, improves glucose tolerance in mice in comparison to casein fed controls.

In summary, whey protein intake appears to acutely stimulate insulin release and improve glucose tolerance and insulin sensitivity in the long-term, even when accompanied by high fat feeding. The available data also suggest that the improvement of insulin sensitivity may be a common feature of α-lactalbumin, β-lactoglobulin, lactoferrin and GMP, with the latter being more potent than α-lactalbumin. The question of whether whey proteins directly influence insulin signalling or mediate their effect indirectly via lipid metabolism and adiposity remains to be determined. Similarly it is also unclear as to how these effects manifest in individual tissues (e.g. muscle, liver and adipose tissue), and whether there are associated changes in inflammatory markers.

1.6.7 Gut microbiota

Given the increasing evidence of the importance of gut microbiota for energy balance regulation (Backhed *et al*, 2004; Murphy *et al*, 2010; Turnbaugh *et al*, 2006), the impact of whey proteins on gut microbiota composition, and associated beneficial effects on weight management, will be an area of great research interest. Currently, a number of studies have shown that whey protein hydrolysate, as well as specific whey proteins, such as β-lactoglubulin, α-lactalbumin, GMP and lactoferrin, to have anti-microbial activity (Freedman *et al*, 1998; Pihlanto-Leppala *et al*, 1999; Shin *et al*, 2000; Yamauchi *et al*, 1993). In addition to the anti-microbial effect, whey proteins have also been associated with the promotion of certain types of gut bacteria, namely *Bifidobacteria* (Petschow & Talbott, 1990). Similar *Bifidobacterium* growth promoting capabilities have been ascribed to both GMP and lactoferrin (Brody, 2000; Petschow *et al*, 1999; Rahman *et al*, 2009).

To date, only three studies have assessed the whey proteins effects on gut microbiota composition, and none of them have gone into any great detail of the specific effects of WPI. Firstly, a study by Sprong *et al,* (2010) demonstrated in a rat model of colitis, that whey protein intake increased the levels of *Lactobacilli* and *Bifidobacteria* in comparison to casein intake. Similar gut microbiota changes were also described in a study that investigated the influence of GMP consumption on gut microbiota composition (Chen *et al*, 2012). However, a more recent study using HFD fed mice failed to find any significant difference in gut microbiota composition between mice consuming casein or whey (Tranberg *et al*, 2013), albeit here the mice were consuming a HFD, which may have affected the ability of whey proteins to influence the gut microbiota. In addition to influencing the gut microbiota, whey proteins, namely lactoferrin, have also been linked to improving gut wall integrity and to suppressing the detrimental effects of bacterial endotoxins, such as LPS (Kruzel *et al*, 2000; Lee *et al*, 1998). This suggests that whey proteins are possibly able to suppress the development of metabolic endotoxemia associated with HFD intake, through changes to the intestinal environment.

In summary, the findings suggest that whey proteins have a pre-biotic effect through promoting the growth of beneficial bacteria (*Lactobacillus* and *Bifidobacteria*), while suppressing pathogenic bacteria such as *E. coli* and *Salmonella* (*Entreobactericaccea*), and also may be able to protect gut integrity and suppress the impact of bacterial entotoxins (e.g. LPS). However, there is a lack of knowledge of how whey proteins effect gut microbial composition and how these changes may relate to the physiological outcomes observed with whey protein intake? Thus, there needs to be further investigation into these matters.

1.7 OVERALL SUMMARY

Body weight is determined by the balance between energy intake and energy expenditure, and obesity develops from the sustained consumption of energy in excess of that spent. The regulation of energy balance is a complex physiological process that requires the interaction between multiple tissues, including the GIT, liver, pancreas, adipose tissue and the brain, with energy status in the peripheral tissues being conveyed to the CNS by hormones produced by the GIT, adipose tissue and pancreas. Integration of these signals in the CNS controls the expression of several neuropeptides important for energy balance regulation, and the resulting responses generated from multiples inputs results in changes in food intake, energy expenditure and lipid metabolism.

Overall the current evidence presented here shows whey protein intake is associated with a range of positive benefits related to weight management. These include an ability to decrease food intake, possibly by altering the plasma levels of hormones (CCK, GLP-1, ghrelin and insulin) important for energy balance regulation. Notably however, evidence suggests that whey protein induced reductions in weight gain and adiposity in HFD fed and obese animals/subjects are unrelated to effects on energy intake, and that whey proteins directly influence lipid and glucose metabolism in tissues such as the liver and adipose tissue. These effects could be correlated to improved insulin sensitivity and glucose tolerance, with evidence demonstrating WPI ability to suppress the development of HFD-induced insulin resistance, to increase energy expenditure and to alter the gut microbiota composition, which could also be important in energy balance regulation and weight management. Finally, the evidence also shows that both the macronutrient composition in the diet and the body energy status of the

animals/subjects involved, appear to greatly impact upon the ability of whey proteins to influence the balance between energy intake and energy expenditure. Thus a better understanding of the specific mechanisms by which whey proteins influence body weight and energy balance may help in the formulation of more effective dietary interventions that could prevent and/or reduce obesity development.

1.8 OBJECTIVES OF THE STUDY

WPI has been increasingly demonstrated to suppress HFD-induced weight gain and adiposity independently of significant changes to energy intake. Therefore, this project tested the hypothesis that WPI directly impacts on adiposity by influencing tissue lipid metabolism.

The specific objectives of the project were:

Objective 1: To ascertain the contribution of energy intake and energy expenditure to WPI effects related to lipid metabolism.

Objective 2: To study the effects of WPI on lipid metabolism and insulin signalling in peripheral tissues (adipose tissue, liver), and to determine the underlying mechanisms of their action.

Objective 3: To utilise the 3T3-L1 pre-adipocyte cell line as a model of fat cells to investigate the functionality of lysophosphatidic acid receptor 5 (LPA_5), as a candidate in mediating whey protein effects on adipose tissue lipid metabolism.

1.9 APPROACH

HFD-fed C57BL/6J mice were used as a model to better understand how WPI alters tissue lipid metabolism without substantially changing energy intake. HFD fed mice display temporal changes in their energy intake pattern compared to LFD fed mice, with no difference in energy intake observed during weeks 1-4, a significant reduction during weeks 5-12, followed by an increase in energy intake which exceeds LFD fed mice beyond week 19 (Lin *et al*, 2000). In contrast, an increase in body weight was observed in these HFD fed mice by week 2, and this continued to increase even during periods of reduced energy intake. Therefore, this project sought to investigate the effect of WPI inclusion into a HFD on lipid metabolism and other energy balance parameters during the periods of hypophagia (weeks 5-12) and hyperphagia (week 19 onwards) in this HFD fed mouse model, by conducting trials over an 8 week (short-term) and 21 week (long-term) period.

In an attempt to further explore whether WPI or its related bioactives could directly affect adipose tissue lipid metabolism, we utilised the 3T3-L1 pre-adipocyte cell line as a model to investigate the functionality of the receptor, LPA_5, as a candidate for mediating WPI effects on lipid metabolism in these cells. The receptor was selected based on its responsiveness to dietary protein hydrolysate (Choi *et al*, 2007a; Choi *et al*, 2007b), and the fact that its endogenous ligand, LPA, had previously been shown to both stimulate pre-adipocyte proliferation and suppress their differentiation into mature lipid laden adipocytes. This initial work was intended to characterise the role of LPA_5 in 3T3-L1 pre-adipocytes growth and differentiation, in order to evaluate its potential to mediate WPI impact on the cellular activity of these cells.

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Chapter 2

Whey protein isolate counteracts the effects of a high fat diet on energy intake and hypothalamic and adipose tissue expression of energy balance-related genes

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2.1 ABSTRACT

Intake of whey protein isolate (WPI) is known to reduce high-fat diet (HFD)-induced body weight gain and adiposity. However, the molecular mechanisms are not fully understood. To this end, we fed C57BL/6J mice for 8 weeks with 10% energy as fat (low fat diet, LFD) or 45% energy as fat (HFD) enriched with either 20% energy as casein (LFD and HFD) or WPI (HF-WPI). Metabolic parameters and the hypothalamic and epididymal adipose tissue expression of energy balance-related genes were investigated. The HFD increased fat mass and plasma leptin levels and decreased dark phase energy intake, meal number, respiratory exchange ratio, and metabolic (VO_2 and heat) and locomotor activity compared with the LFD. The HFD increased hypothalamic mRNA expression of the leptin receptor, insulin receptor (INSR) and carnitine palmitoyltransferase 1b (CPT1b). The HFD also reduced adipose tissue mRNA expression of glucose transporter 4 (GLUT4) and INSR. In contrast, WPI reduced fat mass, normalised dark phase energy intake and increased meal size in HFD fed mice. The dietary protein did not impact on plasma leptin, insulin, glucose or glucagon-like peptide 1 levels, but increased plasma triacylglycerol (TAG) levels in HFD fed mice. At a cellular level, WPI significantly reduced the HFD-associated increase in hypothalamic mRNA expression of the leptin receptor, INSR, and CPT1b. Also, WPI prevented the HFD-induced reduction in adipose tissue mRNA expression of INSR and GLUT4. In comparison to casein, the effects of WPI on energy intake and hypothalamic and adipose tissue gene expression may thus represent a state of reduced susceptibility to weight gain on a HFD.

2.2 INTRODUCTION

Obesity develops when energy consumed in excess of daily metabolic requirements is stored as triacylglycerol (TAG) in the adipose tissue. The prevalence of obesity and associated conditions has reached epidemic proportions worldwide (Bruce & Byrne, 2009; Caterson *et al*, 2004), and therefore there is a growing interest to identify nutritional factors that could influence energy balance and thus could reduce the susceptibility to develop obesity, such as that caused by a high-fat diet (HFD)(Chalkley *et al*, 2002; Hill & Peters, 2002; Lin *et al*, 2000b).

Whey protein isolate (WPI) is a mixture of milk proteins obtained after precipitation of casein during cheese production, and it contains β-lactoglobulin, α-lactalbumin, bovine serum albumin, glycomacropeptide, immunoglobulins, lactoferrin and other minor proteins. WPI has received a lot of attention recently because of its associated health benefits (Krissansen, 2007). Prolonged intake of whey proteins has been found to improve insulin sensitivity and glucose homeostasis (Acheson *et al*, 2011; Gunnarsson *et al*, 2006; Nilsson *et al*, 2007; Shertzer *et al*, 2011). Also, using healthy lean subjects or rodents, a number of studies have reported a short-term reduction in energy intake and an increase in thermogenesis following WPI intake in comparison to other dietary proteins, including casein (Acheson *et al*, 2011; Hall *et al*, 2003; Yu *et al*, 2009; Zhou *et al*, 2011). Although there are a discrepancies (Benton & Swan, 2007; Pichon *et al*, 2008; Royle *et al*, 2008; Veldhorst *et al*, 2009), it is largely recognised that WPI intake influences the balance between energy intake and energy expenditure in the lean state (Luhovyy *et al*, 2007; McAllan *et al*, 2012). In contrast, the effects of WPI on energy intake and energy expenditure in the HFD-induced state are less well known, although WPI has been consistently shown to reduce HFD-induced body

weight gain and adiposity (Pilvi *et al*, 2009; Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2012; Shi *et al*, 2011). However, three studies have reported no effects on energy intake in mice fed a HFD with WPI for 11 (Shertzer *et al*, 2011), 14 (Shi *et al*, 2012) or 21 weeks (Pilvi *et al*, 2007), in comparison to HFD fed controls. In the study by Shertzer *et al,* (2011), WPI was found to increase metabolic activity, while Pilvi *et al,* (2007) found no changes in metabolic activity in the mice fed a HFD with WPI compared with those fed a control diet. It is possible that these discrepancies arise partly due to the difference in the duration of HF feeding, with prolonged feeding-induced neuroendocrine changes having a greater impact on the ability of WPI to regulate energy balance. Thus, one could argue that a shorter duration of HF feeding (in comparison with the above-mentioned studies) may reveal how WPI affects physiological and cellular parameters important for energy balance regulation.

This present study sought to investigate the short-term (8 week) impact of WPI in conjunction with a HFD, specifically focusing on how the dietary protein influences HFD-induced (1) body weight gain and body composition, (2) energy intake (total intake, meal number and meal size) and parameters related to energy expenditure (oxygen consumption, heat production and locomotor activity), (3) plasma levels of hormones and metabolites and (4) the hypothalamic and epididymal adipose tissue expression of genes involved in leptin and insulin signalling, inflammation and lipid metabolism.

2.3 MATERIALS AND METHODS

2.3.1 Animals, diets and reagents

Experiments involving mice were licensed under the Cruelty to Animals Act 1876 and received ethical approval from the University College Cork Animal Experimentation Ethics Committee (no. 2011/005). Male 3-4 week old C57BL/6J mice (Harlan, Middlesex, UK) were maintained at 20-22^{o}C and 45-65 % humidity, with a 12h light 12h dark cycle (06:00-18:00). Mice had *ad libitum* access to food and water throughout the trials and all diets were purchased from Research diets (New Brunswick, NJ, USA). The experimental diets were a low fat diet (LFD; 10% energy as fat and 20% energy as casein; #D12450), a HFD (45% energy as fat and 20% energy as casein; #D12451) and a HFD with WPI (HF-WPI) (Alacen^{tm} 895; NZMP, New Zealand) in place of the casein protein (45% energy as fat and 20% energy as WPI). All reagents were purchased from Sigma unless otherwise stated.

2.3.2 Experimental protocol

The dietary challenges were performed on grouped housed mice (4 per home cage) following a four week acclimatisation period, during which the animals were fed a LFD. As we intended to measure the effect of the above-mentioned diets on energy intake and metabolic parameters using TSE Phenomaster cages (TSE systems, Bad Homburg, Germany), by housing the mice individually in these specialised cages, it was important to first determine the length of time the animals may need to be acclimatised to this new cage environment. This was investigated in Study 1. In Study 2, we assessed the effect of the LFD, the HFD and the HF-WPI diet on energy intake and metabolic parameters using the above-mentioned cages, following the pre-established acclimatisation period.

Study 1: Mice were fed a LFD for 7 weeks (n = 8). In weeks 5 and 6, the animals were individually housed in the TSE Phenomaster cages (TSE systems, Bad Homburg, Germany) for 3 days and energy intake and metabolic parameters were measured on day 2 and 3. At the end of the third day, the animals were placed back in their appropriate home cages. Food intake in the Phenomaster cages was measured using high-precision weighing sensors associated with feeding stations. Oxygen consumption (ml/h/kg; VO_2) and CO_2 production (ml/h/kg; VCO_2) were measured by indirect open-circuit calorimetry. The sensors measured the levels of food consumed and VO_2 and VCO_2 in each cage every 9 minutes. A meal was defined as an intake greater than 0.01g. Energy intake was calculated from 16.10 kJ/g for the LFD. The respiratory exchange ratio (RER) was calculated from VCO_2/VO_2. Locomotor activity was measured using a multi-dimensional infrared beam system, and was defined as the total number of infrared beam breaks in the X- and Y-axis.

Study 2: The experimental protocol is outlined in Figure 2.1. For 8 weeks, three weight-matched groups of mice were fed the LFD, the HFD or the HF-WPI diet (n = 8). Body weights were measured weekly. In weeks 5 and 6, the energy intake and metabolic activity was measured in the individual mice using the TSE Phenomaster cages (Fig 2.1). As mentioned earlier, the animals were housed in the Phenomaster cages for 3 days. Following a 2 day acclimatisation period (see results section for study 1), data was collected on the third day, after which the mice were placed back in their home cages. The same parameters mentioned earlier were measured. Energy intake was calculated as described earlier for the LFD and using 19.80 kJ/g for the HFD and the HF-WPI diet. Heat production

(kcal/h/kg) was calculated using the Weir equation (Weir, 1949)(3.941 × VO_2 + 1.106 × VCO_2) and converted to kJ/h/kg (1kcal = 4.184 kJ). The food quotient (FQ), defined as the ideal diet-specific VCO_2:VO_2 ratio, was calculated for each diet as described previously (Longo *et al*, 2010). In week 8, after a 6-8h fast, body composition was determined by nuclear magnetic resonance (NMR) using the Bruker minispec LF50H (Bruker optics, Ettlingen, Germany). Also, at this time point, plasma was collected from fasted anesthetised mice to measure glucagon-like peptide 1 (GLP-1), insulin, glucose, leptin and TAG levels. The animals were then immediately culled by cervical dislocation, and tissues of interest were dissected and snap-frozen in liquid nitrogen (liver and adipose) or on dry ice (brain).

2.3.3 Plasma hormones, glucose and TAG

Blood collected into BD Vacutainer EDTA tubes (Franklin Lakes, NJ, USA) from fasted anesthetised (65mg/kg ketamine and 13mg/kg xylazine) mice was treated with 500,000 KIU/L aprotinin and 0.1mM diprotin A (Sigma, Arklow, Ireland) to prevent degradation of plasma peptides by dipeptidyl aminopeptidase IV (DPPIV), trypsin and other related proteolytic enzymes. Blood was centrifuged at 2000rpm and 4^{o}C for 15 minutes, and plasma was isolated and stored at -80^{o}C. Enzyme-linked immunosorbent assays (ELISA) were used to measure plasma levels of GLP-1 (Linco Research, St. Charles, MO, USA), leptin and insulin (Crystal Chem, Downers Grove, IL, USA). Plasma glucose and TAG levels were measured using colorimetric assay kits (Glucose: Calibochem, Darmstadt, Germany; TAG: Wako Chemicals, Richmond, VI, USA). Homeostatic model assessment of insulin resistance (HOMA-

IR) values were determined using the formula: fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/L)/ 22.5 (Matthews *et al*, 1985).

2.3.4 Liver TAG

Lipids from frozen liver samples (50mg) were extracted according to the Folch method (Folch *et al*, 1957). Briefly, lipids in each sample were extracted using a 2:1 (v/v) solution of chloroform-methanol and aliquots of the organic phase were collected, dried and re-suspended in duplicate in the infinity TAG lipid stable reagent (Thermo Scientific, Middletown, VA, USA) or the LabAssay TAG kit reagent (Wako Chemicals, Richmond, VI, USA). TAG levels in the samples were determined according the manufacturers' instructions, and for samples resuspended in infinity TAG lipid stable reagent, lipid quantification was performed using a TAG chemistry calibrator (Pointe Scientific, Canton, MI, USA).

2.3.5 RNA extraction and complementary DNA synthesis

Total RNA was isolated from hypothalamic and epididymal adipose tissues into diethylpyrocarbonate-treated distilled water using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. To remove any potential genomic DNA contamination, on-column DNase treatment (Qiagen, Hilden, Germany) was performed during RNA isolation. Complementary DNA (cDNA) was synthesised from 1μg of total RNA using 2.5ng/μl random hexamer primers (Bioline, London, UK), 0.5mM deoxyribonucleotide triphosphate (dNTP) (Promega, Madison, WI, USA), 2U/μl RNase inhibitor (Promega, Madison, WI, USA) and the First Strand Synthesis System containing the Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the

manufacturers' instructions. A parallel reaction without the inclusion of the reverse transcriptase enzyme was also performed as a negative control.

2.3.6 Real-time PCR analysis

The amplification of the cDNA was performed in the Lightcycler 480 system (Roche, Penzberg, Germany) using 0.25μM primers (Eurofins MWG operon, Ebersberg, Germany), 1μl cDNA and the Lightcycler 480 SYBR Green I Master kit (Roche, Penzberg, Germany), according to manufacturers' instructions. Real-time PCR conditions were: 95^{o}C for 10 min followed by 50 cycles at 95^{o}C for 10s, 60 or 55^{o}C for 5s, and 72^{o}C for 15s. Primer sequences used are given in Table 2.1. Melting Curve analysis allowed validation of the authenticity of the real-time PCR products. Automated sequencing was performed to verify the sequences of these PCR products. Data obtained as C_p values were normalised to the expression of 18-S and β-actin according to $\Delta\Delta C_p = \Delta C_p$ target gene – ΔC_p housekeeping gene. 18-S and β-actin have been shown to be appropriate housekeeping genes for both the adipose tissue (Guo *et al*, 2012; Hoggard *et al*, 2009; Mracek *et al*, 2010; Yasmeen *et al*, 2013) and the hypothalamus (Giraudo *et al*, 2010; Hao *et al*, 2010; Sellayah *et al*, 2008). The relative gene expression was calculated using $2^{-\Delta\Delta Cp}$ and it is shown compared to that of the LFD group.

2.3.7 Immunoblot analysis

Adipose tissue samples (approximately 1ml lysis buffer per 200mg; n=3) were homogenized in lysis buffer (50mM HEPES, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Nonident P40 (NP40), 0.5mM dithiothreitol (DTT) and 0.1mM Na_3VO_4), containing protease inhibitors (0.1mM phenylmethylsulphonyl-fluoride (PMSF),

2µg/ml aprotinin, 2µg/ml leupeptin, 0.02mM NaF and 0.025mM NaPPi) and centrifuged at 12,000 g for 10 min to remove insoluble debris. Protein concentrations were analysed using the bicinchoninic acid (BCA) reagent (Pierce Biotechnology, Rockford, IL, USA). Protein lysate (20µg) was mixed with 4X sample buffer (333mM Tris-HCl, 3% SDS, 26.7% Glycerol, 130mM DTT and 0.2% Bromophenol Blue) and heated for 10 minutes at 95°C before loading onto a NuPAGE® 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). Protein bands were separated using SDS-PAGE electrophoresis in MOPS running buffer and transferred to PVDF membranes (BioRad, Hemel Hempstead, UK). Tris-buffered saline solution containing 0.1% Tween-20 was used as wash buffer, which was supplemented with 2% bovine serum albumin (BSA) (Sigma, Arklow, Ireland) and 3% non-fat dry milk (Marvel: Premier foods Ltd., Birmingham, UK) for the blocking solution and with 1% BSA and 1% non-fat dry milk for the antibody diluent. Blocked membranes were exposed to a 1:500 dilution of primary antibody against the insulin receptor β (INSR-β)(sc-711: Santa Cruz Biotechnology, Heidelberg, Germany). Horseradish peroxidise-conjugated (HRP) secondary antibody (1:8000 dilution; Jackson Immuoresearch, Newmarket, Suffolk, UK) was used to visualise the bound primary antibody. Membranes were stained with a 1:15000 dilution of a β-actin-HRP antibody (A3854: Sigma, Arklow, Ireland) to correct for sample loading. Visualisation was performed with the enhanced chemiluminescence (ECL) Western blotting Substrate Kit (Pierce Biotechnology, Rockford, IL, USA).

2.3.8 Statistical analysis

All data are presented as means ± SEM. Differences between the experimental groups were analysed by one-way or two-way ANOVA with *tukey* post hoc

pairwise comparisons. Body weight differences over 8 weeks were analysed by two-way repeated-measures ANOVA with *bonferroni* post hoc pairwise comparisons. Statistical analysis of immunoblot data was performed using a multiple comparisons Kruskal-Wallis test followed by Mann-Whitney U tests for individual comparisons. Significance was accepted at $P < 0.05$, and the statistical analysis was performed using GraphPad Prism version 3.03 (San Diego, CA, USA), Minitab version 15 (State College, PA, USA) and Sigma Stat version 3.1 (Chicago, IL, USA).

2.4 RESULTS

2.4.1 Body weight and composition

Body weight trajectories over the 8-week treatment period showed that mice on the HFD and the HF-WPI diet were significantly heavier than the LFD group from the second and seventh weeks respectively (Fig 2.2A). Importantly, the HF-WPI group gained significantly less body weight than the HFD group ($P < 0.05$; Fig 2.2B). At week 8, the total body fat (%) in the HF-WPI fed mice was significantly lower than in the HFD fed mice ($P < 0.05$; Fig 2.2C), but the total body fat (%) was still greater than in the LFD fed mice ($P < 0.001$). Furthermore, the total lean mass (%) was higher in the HF-WPI group compared to the HFD fed group ($P < 0.05$; Fig 2.2D), and as anticipated it was lower in the HFD and HF-WPI diet fed mice than in the LFD group ($P < 0.001$).

2.4.2 Plasma hormones, glucose and TAG, and hepatic TAG levels

At week 8, plasma leptin concentrations were higher in HFD and HF-WPI groups compared with LFD group ($P < 0.05$), while plasma TAG levels were greater in the

HF-WPI group compared with both the LFD and HFD groups ($P < 0.05$) (Table 2.2). There was no significant difference in plasma glucose, insulin, HOMA-IR and GLP-1 levels amongst the three diet groups (Table 2.2). Hepatic TAG levels (mg/g liver) were determined using the infinity TAG lipid stable reagent or the LabAssay TAG kit following extraction of the lipids. The data generated using the former revealed that HFD-fed mice had a higher hepatic TAG level than the LFD group ($P < 0.05$**;** Table 2.2). Whilst hepatic TAG levels in the HF-WPI diet fed mice were lower than those in the HFD group, the values did not differ significantly from those of either the LFD or HFD group. In contrast, the data generated using the LabAssay TAG kit showed that the hepatic TAG values (mg/g liver) for the HF-WPI diet (58.68 ± 3.92) fed mice were significantly lower than the HFD group (90.9 ± 8.65) ($P < 0.05$), whilst the values for the HF-WPI group were not significantly different from those of the LFD group (46.65 ± 3.09).

2.4.3 Energy intake, energy expenditure and respiratory exchange ratio

Study 1: The LFD fed mice were individually housed in the Phenomaster cages during weeks 5 and 6 of the 7 week dietary challenge. In the three day housing period in the Phenomaster cages, the energy intake, VO_2, RER and locomotor activity in the animals were similar on day 2 and 3 of the housing period (supplementary Fig 2.1). We interpreted this data to suggest that by day 2, the mice had been acclimatised to the new Phenomaster cage environment and that data gathered on day 3 represents an accurate measure of their energy intake and metabolic activity. Previous studies have also used a 48 hour period for acclimatisation, before measuring the metabolic and feeding behaviours in mice using similar specialised cages (Czyzyk *et al*, 2010; Dubois *et al*, 2000).

Study 2: Energy intake and metabolic activity were measured as described earlier in weeks 5 and 6 of the 8 week dietary challenge. Energy intake was lower during the dark phase for the HFD group compared to both HF-WPI and LFD fed mice ($P < 0.05$) (Fig 2.3A). The average meal size over the same period was not significantly different between LFD and HFD fed mice, while the HF-WPI diet fed mice had a greater average meal size than both LFD and HFD groups ($P < 0.05$) (Fig 2.3B). The meal number during the dark phase was reduced for both the HFD and HF-WPI groups compared to the LFD fed mice ($P < 0.001$) (Fig 2.3C). No difference in energy intake-related parameters was found between all groups during the light phase (Fig 2.3A-C). The FQ calculated for LFD, HFD and HF-WPI diet are shown in Fig 2.4A. The respiratory exchange ratio (RER) of both the HFD and HF-WPI fed groups were lower than that of the LFD group in both light and dark phases ($P < 0.05$; Fig 2.4A). The VO_2, heat production and locomotor activity of the HFD fed mice during the dark phase were lower than the LFD group ($P < 0.05$; Fig 2.4B-D). In the HF-WPI diet fed mice, these parameters did not differ significantly from that of either the LFD or HFD groups during the dark phase. No differences in the above-mentioned parameters were observed between all groups during the light phase (Fig 2.4B-D).

2.4.4 Adipose tissue gene and protein expression

The mRNA expression of glucose transporter 4 (GLUT4) (Fig 2.5A), insulin receptor (INSR) (Fig 2.5B), beta-3 adrenergic receptor (β-3AR) (Fig 2.5C) and fatty acid synthase (FASN) (Fig 2.5D) was significantly lower in the HFD group than in the LFD fed mice ($P < 0.05$). The mRNA expression of INSR and GLUT4 was increased in HF-WPI diet fed mice compared with the HFD fed mice ($P < 0.05$),

such that the expression was not significantly different from that of the LFD group (Fig 2.5A-B). Similarly, the mRNA expression of β-3AR in the HF-WPI diet group was increased compared to HFD group, but did not reach statistical significance. The mRNA expression of FASN was not affected by the HF-WPI diet (Fig 2.5D). No significant difference in expression of the following genes was found between all three diet groups: PPARγ (1.00 ± 0.17 in LFD vs. 0.93 ± 0.24 in HFD vs. 1.09 ± 0.18 in HF-WPI), lipoprotein lipase (LPL) (1.00 ± 0.16 in LFD vs. 1.37 ± 0.21 in HFD vs. 1.54 ± 0.20 in HF-WPI), carnitine palmitoyltransferase 1b (CPT1b) (1.00 ± 0.15 in LFD vs. 1.24 ± 0.22 in HFD vs. 1.24 ± 0.14 in HF-WPI), fatty acid transporter protein 1 (FATP1) (1.00 ± 0.13 in LFD vs. 0.83 ± 0.13 in HFD vs. 0.93 ± 0.10 in HF-WPI), tumour necrosis factor alpha (TNF-α) (1.00 ± 0.14 in LFD vs. 2.35 ± 0.82 in HFD vs. 1.43 ± 0.31 in HF-WPI) and cluster of differentiation 68 (CD68) (1.00 ± 0.17 in LFD vs. 2.95 ± 1.05 in HFD vs. 1.37 ± 0.28 in HF-WPI).

The association between diet and INSR gene expression was further explored by investigating the protein expression of INSR-β in the epididymal adipose tissue (Fig 2.6). Intake of the HFD decreased the protein expression of INSR-β compared with the LFD group, similar to mRNA expression, although the decreased protein expression did not reach statistical significance (Fig. 2.6). The protein expression of INSR-β did not differ between the HFD and HF-WPI groups (Fig. 2.6).

2.4.5 Hypothalamic gene expression

Given the key central effects on feeding and on adipocyte metabolism (Nogueiras *et al*, 2010; Nogueiras *et al*, 2007), we next focussed our attention on the hypothalamus and investigated whether the diets affected the expression of energy

balance-related genes in this brain region. The data showed that the HFD group had increased mRNA expression of pro-opiomelanocortin (POMC) (Fig 2.7A), leptin receptor (ObR) (Fig 2.7B), INSR (Fig 2.7C), CPT1b (Fig 2.7D), PPARγ (Fig 2.7E) and CD68 (Fig 2.7F) compared to LFD fed mice ($P < 0.001$). Similarly, an increase in mRNA expression for TNF-α was observed with HFD feeding ($P < 0.05$; Fig 2.7G). The mRNA expression of ObR, INSR, CPT1b and TNF-α was significantly lower in the HF-WPI diet fed mice than in the HFD group (Fig 2.7B-D & G) ($P < 0.05$), with no significant difference in expression being observed for POMC and CD68 between the two groups. The mRNA expression of PPARγ in the HF-WPI diet fed mice also showed a trend towards a decrease compared with the HFD values ($P = 0.06$). The observed mRNA expression of ObR, INSR, CPT1b, PPARγ and CD68 was still elevated in the HF-WPI group compared with the LFD group ($P < 0.05$).

2.5 DISCUSSION

Mammals are able to defend perturbations in their body weight and adiposity by a complex interaction between central and peripheral tissues. The intake of a HFD is known to shift the defended body weight to a higher set point through changes in the mechanisms regulating energy balance (Ryan *et al*, 2012). To better understand how WPI may influence this shift in energy balance during an 8 week HFD feeding trial, we compared energy balance-related parameters in LFD, HFD and HFD with WPI (HF-WPI) fed mice at weeks 5 and 6, and at week 8. In agreement with recent findings (Pilvi *et al*, 2009; Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2011), WPI reduced HFD-induced weight gain and adiposity. Interestingly, HF-WPI diet fed mice had increased energy intake but unaltered energy expenditure-related parameters compared with HFD fed mice. WPI also

influenced hypothalamic and adipose tissue gene expression in the latter mice. To our knowledge, the present study is the first to show that WPI has an impact on energy intake by affecting the meal size and also causes changes to hypothalamic cellular activity in HFD fed mice.

High-fat feeding in mice has been shown to differentially impact upon energy intake in a temporal fashion, with no difference observed in weeks 1-4, a significant reduction in energy intake during weeks 5-12, and a progressive increase in energy intake in week 13 onwards compared with the LFD fed controls (Lin *et al*, 2000b). Consistent with these findings, during weeks 5 and 6, we found a reduced energy intake in the HFD fed mice compared to the mice fed the LFD. We further observed that this reduction is due to the dark phase consummatory behaviour, possibly resulting from the reduction in meal number. The data suggests that mice fed the HFD are attempting to regulate their energy intake at weeks 5 and 6 during the diet-induced shift in the body weight (set point). Intake of WPI, in place of casein, increased HFD-induced dark phase energy intake to a level comparable with that observed in the LFD group, probably by increasing the meal size. The differential effects of WPI and casein on HFD-induced energy intake may arise due to differential kinetics of digestion and/or metabolism of the two proteins and the associated neuroendocrine changes, given that whey proteins are known to be digested fast, thus releasing associated bioactive components into the circulatory system much quicker than that observed with casein digestion (Hall *et al*, 2003; Luhovyy *et al*, 2007). Previous studies have shown that WPI reduces energy intake compared to other dietary proteins in the lean state (Yu *et al*, 2009; Zhou *et al*, 2011), with one study suggesting that whey proteins increase intermeal interval compared to soy proteins (Yu *et al*, 2009). In the obese state, the effects of

WPI on energy intake appear to be lost, yet the dietary proteins still attenuate HFD-induced obesity (Pilvi *et al*, 2009; Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2012; Shi *et al*, 2011). The discrepancies between these data and those of the present study, in particular with regard to energy intake, may be related to the differences in the dietary macronutrient and micronutrient composition (45% energy as fat vs. 60% energy as fat; casein vs. soy as the control; and altered calcium content in the diet) and/or the study duration (8 weeks vs. up to 21 weeks) that were used to assess the energy balance impact of whey proteins (McAllan *et al*, 2012). It is also possible that some of these discrepancies may have arisen due to the fact that we subjected the grouped housed mice to a single housing environment in the TSE Phenomaster cages to measure their energy intake. Indeed, individual housing can induce stress in C57BL/6J mice, resulting in a decreased food intake by 3h following exposure to the new environment (Saegusa *et al*, 2011). However, the effect of the novelty stress is lost after 6h in the new environment as the food intake in these mice becomes similar to that of the controls. To minimise any such effect of single housing on physiological parameters in the present study, we allowed mice a 2 day acclimatisation period in the new TSE Phenomaster cages before measuring their energy balance-related parameters. Indeed, our preliminary investigation (Study 1) revealed that on days 2 and 3 of the housing period, the mice consumed a similar energy content and exhibited similar metabolic activity (Supplementary Fig 2.1).

We, as others (Longo *et al*, 2010; Westerterp, 1993) have found an association between FQ, RER and energy balance. In a state of "perfect" energy and nutrient balance (energy homeostasis), the FQ equals the RER. In the present study, the RER was much lower than FQ for all dietary groups during the light

phase. This is expected given that during this phase, mice consume less calories and have reduced metabolic (VO_2 and heat) and locomotor activities compared to the dark phase. The potential mismatch between energy intake and energy expenditure that causes the fat oxidation, as indicated by the RER being lower than FQ, is likely to represent a mechanism to obtain the required energy to sustain metabolic activity in the mice during the light phase (Westerterp, 1993). During the dark phase, the RER of the HFD group was close to the FQ, but the RER of the LFD group was much higher than the FQ. This indicates that for the HFD group, energy intake is closer to energy utilisation, while for the LFD group, a much greater energy intake is required to meet the energy demands of these animals. This suggestion is supported by the data showing that both energy intake and overall metabolic (VO_2 and heat) and locomotor activity in the mice on the HFD was much lower than those observed in the LFD fed mice. The data support the suggestion that mice fed the HFD for 5 to 6 weeks are attempting to regulate their energy supply with their energy demands to maintain their bodyweight, in the similar way to what the LFD fed mice do, but at a set point much higher than the latter group (Ryan *et al*, 2012). Interestingly, during the dark phase, mice on the WPI diet had a RER almost equal to their theoretical FQ, yet the expected association between energy intake and parameters linked to energy expenditure did not exist, as these mice had a much higher energy intake than the HFD fed mice, but had metabolic and locomotor activities similar to those of the HFD fed mice. The present data suggest that WPI does not have a significant impact upon metabolic activity in HFD fed mice. This is not in agreement with the data from another study (Shertzer *et al*, 2011) in which it was shown that HFD fed mice drinking WPI-supplemented water had increased VO_2 levels and mitochondrial

respiration rates compared with the HFD controls. However, unlike in the present study, the two dietary groups in the Shertzer *et al,* (2011) study were consuming disproportionate amounts of protein due to WPI been present in the drinking water in addition to the protein received in the diet. Therefore, the increased metabolic activity observed in the WPI group in the above-mentioned study may simply have resulted from the increased protein intake-induced metabolism rather than due to an effect of protein source. In the absence of an elevated metabolic activity, energy consumed by HF-WPI fed mice in the present study could be utilised for another biological activity, such as muscle metabolism, resulting in an increased total lean mass (%) in the HF-WPI group compared to the HFD group by week 8.

Feeding the HFD for 8 weeks increased overall fat mass compared to LFD feeding, which concurs with previous studies (Chalkley *et al*, 2002; Lin *et al*, 2000b). Feeding WPI to the HFD fed mice decreased overall fat content in the body, with decreased TAG accumulation in the liver, although the statistical significance of this decrease varied depending upon the type of assay kit used to measure the lipid content in the liver or upon the efficiency of the Folch lipid extraction method (Hanson & Lester, 1980). The results are nonetheless consistent with previous findings reported in the literature (Shertzer *et al*, 2011; Shi *et al*, 2011) that WPI reduces liver TAG levels. The effects of the dietary challenges on tissue lipid metabolism were also reflected at the gene expression level. We showed that HFD feeding decreased the mRNA expression of INSR, GLUT4 and FASN in the epididymal adipose tissue compared with LFD feeding. Given the involvement of these genes in adipocyte lipogenesis (Herman *et al*, 2012; Kersten, 2001), the data suggest that a HFD decreases endogenous TAG production in the epididymal

adipose tissue. Interestingly, we as others (Collins *et al*, 1997; Moraes *et al*, 2003; Sawa & Harada, 2006), observed a reduction in the expression of β3-AR in the epididymal adipose tissue with HFD feeding. As β3-AR plays an important role in energy homeostasis due to its impact on lipolysis and thermogenesis (Collins *et al*, 1997; Moraes *et al*, 2003; Sawa & Harada, 2006), it is likely that this reduction in gene expression coupled with the reduced activity of the protein product that is known to occur in HFD fed mice (Collins *et al*, 1997; Moraes *et al*, 2003; Sawa & Harada, 2006) contributes to the increased TAG accumulation and hence the adiposity in these mice. Similarly, the HFD-induced increase expression of lipid responsive PPARγ (Diano *et al*, 2011; Ryan *et al*, 2011) and CPT1b (Bonnefont *et al*, 2004; Gao *et al*, 2009) in the hypothalamus is consistent with the recent findings that the HFD increases fatty acid accumulation in the brain (Borg *et al*, 2012; Posey *et al*, 2009). Thus, the present data suggests that HFD fed mice have greater lipid storage in multiple tissues compared with the LFD fed mice, potentially leading to the shift in their defended body weight to a higher level. The reduction in overall body and hepatic fat content described here with the HF-WPI diet feeding has been reported by other studies, which have shown the effects of WPI on adipocyte cross-sectional area and liver TAG levels in HFD fed mice (Shi *et al*, 2011). In fact, compared with a diet with casein as the protein source, a whey protein diet fed to rats reduced the expression and activity of key lipogenic enzymes in the liver (Morifuji *et al*, 2005). We further suggest that lipid accumulated in the hypothalamus may also be decreased with WPI intake because of the significantly lower hypothalamic CPT1b expression coupled with a trend towards a decreased expression of PPARγ (P = 0.06). These cellular changes occurred in a background of elevated plasma TAG levels, suggesting a reduced

plasma lipid uptake and/or storage in the tissues in HF-WPI diet fed mice compared with HFD controls. The present investigation revealed that WPI does not affect expression of LPL or FATP1 that are involved in fatty acid extraction (Fielding & Frayn, 1998) and transport (Gimeno, 2007) to the epididymal adipose tissue, respectively, although we cannot rule out the possibility that WPI may have affected the protein expression or activity of the above-mentioned genes, causing the HF-WPI fed mice to have elevated plasma TAG but reduced adiposity compared to HFD fed mice. To explore the possibility that WPI may have influenced lipid storage, we investigated genes involved in lipid metabolism within the adipose tissue. The lack of a WPI effect on adipose expression of FASN and CPT1b, may suggest that adipose tissue-specific lipogenesis and β-oxidation pathways (Bonnefont *et al*, 2004) are unaffected by WPI intake. Interestingly, we observed a trend toward an increase in β3-AR expression in epididymal adipose tissue of the HF-WPI fed mice. A longer duration of WPI intake (21 weeks) was found to significantly increase the adipose expression of β3-AR compared to the control HFD group (Pilvi *et al*, 2008). These data suggest the possibility that WPI increases lipolysis in adipocytes via activation of β3-AR, possibly arising as a consequence of effects of the dietary protein on the central nervous system (CNS), given that CNS outflow to the adipose tissue activates β3-AR (McAllan *et al*, 2013; Nogueiras *et al*, 2010; Nogueiras *et al*, 2009; Nogueiras *et al*, 2007).

HFD-induced development of obesity is associated with increased inflammation (Gustafson *et al*, 2009; Harford *et al*, 2011; Isakson *et al*, 2009) and is accompanied by functional peripheral and central resistance to both leptin (Levin & Dunn-Meynell, 2002; Lin *et al*, 2000a; Lin *et al*, 2000b; Munzberg & Myers, 2005; Van Heek *et al*, 1997) and insulin (Chalkley *et al*, 2002; Felig, 1984; Kahn & Flier,

2000; Pedersen *et al*, 1991; Posey *et al*, 2009). Evidence from the present study also suggests that the components of the leptin and insulin signalling pathway acting in the hypothalamus and the adipose may be affected by the HFD. Feeding of the HFD for 8 weeks increased the expression of CD68 and TNF-α in the hypothalamus. In parallel, we observed an increased expression of INSR in the hypothalamus, and a reduced expression of INSR and GLUT4 in the adipose tissue with HFD feeding. These findings are consistent with the suggestion that insulin resistance is partly instigated by an inflammatory response to a HFD (De Souza *et al*, 2005; McGillicuddy *et al*, 2011; Pedersen *et al*, 1991). The increased expression of the leptin receptor and its downstream target POMC in the hypothalamus of HFD fed mice compared to the LFD fed controls, corroborates with previous data (Lin *et al*, 2000a; Nilaweera *et al*, 2007; Townsend *et al*, 2008). The changes in the expression of the leptin receptor may be a homeostatic mechanism attempting to defend against HFD-induced body weight gain, in a background of elevated plasma leptin, hypothalamic inflammation and increased PPARγ expression, which all contribute to central leptin resistance (Lu *et al*, 2011; Ryan *et al*, 2011). The increased hypothalamic expression of genes linked to the leptin and insulin signalling pathways in response to a HFD may thus represent an elevated threshold needed to initiate physiological responses of these hormones (Posey *et al*, 2009; Ryan *et al*, 2012; Van Heek *et al*, 1997). In the epididymal adipose tissue and in the hypothalamus, WPI reversed the HFD-associated effects on the mRNA expression of INSR (both tissues), GLUT4 (adipose) and leptin receptor (hypothalamus), although the expression of INSR-β isoform in the adipose tissue did not differ from that of the HFD group. Differences in INSR mRNA and INSR-β protein expression could have arisen due to effects of the diet on transcription,

translation and/or processing of the mature precursor protein or may also have arisen due to the differences in the sensitivity of the techniques (real-time PCR vs immunoblotting) used to measure expression. Despite these differences, WPI-induced changes in the expression of genes linked to the leptin and insulin signalling pathways were observed to occur in concert with reduced tissue lipid accumulation and potentially lower inflammation, specifically in the hypothalamus, since the expression of TNF-α in the hypothalamus was significantly lower in the HF-WPI diet fed mice compared to the HFD fed mice. The gene expression profile in the WPI-fed mice may thus represent a lower threshold (or higher sensitivity) to leptin and insulin action, which may allow the CNS outflow to the adipose tissue to activate and normalise the expression of β3-AR as that observed in week 8 of the HF-WPI dietary challenge. The investigation of gene expression in specific nuclei within the hypothalamus may allow future investigators to further define the role of this brain region in mediating the effects of WPI intake on the adipose tissue.

In summary, we showed that WPI causes an overall reduction in fat mass in HFD fed mice, normalises their energy intake, possibly by affecting meal size, and influences their hypothalamic and adipose tissue expression of genes linked to leptin and insulin signalling pathways and lipid metabolism. Further work is needed to determine how WPI appears to prevent plasma TAG storage in the tissues and whether this contributes to potentially increased leptin and insulin sensitivity in the hypothalamus and the adipose tissue. Overall, the present data collected at two different time points (at weeks 5/6 and week 8) are consistent with the suggestion that WPI partially or completely reverses the HFD-induced

physiological and cellular changes, which may therefore represent a state of reduced susceptibility to weight gain on a HFD.

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2.7 AUTHOR CONTRIBUTIONS

The authors' contributions are as follows: LM, KNN, JFC, HMR and RK conceived and designed the research; LM performed the research; DK performed plasma analysis; HS performed immunoblot analysis; LM wrote the paper; KNN, JFC, HMR, RK and HS corrected the manuscript. The authors declare that there are no conflicts of interest.

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Chapter 2

TABLES & FIGURES

Table 2.1 Sequences of primers used for Real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
PPARγ	5'-tcaggtttgggcggatgc-3'	5'-tcagcgggaaggactttatgtatg-3'
FASN	5'-tccacctttaagttgccctg-3'	5'-tctgctctcgtcatgtcacc-3'
LPL	5'-tgtctaactgccacttcaaccac-3'	5'-gggcacccaactctcatacattc-3'
GLUT4	5'-ggcctgcccgaaagagtc-3'	5'-aggagctggagcaaaggac-3'
β-3 AR	5'-cgccttcaacccggtcatctactg-3'	5'-ggtggactctgcctggcttcaac-3'
INSR	5'-gatttccccaacgtgtcctctac-3'	5'-caatgcggtacccagtgaagtg-3'
CPT1b	5'-cgagaggggcggactgagactg-3'	5'-ggctaggcggtacatgttttggtg-3'
POMC	5'-gggcaagcgctcctactccatg-3'	5'-ctcgccttccagctccctcttg-3'
ObR	5'-gaccgccgaacacaaccgatgac-3'	5'-acacctagctggcgaaaaactgaag-3'
TNF-α	5'-tggcctccctctcatcag-3'	5'-acttggtggtttgctacgac-3'
CD68	5'-cacttcgggccatgtttctcttg-3'	5'-aggggctggtaggttgattgtcgtc-3'
FATP1	5'-ccggtgtggtggctgctcttctc-3'	5'-gctgccatctccccgccataaatg-3'
β-actin	5'-agagggaaatcgtgcgtgac-3'	5'-caatagtgatgacctggcgt-3'
18-s	5'-aggaccgcggttctattttgttgg-3'	5'-atgctttcgctct-ggtccgtcttg-3'

PPARγ, Peroxisome proliferator activated receptor gamma; FASN, Fatty acid synthase; LPL, Lipoprotein lipase; GLUT4, Glucose transporter 4; β-3 AR, Beta-3 adrenergic receptor; INSR, Insulin receptor; CPT1b, Carnitine palmitoyltransferase 1b; POMC, Pro-opiomelanocortin; ObR, Leptin receptor; TNF-α, Tumour necrosis factor alpha; CD68, Cluster of differentiation 68; FATP1, Fatty acid transporter 1.

Table 2.2 Plasma levels of hormones and TAG, and hepatic TAG accumulation (Mean values with standard error of the means; n = 7-8)

Group	Low fat		High fat		High fat WPI	
	Mean	SEM	Mean	SEM	Mean	SEM
Leptin (ng/ml)	3.07[a]	0.34	33.78[b]	6.27	18.79[b]	5.05
GLP-1 (pM)	6.63	0.28	6.00	0.13	6.15	0.32
Insulin (ng/ml)	0.21	0.01	0.31	0.04	0.26	0.04
Glucose (nmol/µl)	10.65	1.40	11.60	1.00	12.18	0.49
HOMA-IR	2.67	0.39	4.17	0.62	3.46	0.32
Plasma TAG (mg/dl)	94.93[a]	7.54	112.70[a]	5.18	146.47[b]	7.73
Liver TAG (mg/g liver)	48.61[a]	5.55	91.48[b]	20.06	59.35[ab]	5.29

WPI, Whey protein isolate; GLP-1, Glucagon-like peptide 1; TAG, Triacylglycerol; HOMA-IR, homeostatic model assessment-insulin resistance.

[a,b] Mean values in each row with different superscript letters were significantly different ($P < 0.05$).

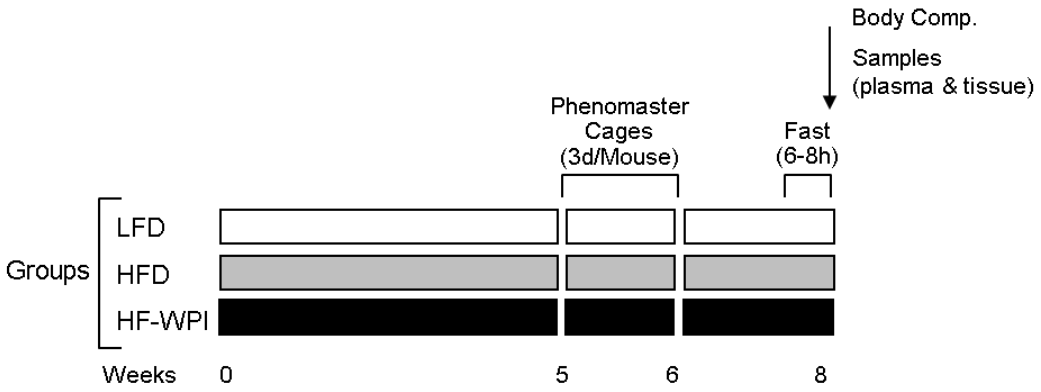


Figure 2.1 Timeline of dietary treatments and experimental measurements. C57BL/6J mice were fed for 8 weeks with diets containing 10% energy as fat (low fat diet, LFD) or 45% energy as fat (high fat diet, HFD) with either 20% energy as casein (LFD and HFD) or whey protein isolate (WPI) (HF-WPI). To measure metabolic parameters at weeks 5 to 6, mice were individually housed in TSE phenomaster cages for 3 days with data being collected in the final 24h of the housing period. In week 8, the body composition was measured following a 6-8 h fast, and then plasma and tissue samples were isolated.

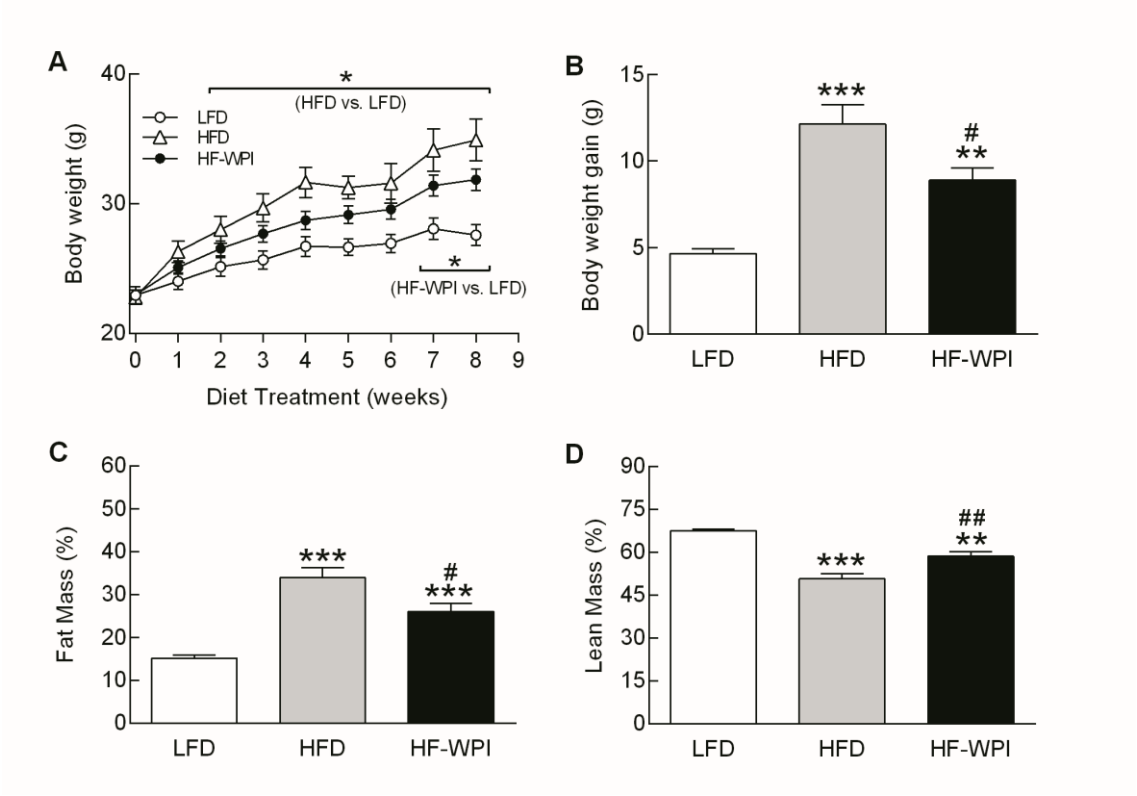


Figure 2.2 Effect of feeding a low-fat diet (LFD, —○—), a high-fat diet (HFD,—△—) or a HFD with whey protein isolate (HF-WPI, —●—) for 8 weeks upon (A) body weight, (B) body weight gain, (C) total fat mass (%) and (D) total lean mass (%) in C57BL/6J mice. Values are means (n = 8 per group), with standard errors represented by vertical bars. Mean value was significantly different from that of the group fed the LFD diet: $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. Mean value was significantly different from that of the group fed the HFD diet: $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$.

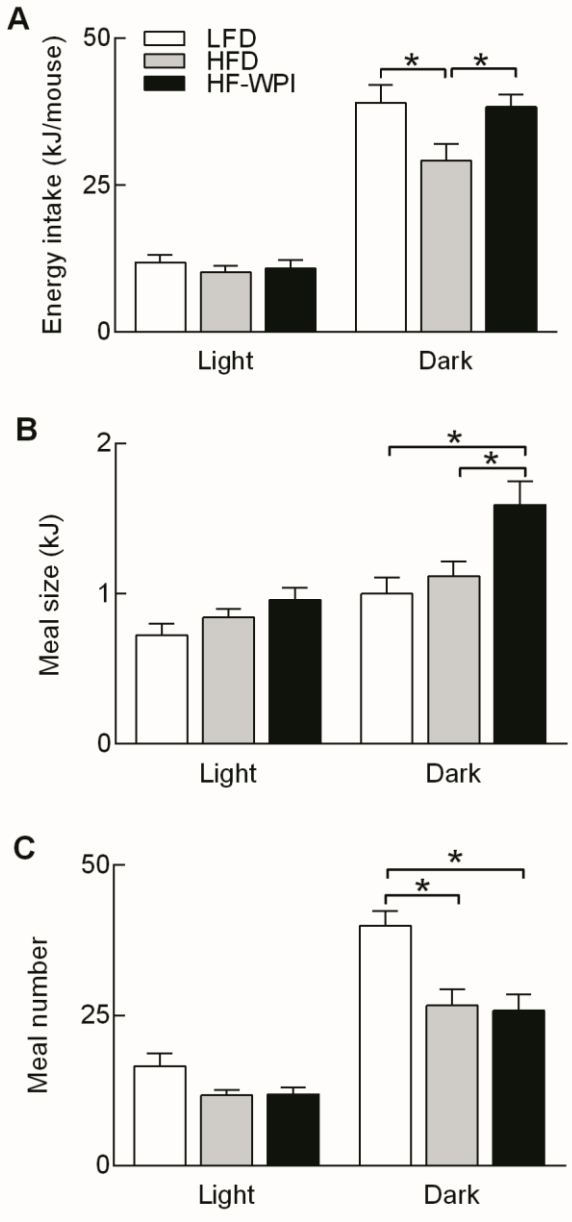


Figure 2.3 Effect of feeding a low fat diet (LFD), high fat diet (HFD) or HFD with whey protein isolate (HF-WPI) for 5 to 6 weeks upon (A) energy intake (B) meal size and (C) meal number in C57BL/6J mice. Experimental data, collected from individual mice at 9 minute intervals over a 24 hour period using TSE phenomaster cages, are shown for light and dark phases. Values are means (n = 8 per group), with standard errors represented by vertical bars: * $P < 0.05$.

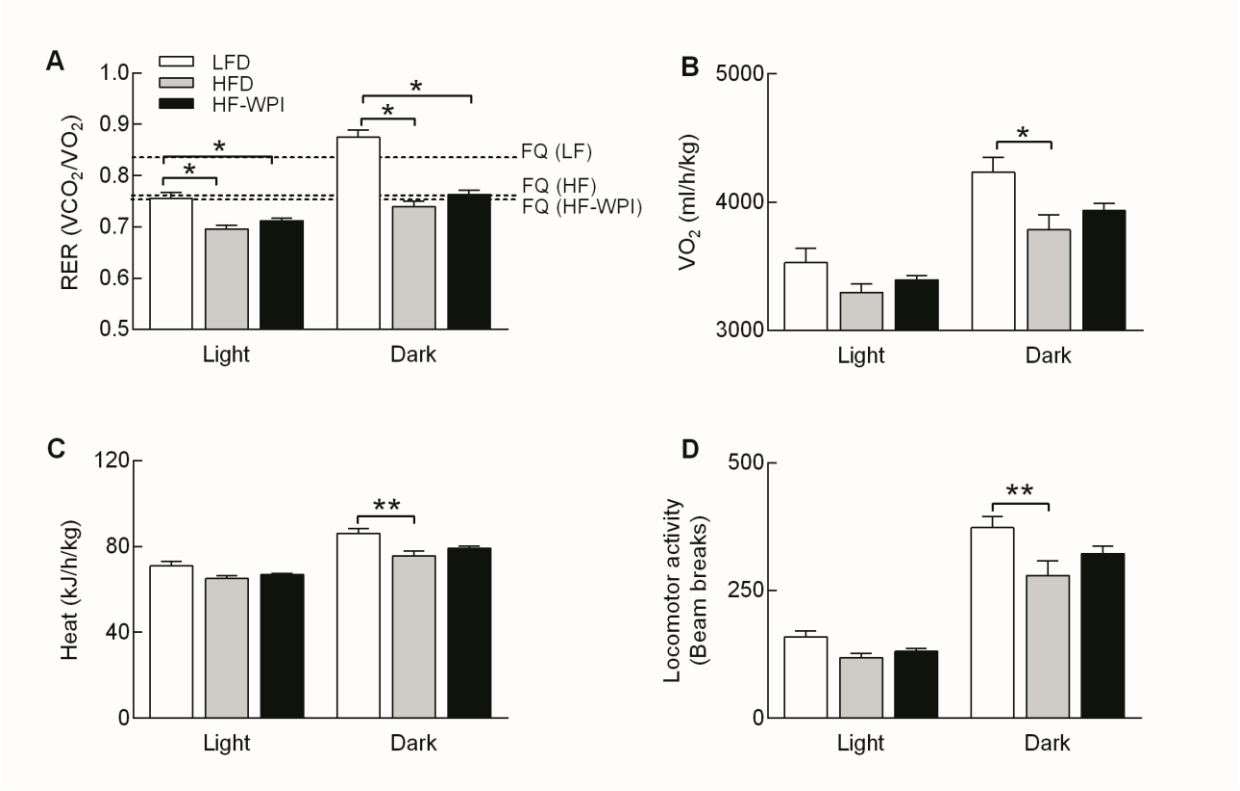


Figure 2.4 Effect of feeding a low fat diet (LFD), high fat diet (HFD) or HFD with whey protein isolate (HF-WPI) for 5 to 6 weeks upon (A) respiratory exchange ratio (RER), (B) oxygen consumption (VO_2), (C) heat production and (D) locomotor activity in C57BL/6J mice. Experimental data, collected from individual mice at 9 minute intervals over a 24 hour period using TSE phenomaster cages, are shown for the light and dark phases. The food quotient (FQ; -----) used in (A) is defined as the ideal diet-specific VCO_2:VO_2 ratio, and was calculated for each diet as described previously (Longo *et al*, 2010). Values are means (n = 8 per group), with standard errors represented by vertical bars: * $P < 0.05$, ** $P < 0.01$.

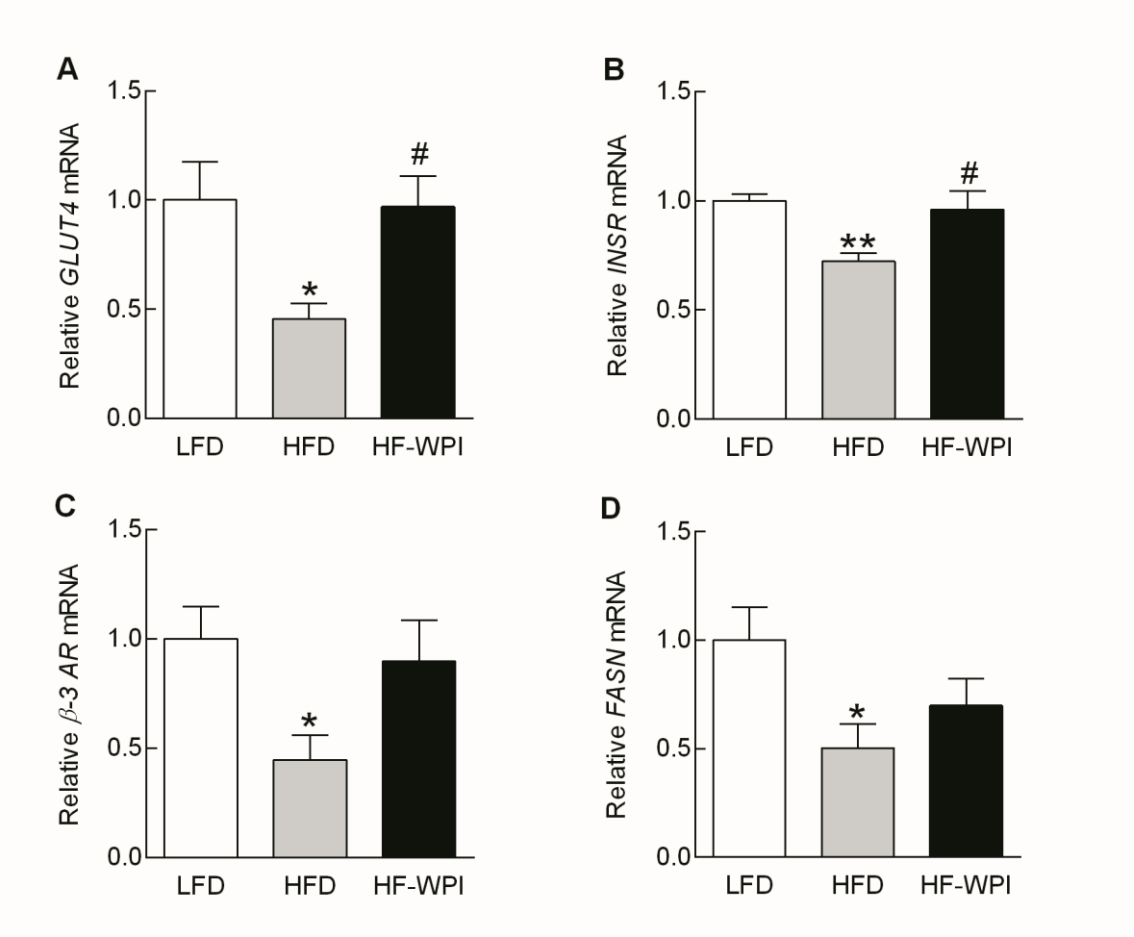


Figure 2.5 Effect of feeding a low fat diet (LFD), high fat diet (HFD) or HFD with whey protein isolate (HF-WPI) for 8 weeks upon epididymal adipose tissue mRNA expression of (A) glucose transporter 4 (GLUT4), (B) insulin receptor (INSR) (C) beta-3 adrenergic receptor (β-3AR) and (D) fatty acid synthase (FASN) in C57BL/6J mice. The mRNA expressions were normalised using 18-S and β-actin according to $\Delta\Delta C_p$ = ΔC_p target gene – ΔC_p housekeeping gene. The gene expressions were calculated using $2^{-\Delta\Delta Cp}$ and are shown in comparison to that of the LFD group. Values are means (n = 7-8 per group), with standard errors represented by vertical bars. Mean value was significantly different from that of the LFD diet group: $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. Mean value was significantly different from that of the HFD diet group: $^{\#}P < 0{\cdot}05$.

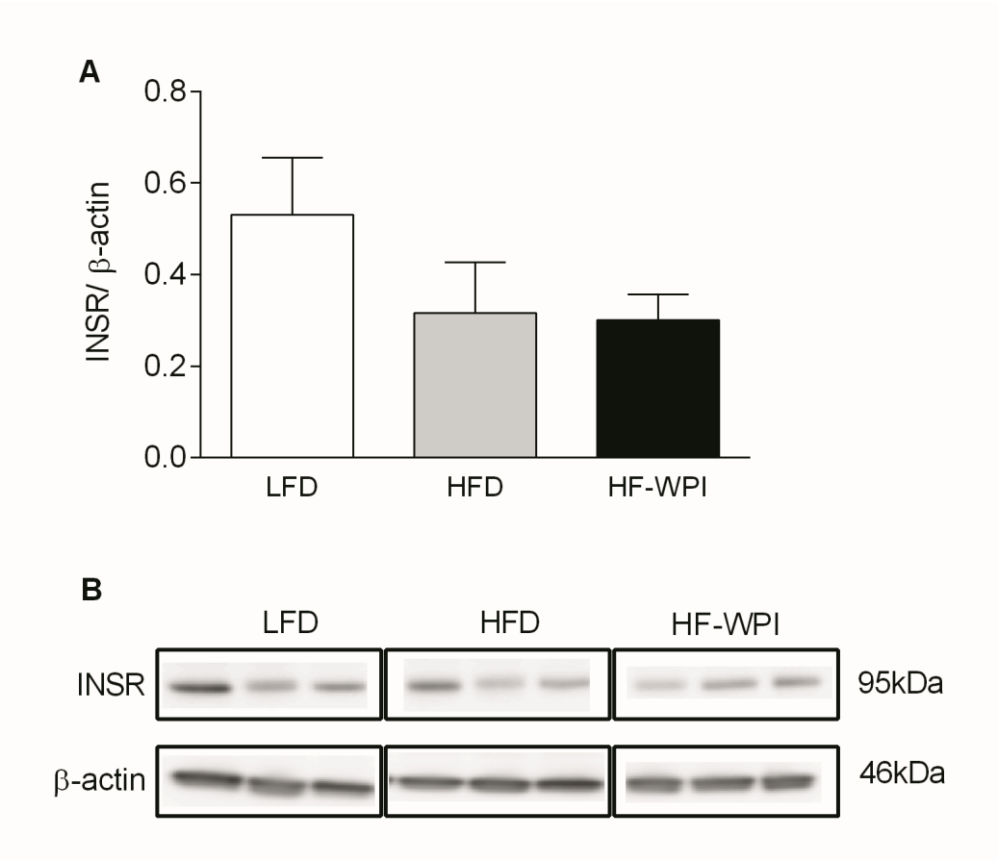


Figure 2.6 Effect of feeding a low fat diet (LFD), high fat diet (HFD) or HFD with whey protein isolate (HF-WPI) for 8 weeks upon epididymal adipose tissue protein expression of insulin receptor-β (INSR-β) in C57BL/6J mice, as measured by immunoblot. The protein expression of INSR-β relative to the expression of β-actin is shown in (A) and the images of the corresponding immunoblot are shown in (B). Values are means (n = 3 per group), with their standard errors represented by vertical bars.

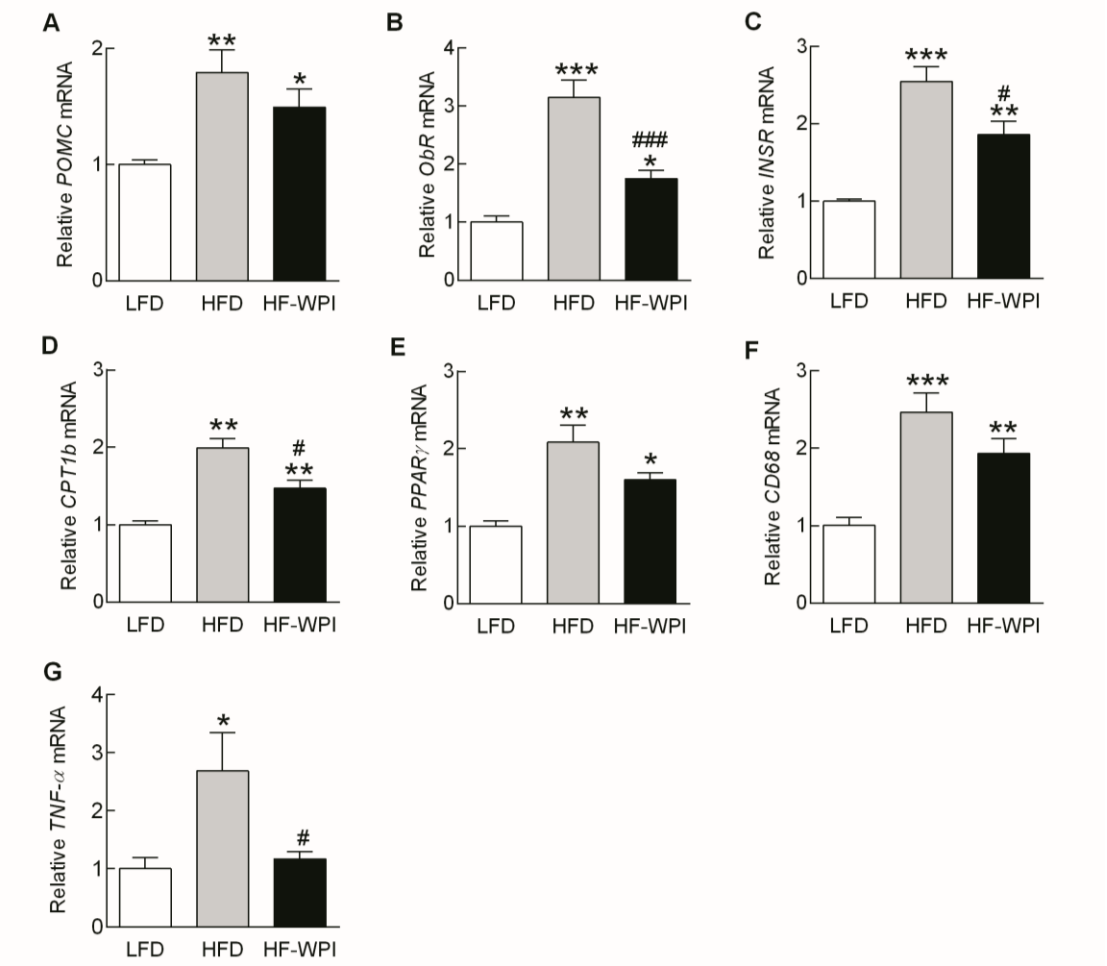
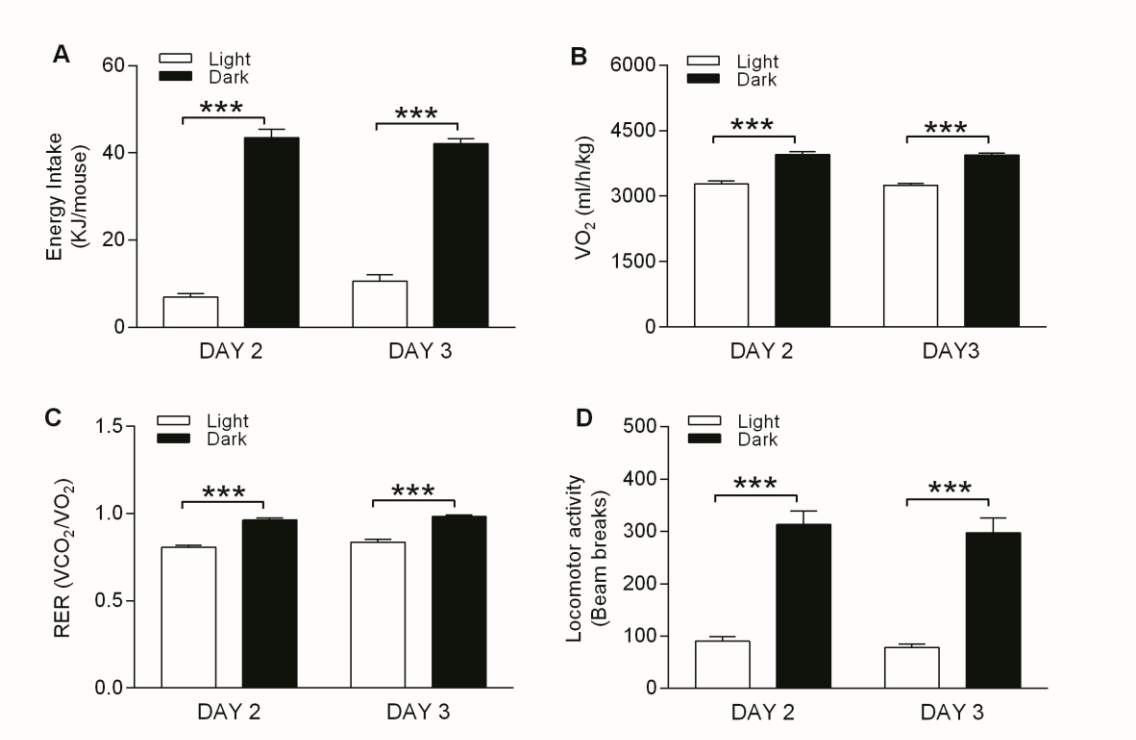


Figure 2.7 Effect of feeding a low fat diet (LFD), high fat diet (HFD) or HFD with whey protein isolate (HF-WPI) for 8 weeks upon hypothalamic mRNA expression of (A) pro-opiomelanocortin (POMC), (B) leptin receptor (ObR), (C) insulin receptor (INSR), (D) carnitine palmitoyltransferase 1b (CPT1b), (E) PPARγ , (F) cluster of differentiation 68 (CD68) and (G) TNF-α, in C57BL/6J mice. The mRNA expressions were normalised using 18-S and β-actin according to $\Delta\Delta C_p = \Delta C_p$ target gene – ΔC_p housekeeping gene. The relative gene expressions were calculated using $2^{-\Delta\Delta Cp}$ and are shown compared to the LFD group. Values are means (n = 6-8 per group), with their standard errors represented by vertical bars. Mean value was significantly different compared to LFD group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; and significantly different compared to HFD group: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

Chapter 2

SUPPLEMENTARY INFORMATION



Supplementary figure 2.1 The effect of feeding a low fat diet for 5-6 weeks on (A) energy intake, (B) oxygen consumption (VO_2), (C) respiratory exchange ratio (RER) and (D) locomotor activity in C57BL/6J mice. Metabolic parameters were measured in mice (n = 8) housed individually for 3 days in the TSE phenomaster cages. Experimental data collected at 9 minute intervals are shown for light and dark phases of days 2 and 3. Data represent mean values ± SEM (n = 8). *** $P <$ 0.001.

Chapter 3

Protein quality and the protein to carbohydrate ratio within a high fat diet influences energy balance and the gut microbiota in C57BL/6J mice

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3.1 ABSTRACT

Macronutrient quality and composition are important determinants of energy balance and the gut microbiota. Here, we investigated how changes to protein quality (casein versus whey protein isolate; WPI) and the protein to carbohydrate (P/C) ratio within a high fat diet (HFD) impacts on these parameters. Mice were fed a low fat diet (LFD; 10% kJ) or a high fat diet (HFD; 45% kJ) for 21 weeks with either casein (20% kJ) or WPI at 20%, 30% or 40% kJ. In comparison to casein, WPI at a similar energy content normalised energy intake, increased lean mass and caused a trend towards a reduction in fat mass (P = 0.08), but the protein challenge did not alter oxygen consumption or locomotor activity. WPI reduced HFD-induced plasma leptin and liver triacylglycerol, and partially attenuated the reduction in adipose FASN mRNA in HFD-fed mice. High throughput sequence-based analysis of faecal microbial populations revealed microbiota in the HFD-20% WPI group clustering closely with HFD controls, although WPI specifically increased *Lactobacillaceae/Lactobacillus* and decreased *Clostridiaceae/Clostridium* in HFD-fed mice. There was no effect of increasing the P/C ratio on energy intake, but the highest ratio reduced HFD-induced weight gain, fat mass and plasma triacylglycerol, non-esterified fatty acids, glucose and leptin levels, while it increased lean mass and oxygen consumption. Similar effects were observed on adipose mRNA expression, where the highest ratio reduced the HFD-associated expression of UCP-2, TNFα and CD68 and increased the diet-associated expression of β3-AR, LPL, IR, IRS-1 and GLUT4. The P/C ratio also impacted on gut microbiota, with populations in the 30/40% WPI groups clustering together and away from the 20% WPI group. Taken together, our data show that increasing the P/C ratio

has a dramatic effect on energy balance and the composition of gut microbiota, which is distinct from that caused by changes to protein quality.

3.2 INTRODUCTION

It is widely recognised that levels of obesity and related clinical conditions such as diabetes, stroke, hyperlipidemia and cardiovascular disease are increasing worldwide (Caterson *et al*, 2004). Importantly, the development of obesity increases the set point at which the body weight, and more specifically body fat, is defended, thus making its reversal difficult to achieve (Guo *et al*, 2009; Ryan *et al*, 2012). As such, there is an increased research interest to develop effective treatments for this disease.

Dairy proteins belonging to the whey fraction (a by-product of cheese manufacture) have been increasingly tested for their potential anti-obesity effect, and specifically for their ability to reduce high fat diet (HFD)-associated body weight and fat mass gain (Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2011). Shi *et al.,* (2012) showed that replacing 5%, 50% or 100% of the dietary casein protein-derived energy content with a lactoperoxidase and lactoferrin-enriched whey protein isolate (WPI) caused a proportional suppression of body weight gain in HFD fed mice. We have previously demonstrated that a WPI-related reduction in body weight and fat mass gain in HFD fed mice was accompanied by a normalisation of energy intake and the complete or partial reversal of energy balance-related gene expression in the adipose tissue and the hypothalamus (McAllan *et al*, 2013b). While these data suggest that whey proteins have specific-effects on energy balance, such effects appear be modified by the macronutrient composition in the diet (Pichon *et al*, 2008). In the latter study, it was shown that

increasing the lipid to carbohydrate ratio within a whey protein-rich diet significantly reduced energy intake and bodyweight gain in rats. Collectively, these data suggest that protein quality and macronutrient composition are important determinants of energy balance.

Interestingly, diet is also an important factor in determining the composition of the gut microbiota (Turnbaugh *et al*, 2009; Wu *et al*, 2011) and specific gut microbiota signatures are associated with obesity phenotypes in animals and humans (Backhed *et al*, 2004; Murphy *et al*, 2010; Turnbaugh *et al*, 2006). Notably, studies have shown specific whey proteins to possess anti-microbial activity (Freedman *et al*, 1998; Shin *et al*, 2000; Yamauchi *et al*, 1993), and that the digestive process itself facilitates the formation of potent antimicrobial whey-derived peptides, such as pepsin catalysed lactoferrin to lactoferricin (Hoek *et al*, 1997). A study by Sprong *et al.,* (2010) demonstrated that in comparison to casein, whey protein intake increased levels of *Lactobacilli* and *Bifidobacteria* in a rat model of colitis. However, in a more recent study whey protein intake was found to have no influence on gut microbiota composition in mice fed a HFD for 7 or 13 weeks (Tranberg *et al*, 2013). Key unanswered questions are; could whey proteins specifically influence the gut microbiota composition associated with prolonged high fat feeding, and would any changes relate to energy balance? Could changes to the protein to carbohydrate ratio within a HFD vary the gut microbiota profile and energy balance in a different way to changes to protein quality?

To assess WPI specific effects on above parameters, we subjected male C57BL/6J mice to 21 weeks of either a low fat diet (LFD) with 20% kJ casein or a HFD with 20% kJ casein or WPI. In addition, using two additional HFD dietary

groups containing 30 or 40% kJ WPI, we evaluated the impact of increasing the protein to carbohydrate (P/C) ratio within the HFD on parameters of interest. The present data showed that WPI had a specific effect on energy balance and gut microbiota, while increasing the P/C ratio within the HFD led to dramatic alterations in energy balance, body composition, metabolic health and the composition of the gut microbiota.

3.3 MATERIALS AND METHODS

3.3.1 Ethics Statement

All research involving mice was licensed under the Cruelty to Animal Act 1876 and received ethical approval from the University College Cork Animal Ethics Review Committee (#2011/005).

3.3.2 Animals

Male 3-4 week old C57BL/6J mice (Harlan, Oxon, UK) were group housed either 5 per cage (Study 1) or 4 per cage (Study 2) in individually ventilated cages and acclimatised for four weeks in a light (06:00-18:00), temperature (21 ± 1^{o}C) and humidity (45-65%) controlled environment with free access to water and a low fat diet (LFD; 10% kJ fat and 20% kJ casein; #D12450, Research diets; New Brunswick, NJ, USA).

3.3.3 Experimental protocol

Two studies were performed to assess how the WPI-derived energy content within a HFD (Study 1) or LFD (Study 2) impacts on energy balance-related parameters in mice over a 21 week (Study 1) or 7 week period (Study 2).

Study 1: Following the acclimatisation period, weight-matched dietary groups were maintained on the LFD or switched to either a HFD (45% kJ fat and 20% kJ casein; #D12451) or a HFD with WPI (Alacen^{tm} 895 NZMP, New Zealand) at an energy content of 20% kJ (HFD-20% WPI), 30% kJ (HFD-30% WPI) or 40% kJ (HFD-40% WPI) (Supplementary table 1) (n = 10) for a total of 21 weeks. Body weights were measured weekly. Energy intake in group housed mice was measured by weighing the food hopper each week until week 16. During weeks 17-20, energy intake and metabolic activity in individual mice was measured using TSE Phenomaster cages (TSE systems, Bad Homburg, Germany). Following this analysis and prior to re-housing the mice in their home cages, faecal pellets were collected from individual mice for examination of the microbial composition via pyrosequencing and subsequent bioinformatic analysis. At the end of the experimental period, mice were fasted for 6 hours and the body composition was measured by NMR using the Bruker minispec LF50H (Bruker optics, Ettlingen, Germany). The mice were then anesthetised using ketamine (65mg/kg bodyweight) and xylazine (13mg/kg bodyweight). Blood was collected from these anesthetised mice into vacutinater EDTA tubes (BD, USA) and treated with Aprotinin (500,000 KIU/L final concentration; Sigma, Ireland) and Diprotin A (0.1mM final concentration; Sigma, Ireland) to protect plasma peptides from proteolytic degradation. Plasma was isolated from blood by centrifugation at 2000rpm and 4^{o}C for 15 minutes. The mice were then sacrificed by cervical dislocation, and tissues of interest were dissected and snap frozen in liquid nitrogen (liver, adipose and stomach) or on dry ice (brain). Plasma and tissue samples were stored at -80^{o}C until analysis.

Study 2: Weight matched mice were provided with either the LFD or a LFD with WPI replacing the casein protein (LFD-WPI; 10% kJ fat and 20% kJ WPI) (n = 8) for 7 weeks. Body weights were measured weekly. Energy intake and metabolic activity in individual mice was measured during weeks 5 and 6 using the TSE Phenomaster system. After the analysis, mice were re-housed, as before, in the home cages and the experiment was terminated at the end of week 7.

3.3.4 Analysis of metabolic parameters

The TSE Phenomaster cages comprised an open-circuit indirect calorimetry system with gas sensing units to measure oxygen consumption (ml/h/kg) (VO_2) and CO_2 production (ml/h/kg) (VCO_2). The cages also contained high-precision sensor-associated feeding baskets to accurately measure food intake (g), with a meal defined as intake over 0.01g. A multi-dimensional infrared beam system allowed the measurement of locomotor activity, which was defined as the total number of infrared beam breaks in the *X*- and *Y*-axis. The mice were singly housed in TSE Phenomaster cages for a total of 3 days, with data collected during the final 24 hours, following a 2 day acclimatisation to the new cage environment. The acclimatisation period was established based on the data from our previous study (McAllan *et al*, 2013b). Heat production (kcal/h/kg) in individual mice was calculated using the Weir equation ($3.941 \times VO_2 + 1.106 \times VCO_2$)(Weir, 1949), and this was converted to kJ/h/kg using 1kcal = 4.184 kJ. The respiratory exchange ratio (RER) was calculated by VCO_2/VO_2. Energy intake was calculated from food intake measurements using the energy content of the diets supplied by the manufacturer.

3.3.5 Microbial DNA extraction, amplification and high throughput DNA sequencing

Total metagenomic DNA was extracted from individual faecal samples using QIamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), after an additional bead-beating step. Bacterial composition was determined by sequencing of 16S rRNA amplicons (V4-V5 region; 408nt long) generated by a separate PCR reaction for each sample (in triplicate) using universal 16S primers, where, the forward primer (5'-AYTGGGYDTAAAGNG) with attached molecular identifier tags between 454 adapter sequence and target-specific primer sequence, and the reverse primer V5 (5'-CCGTCAATTYYTTTRAGTTT) (Claesson *et al*, 2010), were used along with Biomix Red (Bioline, London UK). The template DNA was amplified under the following PCR conditions for a total of 35 cycles: 94˚C for 2 minutes and 1 minute respectively (initialization and denaturation), 56˚C for 60 seconds (annealing) and 72˚C for 60 seconds (elongation), proceeded by a final elongation stage of 2 minutes. Negative control reactions with PCR grade water in place of template DNA were used to confirm a lack of contamination. Amplicons were pooled and cleaned using the AMPure XP purification system (Beckman and Coulter, Takeley, UK) and DNA concentration was determined using the NANODROP 3300 Fluorospectrometer (Thermo Scientific, USA) coupled with the Quant-it™ Picogreen® dsDNA Assay Kit (Invitrogen, Paisley, UK). Equal volumes of each sample were then pooled together and underwent a final cleaning and quantification stage. Amplicons were sequenced in-house on a Roche GS FLX Titanium platform.

3.3.6.Bioinformatics

Raw sequencing reads were 'de-noised' using traditional techniques implemented in the Ribosomal Database Project Pyrosequencing (RDP) Pipeline with ambiguous bases, non exact primer matches and reads shorter than 150bp being excluded. Trimmed FASTA files were then BLASTed against a previously published 16S-specific database using default parameters. The resulting files were then parsed using the MEGAN software package, which assigns reads to the National Centre for Biotechnology Information (NCBI) taxonomies via the lowest common ancestor algorithm. Results were filtered prior to tree construction and summarisation by the use of bit scores from within MEGAN where a cut-off bit score of 86 was employed (Rea *et al*, 2011; Urich *et al*, 2008). The QIIME software suite was employed to achieve clustering of sequence reads into operational taxonomic units (OTUs) (Caporaso *et al*, 2010). Chimeric OTUs were removed using the ChimeraSlayer program (Haas *et al*, 2011) and phylogenetic trees were constructed using the FastTreeMP tool (Price *et al*, 2010). Beta diversity values were calculated based on Bray Curtis, weighted and unweighted UniFrac distances, and the KING viewer was used to visualise resulting PCoA plots (Chen *et al*, 2009; Huson *et al*, 2007). Sequence reads were deposited in the European Nucleotide Archive (EHA) under the accession number PRJEB4636.

3.3.7 Plasma analysis

Colorimetric assays were used to measure plasma levels of glucose (Calibochem, Darmstadt, Germany), triacylglycerol (TAG; Wako Chemicals, Richmond, VI, USA) and non-esterified fatty acids (NEFA; Abcam, Cambridge, UK). Commercially available ELISA kits were used to analyse plasma levels of insulin, leptin (Crystal Chem, Downers Grove, IL, USA), glucagon-like peptide 1 (GLP-1; Millipore, St.

Charles, MO, USA) and corticosterone (Enzo Life sciences, Farmingdale, NY, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was determined using the formula: fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/L)/ 22.5 (Matthews *et al*, 1985). To measure plasma amino acid levels, samples were first deproteinised by mixing with equal volumes of 24% (w/v) tri-chloroacetic acid. The samples were then allowed to stand for 10 minutes before been centrifuged at 14400 x g (Microcentaur, MSE, UK) for 10 minutes. Supernatants were mixed with 0.2 M sodium citrate buffer, pH 2.2, and the plasma concentration of amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, UK) fitted with a Jeol Na^+ high performance cation exchange column.

3.3.8 Liver TAG analysis

Total lipids from liver samples (approx. 50mg) were extracted as described previously (McAllan *et al*, 2013b) using the Folch extraction method (Folch *et al*, 1957). Briefly, total lipids were extracted using a 2:1 (v/v) chloroform-methanol solution, into which a 0.88% NaCl solution was added before centrifugation at 2000rpm and 4^{o}C for 30mins. Aliquots of the organic phase were collected, dried and re-suspended in the LabAssay TAG reagent (Wako Chemicals, Richmond, VI, USA) to measure TAG levels using the LabAssay TAG kit according the manufacturer's protocol.

3.3.9 Real-Time PCR analysis

Total RNA was isolated from tissues using RNeasy mini (liver and stomach) or RNeasy lipid mini (adipose and hypothalamus) kits (Qiagen, Hilden, Germany)

according to manufacturers' instructions. RNA was treated with DNase (Qiagen, Hilden, Germany) during RNA isolation to eliminate any potential genomic DNA contamination. The reverse transcription of 1µg of RNA into complementary DNA (cDNA) was performed using 2.5ng/µl random hexamer primers (Bioline, London, UK), 0.5mM dNTP (Promega, Madison, VI, USA), 2U/µl RNase inhibitor (Promega, Madison, VI, USA), and the Superscript II first stand system (Invitrogen, Carlsbad, CA, USA) according to the manufactures' instructions. Gene expression was measured by the Roche Lightcycler 480 system (Rotkreuz, Switzerland) via amplification of 1µl cDNA using the Lightcycler SYBR Green I Mastermix kit (Roche, Penzberg, Germany) and 2.5µM gene-specific primers (Eurofins MWG operon, Ebersberg, Germany) in a 10µl total reaction volume. Primer sequences used are listed in supplementary table 2. PCR conditions were; 10mins at 95^{o}C, followed by 50 cycles of 95^{o}C for 10s, 58-65^{o}C for 5s and 72^{o}C for 15s. Authenticity of PCR products was determined by melting curve analysis and by automated sequencing. Crossing point (Cp) of fluorescence signals were used to calculate target gene expression by $2^{-\Delta\Delta Cp}$, following normalisation against housekeeping genes according to ΔΔCp = ΔCp target gene – ΔCp housekeeping gene. Housekeeping genes used were β-actin (liver, stomach and hypothalamus), YWHAZ (liver and hypothalamus), 18-S (adipose) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (adipose and stomach). Relative gene expression is shown in comparison to the LFD group.

3.3.10 Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between experimental dietary groups were analysed by one-way or two-way

ANOVA followed by pairwise comparisons using *tukey* or *bonferroni* post hoc tests, respectively. Body weight trajectories were analysed by two-way repeated measures ANOVA with *bonferroni* post hoc tests. Non-parametric data was compared by Kruskal-Wallis ANOVA followed by *Dunn's* pairwise comparisons. Significance levels were set at $P \leq 0.05$, and statistical analysis was performed using Graphpad prism (ver. 5.04; San Diego, CA, USA) and Minitab (ver.15; State College, PA, USA).

3.4 RESULTS

3.4.1 WPI inclusion or increasing the P/C ratio within a HFD alters body composition and plasma amino acids

Fig. 3.1A-B demonstrates that body weight gain of HFD-20% WPI fed mice was similar to HFD controls. However, intake of WPI was seen to have a specific effect on body composition, with HFD-20% WPI fed mice having an increased lean mass (%) ($P < 0.05$), and a trend towards a reduction in fat mass (%) ($P = 0.08$) compared to the HFD control group (Fig. 3.1C). Increasing the WPI derived energy content in the HFD to 40% and proportionally reducing the carbohydrate energy content led to a significant reduction in body weight gain compared to all other HFD-WPI groups ($P < 0.001$), with observed values similar to that seen for the LFD group. This was accompanied by significantly reduced body fat mass and increased lean mass levels in the HFD-40% WPI fed mice compared to HFD control and other WPI diets groups ($P < 0.001$), while the body composition of the HFD-30% WPI group did not differ from that of the HFD-20% WPI group (Fig. 3.1C).

Comparison of the plasma amino acid profiles, including those that could influence lean and fat mass, revealed an impact of WPI and the P/C ratio (Table

3.1). WPI specific effects were observed on glutamic acid, aspartic acid and glycine, which either decreased (glutamic acid and aspartic acid) or increased (glycine) compared to HFD fed mice ($P < 0.05$) (Table 3.1). Changes in macronutrient ratio in HFD-40% WPI decreased plasma histidine, phenylalanine, serine and threonine levels compared to the lowest P/C ratio (20% WPI) ($P < 0.01$) (Table 3.1).

3.4.2 The WPI-enriched HFD normalised energy intake, while increasing the P/C ratio accentuated metabolism

The cumulative energy intake (MJ) for the dietary groups (2 cages/group, all with n = 5 mice), measured over the first 16 weeks did not significantly differ between LFD, HFD and HFD-20% WPI groups (24.73 ± 2.70 vs. 27.69 ± 1.54 vs. 29.40 ± 0.31, respectively). In contrast, data gathered by individually housing the mice in TSE Phenomaster cages in weeks 17-20 demonstrated that the energy intake of the HFD-WPI groups was greater than that of the HFD control group during both the light and dark phases ($P < 0.05$), while being similar to that of the LFD group (Fig. 3.2A). Increasing the P/C ratio had no significant effect on cumulative energy intake (MJ) in HFD fed mice (20% WPI, 29.40 ±0.31 vs. 30% WPI, 30.01 ± 0.62 vs. 40% WPI, 26.71 ± 0.15). Energy intake measurements from TSE Phenomaster cages corroborated this data (Fig. 3.2A). There was also no significant effect on meal number or meal size of altering the P/C ratio (i.e. between WPI groups; Fig. 3.2B-C).

The HFD-20% WPI diet had no impact on VO_2, heat production, locomotor activity or respiratory exchange ratio (RER) when compared to HFD fed mice (Fig. 3.3A-D). Increasing the P/C ratio was found to impact on energy expenditure with HFD-40% WPI fed mice having significantly increased levels of dark phase VO_2

compared to HFD-20% and 30% WPI fed mice ($P < 0.001$) (Fig. 3.3A). A similar change in heat production was observed between the groups, albeit data was only significant between HFD-40% and HFD-30% WPI groups ($P < 0.05$) (Fig. 3.3B). There was no effect of WPI or P/C ratio on locomotor activity (Fig. 3.3C). RER values of all HFD groups were lower than the LFD group in both the light and dark phases, consistent with increased fat metabolism ($P < 0.001$) (Fig. 3.3D).

Investigation of the above parameters in mice fed a LFD with WPI or casein for 7 weeks (Study 2) revealed that WPI does not influence body weight, energy intake, VO_2, locomotor activity or RER in a low fat background (Supplementary Fig. 3.1A-D).

3.4.3 Increasing the protein to carbohydrate ratio attenuated the adverse metabolic impact of HFD

Specific effects of WPI and the P/C ratio were observed on lipid metabolism-related gene expression and on tissue lipid deposition. Firstly, the decrease in epididymal adipose tissue fatty acid synthase (FASN) mRNA expression with HFD feeding, was somewhat attenuated by WPI challenge ($P < 0.05$), with no added benefit of increasing the P/C ratio on the expression of this gene (Fig. 3.4A). Notably, the epididymal mRNA expression of a number of other genes were altered by the P/C ratio, specifically, fatty acid transporter 1 (FATP1), beta-3 adrenergic receptor (β3-AR), peroxisome proliferator-activated receptor gamma (PPARγ), uncoupling protein 2 (UCP-2) and lipoprotein lipase (LPL) ($P \leq 0.05$) (Fig. 3.4A-B). In the liver, WPI specifically reduced TAG levels (Table 3.2) and the mRNA expression of fatty acid binding protein 1 (FABP1) compared to HFD fed mice (Table 3.3). The highest P/C ratio (40% WPI) significantly decreased mRNA levels

of cluster of differentiation 36 (CD36) and PPARγ ($P < 0.05$) (Table 3.3), dramatically reduced liver TAG levels compared to 20/30% WPI fed mice (Table 3.2), and normalised the elevated plasma levels of TAG and NEFA observed with HFD feeding ($P \leq 0.05$) (Table 3.2). Finally, the mRNA levels of lipid metabolism-related carnitine palmitoyltransferase 1a-c (CPT1a-c), fatty acid transport protein 5 (FATP5) and PPARα, in tissues of interest, were not influenced by the dietary challenges (Fig.3.4A & Tables 3.3-3.4).

Increasing the P/C ratio reduced plasma glucose levels, particularly in the 40% WPI group ($P < 0.05$) (Table 3.2). In parallel HOMA-IR values were also reduced ($P < 0.05$), but the change in plasma insulin concentration did not reach statistical significance (Table 3.2). At a cellular level, the highest P/C ratio normalised the HFD-induced reduction in epididymal adipose tissue mRNA expression of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1), and partially prevented the HFD-induced reduction in glucose transporter 4 (GLUT4) (Fig. 3.4B) ($P < 0.001$). In the hypothalamus, WPI specifically increased IR mRNA expression ($P < 0.05$), as did P/C ratio, with the highest P/C ratio having the greatest impact (Table 3.4). Epididymal adipose tissue mRNA expression of inflammatory markers, namely tumour necrosis factor (TNF)-α and cluster of differentiation (CD) 68 only responded to the highest P/C ratio, which significantly reduced the expression of both in a HFD background (Fig. 3.4B) ($P < 0.001$). In the hypothalamus, whilst TNF-α mRNA expression was elevated by HFD feeding, neither WPI nor the P/C ratio influenced its levels, although there was a trend towards a decrease for the highest P/C ratio (40% WPI) (Table 3.4). None of the dietary challenges influenced hepatic glucose transporter 2 (GLUT2), IRS-1 and

TNF-α mRNA expression (Table 3.3) or hypothalamic IRS-1 mRNA expression (Table 3.4).

The increased plasma leptin concentration in response to the HFD was significantly blunted by WPI intake with dramatic reductions seen at the highest P/C ratio ($P < 0.001$) (Table 3.2). Yet, the hypothalamic expression of genes known to be responsive to plasma leptin levels were unaffected, specifically mRNA levels of leptin receptor (ObR), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and growth hormone secretagogue receptor (GHS-R) (Table 3.4). In addition, gastric mRNA expression for the orexigenic hormone ghrelin was not found to significantly differ between all dietary treatment groups (1.00±0.37, LFD vs. 1.11 ±0.40, HFD vs. 0.73±0.35, 20% WPI vs. 0.29±0.28, 30% WPI vs. 1.07 ± 0.45, 40% WPI). Plasma corticosterone levels were also elevated with HFD feeding, but were not influenced by protein source (WPI or casein) or P/C ratio (Table 3.2). Similarly, there was no effect of WPI on the HFD-associated suppression of adipose 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Fig. 3.4B), or the HFD-induced increase in glucocorticoid receptor (GCCR) in the hypothalamus (Table 3.4).

3.4.4 WPI inclusion or increasing the P/C ratio within a HFD altered the composition of gut microbiota

A total of 251,395 V4-V5 16S rRNA sequence reads were generated which corresponded to an average of 50,279 reads per diet group or 5,130 reads per animal. α-diversity values were calculated for biodiversity (Shannon index), species richness (Chao1) and the number of species relative to the abundance in the sample (Simpson diversity index). When α-diversity values were compared by diet group, the only difference observed was a significantly higher microbial

richness (Chao1) within the HFD microbiota compared to the HFD-30% WPI (*P* = 0.028). Principal coordinate analysis (based on unweighted UniFrac distances) (Fig. 3.5) of the sequence data highlighted a clustering of the LFD, HFD and HFD-20% WPI group microbial populations, while HFD-30% and 40% WPI groups clustered in a close proximity to each other and distinctly from the LFD, HFD and HFD-20% WPI group clusters. Indeed, the LFD diet with casein as a protein source clustered most closely with the HFD containing the casein protein.

Phylogenetic analysis revealed several significant microbial population shifts between the HFD control and WPI groups (Table 3.5). At the family level, all WPI diet groups had significantly increased proportions of *Lactobacillaceae* and significantly decreased proportions of *Clostridiaceae* compared to the HFD control group. *Bifidobacteriaceae* populations were increased in both the HFD-20% WPI and HFD-30% WPI diet groups compared to the HFD control, while in contrast they were significantly lower in the HFD-40% WPI group compared to the HFD control. The aforementioned patterns were also observed with respect to the corresponding genera (*Lactobacillus, Clostridium* and *Bifidobacterium* respectively) (Table 3.5). Also at the genus level, proportions of *Rikenella* were significantly higher in the HFD-40% WPI group compared to the HFD-20/30% WPI groups, while proportions of *Peptostreptococcus* were significantly higher in the HFD-40% WPI group than in any other diet group (Table 3.5). Specific comparison of the microbiota of the HFD-20% WPI and HFD control was deemed particularly important given that the changes occurring here reflected changes resulting specifically from the presence of whey protein, rather than casein, in the diet, and not simply a change in the P/C ratio in the diet. In addition to the changes in the *Lactobacillus*, *Clostridium* and *Bifidobacterium* populations (and associated

families) referred to above, it was also noted that the proportions of *Desulfovibrio* and *Mucisprillum* (genus) were increased in the HFD-20% WPI, relative to the HFD control animals.

3.5 DISCUSSION

The key findings of this study are that WPI has a specific effect on HFD-induced energy intake, metabolic health and gut microbiota composition. Additionally, with the exception of energy intake, increasing the P/C ratio, by increasing WPI dietary content, was seen to dramatically alter the above-mentioned parameters.

3.5.1 Energy intake

Cumulative energy intake measured up to week 16 did not significantly differ between LFD and HFD fed mice. Whilst this is consistent with data reported elsewhere (Tranberg *et al*, 2013), HFD feeding has also been shown to increase or decrease energy intake in rodents (Lin *et al*, 2000; Sclafani *et al*, 1993). Differences between these reported data may relate to variances in diet composition, including fat source/composition, or it may be due to differences in the palatability of the LFD used as the control (Sclafani *et al*, 1993; West & York, 1998).

In this study, there was a discrepancy in energy intake in HFD fed mice depending on the housing environment (single or group housed). In contrast to the group housed environment up to week 16, HFD fed mice when individually housed in metabolic cages during weeks 17-20 showed a hypophagic response compared to LFD fed mice. It is possible that these differences may be related to the accuracy of the method used to measure food intake in group versus single housed mice, although if this was an error due to methodology, then it would likely to have

influenced all dietary groups equally and not just the HFD group. Alternatively, the different behavioural responses could be result of social isolation, which has been shown to decrease energy intake and elevate plasma corticosterone levels (Saegusa *et al*, 2011; Yamada *et al*, 2013). However, socially isolated mice adapt to their new environment and consume similar amounts of food as pre-adapted singularly housed mice 6 hr post-novelty stress, yet interestingly 24hr later their food intake reduces significantly once again in the new environment, suggesting that stress of social isolation could extend up to 24h (Yamada *et al*, 2013). We showed that group housed mice on a LFD when placed in isolation adapt to the new environment and continue to consume similar amounts of food by day 2 and 3 in the new location (McAllan *et al*, 2013b), and consequently we used the day 3 time point to measure energy intake in this study. Rodents on a HFD have been shown to display increased anxiety (Buchenauer *et al*, 2009), and have an over-active hypothalamic-pituitary-adrenal axis (Dallman, 2010; Sharma & Fulton, 2013) resulting in elevated plasma corticosterone levels, as demonstrated here. This could explain why HFD fed mice are more susceptible to stress stimuli with more pronounced reductions in energy intake compared to LFD fed counterparts subjected to the same stress levels (Finger *et al*, 2011; Harris *et al*, 1998; Zhou *et al*, 1999). Consequently, in a HFD background, it's possible that social isolation-induced stress responses could have had a greater impact on energy intake with effects lasting up to the 3 day housing period as used in this study. Given the finding that whey proteins such as lactoferrin and α-lactalbumin and native whey protein itself reduce stress (Kamemori *et al*, 2004; Markus *et al*, 2002; Markus *et al*, 2000; Takeuchi *et al*, 2003; Vekovischeva *et al*, 2013), it is possible that replacing the casein protein with an equivalent WPI content caused normalisation

of energy intake in HFD-fed mice by affecting a specific feeding behaviour related mechanism(s), with increasing the WPI protein-derived bioactives having no further effect. This WPI effect on energy intake appeared be specific to the HFD-induced neuroendocrine state because mice on the LFD with WPI showed similar energy intake to casein diet-fed controls. Since neither WPI nor increasing the P/C ratio influenced plasma corticosterone levels, adipose tissue expression of 11β-HSD1 or hypothalamic expression of GCCR in HFD fed mice, it is possible that WPI may have affected other central mechanisms mediating stress responses not investigated in this study (Ghosal *et al*, 2013; Herman & Cullinan, 1997; Viau *et al*, 1999), either independently or in combination with key mechanisms regulating energy balance. Given that leptin decreases meal size and number (Bady *et al*, 2006; Brown *et al*, 2006; Hulsey *et al*, 1998), and WPI reduced the HFD-induced increase in plasma leptin levels, it's possible that WPI-derived bioactives could have specifically influenced the circadian rhythm of leptin production and/or action within the neuroendocrine state of HFD-fed mice in a socially isolated environment. Additionally, the reduction in plasma amino acids associated with WPI intake (see below), could also have acted as a possible central trigger to increase energy intake in WPI groups compared to the HFD control in the single house environment.

HFD feeding has been shown to cause a gain of weight in rats up to the duration of a test period lasting 76 weeks, with animal's body weight gain responding to changes to the dietary fat content introduced at various time points (Peckham *et al*, 1962). Lin *et al,* (2000) also demonstrated that mice on a HFD for 19 weeks are still responsive to intracerebroventricular administration of leptin. These data suggest that energy balance-related mechanisms are able to respond to

energetic challenges even after prolonged high fat intake. High protein intake within a HFD suppresses energy intake (Freudenberg *et al*, 2012; Pichon *et al*, 2006) albeit not consistently (Schwarz *et al*, 2012; Shertzer *et al*, 2011), and in our study, the P/C ratio did not alter energy intake in either housing environment. This could be a result of the quantity or composition of the macronutrient used in the test diets. Indeed, data from human trials showed that increasing the protein dietary content (10/15% to 30%) only decreased energy intake when the carbohydrate content was kept constant (Blatt *et al*, 2011; Weigle *et al*, 2005). This further highlights the importance of designing appropriate experimental diets with the correct macronutrient composition for uncovering the energy balance-related impact of the dietary component under investigation.

3.5.2 Metabolic health

Replacement of the casein protein with an equivalent energy content of WPI (i.e. 20%) did not specifically alter metabolic activity, heat production or locomotor activity in HFD or LFD fed mice. In contrast, Acheson *et al,* (2011) showed that whey has a greater thermic effect than casein or soy in humans. These differences in data may be related to the fact that the latter study only investigated an acute post-prandial response to a defined test meal, or it may relate to how different species (humans versus mice) digest and metabolise dietary proteins. Shetzer *et al,* (2011) found that mice consuming a HFD and WPI supplemented drinking water have enhanced oxygen consumption compared to mice drinking unsupplemented water. In this instance, the increased metabolic activity may have arisen simply due to the increased protein intake (proteins from diet and from WPI

supplemented water). In fact, this corroborates with the data presented here, which show that increasing the P/C ratio resulted in increased energy expenditure (VO_2 and heat production) and darkphase locomotor activity, resulting presumably from the increased catabolism of ingested dietary protein, coupled with thermic effects of WPI compared to casein (Acheson *et al*, 2011) and/or due to increased deposition of lean mass with WPI content (Kanda *et al*, 2013; Pennings *et al*, 2011; Tipton *et al*, 2007). Interestingly, Zhang *et al,* (2007) showed that HFD fed mice on leucine supplemented drinking water have reduced fasting plasma levels of aspartic acid, glutamic acid, and phenylalanine, as well as increased VO_2 and reduced adiposity compared to HFD controls. Given the influence of leucine on WPI-induced muscle hypertrophy (Norton *et al*, 2012; Rieu *et al*, 2007) and its unique ability to regulate the translation of protein synthesis (Norton & Layman, 2006), it is possible that the elevated leucine content found normally in the WPI diets may have enhanced muscle protein synthesis by directing other amino acids towards protein synthesis and/or catabolism (Bender, 2012; Garlick *et al*, 1999), with the required energy been derived possibly from fat catabolism (McAllan *et al*, 2013a). Consistent with the latter suggestion, we found an increased lean mass and a trend towards a reduction in fat mass with decreased plasma levels of several amino acids, but not leucine, when the casein protein in a HFD was replaced with WPI or when the P/C ratio in the HFD was increased.

WPI intake appeared to cause a trend towards a reduction in fat mass, and in the liver this manifested as a WPI specific reduction in TAG levels, which was accompanied by the suppression of FABP1 mRNA expression, similar to previous findings (Hamad *et al*, 2011; Pilvi *et al*, 2008; Shertzer *et al*, 2011; Shi *et al*, 2011). In the epididymal adipose tissue, WPI prevented the HFD-induced reduction in

FASN gene expression, albeit a recent study reported that WPI may not meaningfully affect the epididymal tissue in HFD fed mice, but instead impact on the subcutaneous fat pad to a greater extent (Tranberg *et al*, 2013). These data suggest that WPI affects cellular activity in the liver and in specific adipose tissue depots. While it has been suggested that whey protein may facilitate enhanced post-prandial chylomicron clearance via an alteration in LPL expression/activity (Mortensen *et al*, 2009; Pal *et al*, 2010), here we did not find a WPI specific effect on LPL expression or plasma TAG levels, but we did observe that intake of the highest P/C ratio (40%-WPI) led to an increase adipose tissue LPL mRNA expression which was accompanied by significant reduction in plasma TAG levels, and a complete reversal of HFD effects on genes involved in lipid accumulation (PPARγ), fatty acid transport (FATP1), and lipolysis (β3-AR). Given that HF feeding/obesity down-regulates β3-AR mRNA expression (Collins *et al*, 1999), our data suggests an increased adipocyte lipolysis, and a reduction in adipose TAG storage in HFD-40% WPI fed mice. Yet the endogenous CPT1b-associated β-oxidation pathway and the UCP-2-associated pathway in epididymal adipose tissue seemed to be unaffected (CTP1b) or suppressed (UCP-2) by raising the P/C ratio. This data raises the possibility that the free fatty acids generated from the potentially increased availability of β3-AR in the adipose may have been re-directed for utilisation by other physiological processes active in the HFD-40% group, possibly leading to the increased metabolic activity (VO_2) observed in the animals. It is also noteworthy that WPI has been shown to increase faecal fat excretion compared to casein (Pilvi *et al*, 2007), which may have also contributed to the decreased plasma TAG and NEFA seen here with intake of the highest P/C ratio diet (HFD-40% WPI).

Given the link between HFD-induced obesity, low-grade inflammation and insulin resistance (Gustafson *et al*, 2009; McGillicuddy *et al*, 2011), one could argue that the dramatic reduction in fat mass observed with the highest P/C ratio may underlie the effects on inflammatory markers in the adipose tissue (TNF-α and CD68) and the hypothalamus (TNF-α), along with the simultaneous changes in expression of genes involved in insulin signalling (IR, IRS-1 and GLUT4 in the adipose, IRS-1 in liver and IR in the hypothalamus), and the reduction in plasma glucose in these mice. Improvements to insulin sensitivity with WPI have been reported previously (Belobrajdic *et al*, 2004; Pichon *et al*, 2008), but our data suggested that only an increased P/C ratio in the HFD facilitated improvements to insulin signalling pathway associated gene expression, particularly in the adipose, in parallel with reduced HOMA-IR values.

3.5.3 Composition of gut microbiota

While many of the effects described above may be due to direct WPI or P/C ratio-host interactions, the effect of WPI and P/C ratio on the composition of the gut microbiota may also play an important role in adiposity and weight gain in these animals. Here, high-throughput sequencing based analyses of faecal microbial populations revealed the clustering of the microbiota from animals in receipt of the 30% and 40% WPI diets away from those in receipt of 20% WPI or HFD-casein diets. Tranberg *et al,* (2013) recently suggested that the efficient absorption of dairy whey proteins in the small intestine may explain the absence of changes in the faecal microbiota. This may explain the clustering of the microbiota from animals fed 20% WPI or HFD-casein diets in our study. However, it is apparent that the high concentrations of WPI present in the 30% and 40% WPI diets

employed in our study had a more profound effect, possibly due to additional whey proteins finding their way to the large intestine and/or the overall change in the P/C ratio in the diet. Consumption of the 30% and 40% WPI diets did not result in a shift in the microbiota toward that of the LFD animals and thus the effects on weight gain are not simply due to an overall conversion to a LFD-like microbiota.

Specific taxonomic changes were also noted in response to the different diets. In all cases dietary WPI resulted in significant increases in *Lactobacillaceae*/*Lactobacillus* and decreases in *Clostridiaceae*/*Clostridium*. Increased proportions of *Lactobacillus* have previously also been observed in a study of individuals following a regime of calorie restriction and exercise (Santacruz *et al*, 2009). However, in contrast, increased proportions of *Lactobacillus* have also been noted in HFD fed rats (Parnell & Reimer, 2012) and diet-induced obese mice (Clarke *et al*, 2013). Whilst specific species of *Lactobacillus* have been associated with both lean and obese gut microbiota profiles and also to play a role in obesity and immune response regulation (Armougom *et al*, 2009; Erickson & Hubbard, 2000; Ouwehand *et al*, 1999), due to the length of the 16S sequences generated and the high degree of sequence homology, we cannot assess changes in proportions of *Lactobacillus* at the species level. An increase in the proportions of *Bifidobacteriaceae/Bifidobacterium* was also observed in both the HFD-20% WPI and HFD-30% WPI compared to the HFD group. This result, combined with the aforementioned increases in *Lactobacillaceae/Lactobacillus*, mirror those reported by Sprong *et al,* (2010) who suggest that whey proteins act as grow factors for certain species of bacteria by an amino acid composition mediated mechanism. This pattern did not extend to the HFD-40% WPI group suggesting that, at these high protein levels, other factors are at play. Our observations are also consistent

with previous findings that high proportions of the class *Clostridiales* are associated with the gut microbiota of animals fed a HFD (Hildebrandt *et al*, 2009), while fasting reduces the levels of *Clostridium* (Sonoyama *et al*, 2009). Notably, *Clostridiaceae* can produce short chain fatty acids as a product of their metabolism (Sousa *et al*, 2007), which can play an important role in the regulation of immune cells and has been associated with inflammation and obesity (Schwiertz *et al*, 2010). These differences, as well as others in the *Proteobacteria, Actinobacteria, Deferribacteres* (phylum), *Desulfovibrionaceae, Deferribacteraceae, Veillonellaceae* (family), *Desulfovibrio* and *Mucispirillum* (genus) taxa in the HFD-20% whey protein, relative to HFD controls (20% casein) are particularly notable as these reflect changes resulting from the specific presence of whey proteins in the diet, in place of casein, rather than changes in the P/C ratio. Changes in relative proportions may be attributed to (a) the ability of bacteria to utilise whey proteins as a growth medium, (b) the anti-microbial activity of whey protein/peptide components, (c) decreased competition as a result of the whey proteins/peptides antimicrobial activity or (d) whey protein mediated changes in the host. Ultimately, the question of cause versus effect remains unanswered, and so while the changes to the microbiota observed may contribute to the mechanisms involved in controlling weight gain, further studies, with for example germ free animals, will be required to determine this definitively.

3.5.4 WPI effects on energy balance from a whole animal context

Focusing on the experimental data gathered between weeks 17-21, during which we measured metabolic parameters, faecal microbial population, body composition and tissue and plasma levels of energy balance-related parameters, it

is clear that WPI intake increased energy intake associated with the HFD without altering energy expenditure, as measured by VO_2 and locomotor activity. However, the body composition and body weight in the HFD-WPI group does not appear to reflect a positive energy balance, as animals showed a trend towards a reduction in fat mass and increased lean mass. It is noteworthy in this regard that WPI has been shown to increase faecal fat excretion (Pilvi *et al*, 2007) and we also observed some subtle changes in the gut microbiota at a phylogenic level that are associated with non-obese states, raising the possibility of a reduced intestinal TAG absorption in the HFD-WPI groups with increased energy intake, leading to similar body weight trajectories as HFD controls. Increasing the P/C ratio by changing WPI from 30% to 40% did not alter energy intake but significantly accentuated energy expenditure with a concurrent dramatic change in physiology.

In summary, our results show that WPI specifically normalises energy intake, increases lean mass and causes a trend towards a reduction in fat mass associated with prolonged high fat feeding. Raising the P/C ratio had no effect on energy intake but augmented metabolic activity and beneficially altered gene expression profiles for lipid metabolism, inflammation and insulin signalling, particularly in the adipose tissue. High-throughput analyses of gut microbiota revealed distinct changes in microbial populations, with an increased P/C ratio causing clustering of 30/40% WPI groups together and distinct from those of HFD and 20% WPI groups, but with specific phylogenetic differences existing between the latter groups. These data indicate that changes to P/C ratio have a dramatic effect on energy balance and the composition of the gut microbiota distinct from that seen with changes to protein source. Future studies should focus on

determining whether the effects demonstrated for the highest P/C ratio are specific to the WPI content, a consequence of macronutrient change, or both.

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3.7 AUTHOR CONTRIBUTIONS

The authors' contributions are as follows: LM, KNN, JFC, PDC, PS, RPR, GF and HMR conceived and designed the research; LM performed the majority of the research; PS performed gut microbiota DNA extraction, sequencing and analysis; POC performed amino acid analysis; LM wrote the majority of the paper; PS wrote the sections on the gut microbiota; KNN, JFC, PDC, POC, RPR, GF and HMR corrected the manuscript. The authors declare that there are no conflicts of interest.

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Chapter 3

TABLES & FIGURES

Table 3.1 Plasma amino acid levels (μmol/L) in mice fed a 45% kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1]

	HFD	20% WPI	30% WPI	40% WPI	P value
Alanine	170.91 ± 8.82^{a}	163.83 ± 11.59^{ab}	137.44 ± 9.72^{ab}	127.15 ± 8.46^{b}	<.05
Arginine	41.74 ± 4.82	38.41 ± 7.16	56.39 ± 7.60	39.08 ± 4.34	NS
Aspartic acid	7.47 ± 1.25^{a}	3.63 ± 0.51^{b}	3.49 ± 0.51^{b}	4.85 ± 1.02^{ab}	<.05
Cysteine	11.95 ± 1.34	8.87 ± 1.37	8.93 ± 1.33	7.86 ± 1.72	NS
Glutamic acid	90.76 ± 3.42^{a}	74.74 ± 1.89^{b}	75.25 ± 1.86^{b}	71.25 ± 2.69^{b}	<.001
Glycine	106.22 ± 4.28^{a}	131.43 ± 3.57^{b}	116.45 ± 4.52^{ab}	131.92 ± 2.69^{b}	<.001
Histidine	75.43 ± 2.12^{a}	74.93 ± 2.98^{a}	68.21 ± 3.35^{ab}	64.21 ± 2.51^{b}	<.05
Isoleucine	213.81 ± 8.87	207.87 ± 9.09	195.27 ± 5.74	186.78 ± 10.17	NS
Leucine	91.86 ± 9.66	81.36 ± 6.65	66.93 ± 6.69	69.71 ± 10.21	NS
Lysine	113.81 ± 5.88	108.18 ± 8.30	102.57 ± 8.39	88.29 ± 4.07	NS
Methionine	23.94 ± 1.77	23.65 ± 0.75	21.05 ± 0.97	20.40 ± 1.08	NS
Phenylalanine	46.92 ± 1.70^{a}	41.13 ± 1.13^{ab}	38.23 ± 1.52^{bc}	33.30 ± 2.35^{c}	<.001
Proline	118.26 ± 10.14	127.63 ± 9.63	106.04 ± 11.02	104.39 ± 11.93	NS
Serine	68.18 ± 5.69^{ab}	74.98 ± 2.29^{a}	62.42 ± 2.57^{ab}	55.73 ± 2.12^{b}	<.01
Threonine	92.71 ± 2.93^{a}	87.63 ± 2.49^{a}	78.29 ± 2.95^{ab}	69.28 ± 3.96^{b}	<.01
Tyrosine	40.29 ± 2.47^{a}	36.37 ± 1.12^{ab}	33.76 ± 1.54^{ab}	29.63 ± 2.77^{b}	<.01
Valine	154.51 ± 8.65^{a}	130.59 ± 5.99^{ab}	118.40 ± 6.69^{b}	113.16 ± 9.49^{b}	<.01

[1] Data are means ± SEM (n = 7-10). In each row, values without a common letter significantly differ, $P < 0.05$; NS, non-significant.

Table 3.2 Hepatic TAG accumulation and plasma levels of hormones and metabolites in mice fed a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1]

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
Liver TAG (mg/g tissue)	71.47 ± 9.17^{a}	139.03 ± 6.92^{b}	104.94 ± 5.88^{c}	107.21 ± 8.83^{c}	70.82 ± 7.64^{a}	<.001
Leptin (ng/ml)	4.41 ± 0.75^{a}	84.69 ± 3.17^{b}	53.78 ± 5.67^{c}	51.12 ± 4.69^{c}	15.01 ± 4.07^{a}	<.001
Plasma TAG (mg/dl)	39.85 ± 2.91^{a}	56.90 ± 6.31^{b}	45.15 ± 4.32^{ab}	52.36 ± 3.91^{ab}	40.19 ± 3.96^{a}	<.05
Plasma NEFA (mmol/L)	0.37 ± 0.09^{a}	0.67 ± 0.07^{b}	0.64 ± 0.05^{b}	0.63 ± 0.06^{b}	0.36 ± 0.05^{a}	<.01
Corticosterone (ng/ml)	150.0 ± 21.7^{a}	319.0 ± 48.6^{b}	286.8 ± 31.9^{ab}	334.1 ± 36.7^{b}	277.1 ± 35.5^{ab}	<.01
GLP-1 (pM)	25.03 ± 2.47	33.15 ± 2.83	28.07 ± 2.3	27.88 ± 1.98	24.03 ± 2.25	NS
Glucose (mmol/L)	9.14 ± 1.24^{a}	15.68 ± 0.83^{b}	13.92 ± 0.95^{b}	14.08 ± 1.05^{b}	9.25 ± 0.77^{a}	<.001
Insulin (ng/ml)	0.29 ± 0.05	0.55 ± 0.08	0.40 ± 0.10	0.37 ± 0.04	0.31 ± 0.06	NS
HOMA-IR	2.48 ± 0.67^{a}	9.79 ± 1.07^{b}	6.81 ± 2.58^{ab}	6.20 ± 0.95^{ab}	3.04 ± 0.64^{a}	<.01

[1] Data are means ± SEM (n = 5-10). In each row, values without a common letter significantly differ, $P \leq 0.05$; NS, non-significant. TAG, triacylglycerol; NEFA, non-esterified fatty acids; GLP-1, glucagon-like peptide 1; HOMA-IR, homeostasis model assessment of insulin resistance.

Table 3.3 Relative hepatic gene expression in mice fed a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1]

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
CD36	1.00 ± 0.20^{a}	2.85 ± 0.34^{b}	2.98 ± 0.45^{b}	2.17 ± 0.25^{ab}	1.50 ± 0.25^{a}	<.05
PPARγ	1.00 ± 0.12^{a}	3.00 ± 0.57^{b}	2.30 ± 0.49^{b}	1.88 ± 0.46^{b}	0.85 ± 0.13^{a}	<.001
FABP1	1.00 ± 0.09^{ab}	1.27 ± 0.10^{b}	0.86 ± 0.06^{ac}	1.27 ± 0.17^{bc}	0.71 ± 0.09^{a}	<.001
IRS-1	1.00 ± 0.15^{a}	0.58 ± 0.04^{b}	0.69 ± 0.05^{ab}	0.68 ± 0.06^{ab}	0.79 ± 0.06^{ab}	<.01
CPT1a	1.00 ± 0.08	0.85 ± 0.08	0.97 ± 0.06	0.90 ± 0.13	0.86 ± 0.10	NS
GLUT2	1.00 ± 0.09	0.76 ± 0.04	1.02 ± 0.08	0.97 ± 0.09	0.89 ± 0.05	NS
FATP5	1.00 ± 0.21	0.89 ± 0.07	0.88 ± 0.07	1.16 ± 0.07	1.24 ± 0.12	NS
FASN	1.00 ± 0.36	0.46 ± 0.14	0.54 ± 0.19	0.40 ± 0.06	0.32 ± 0.04	NS
PPARα	1.00 ± 0.04	1.13 ± 0.05	1.21 ± 0.05	1.17 ± 0.10	1.28 ± 0.11	NS
TNF-α	1.00 ± 0.26	0.84 ± 0.15	1.12 ± 0.18	0.80 ± 0.09	0.71 ± 0.10	NS

[1]Data are means ± SEM (n = 7-10). In each row, values without a common letter significantly differ, $P < 0.05$; NS, non-significant. Gene expression shown relative to the LFD control group set at 1.00.

CD36, Cluster of differentiation 36; PPARγ, Peroxisome proliferator-activated receptor gamma; FABP1, Fatty acid binding protein 1; IRS-1, Insulin receptor substrate 1; CPT1a, Carnitine palmitoyltransferase 1a; GLUT2, Glucose transporter 2; FATP5, Fatty acid transporter 5; FASN, Fatty acid synthase; PPARα, Peroxisome proliferator-activated receptor alpha; TNF-α, Tumour necrosis factor alpha.

Table 3.4 Relative hypothalamic gene expression in mice fed a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1]

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
IR	1.00 ± 0.03^{ab}	0.97 ± 0.07^{a}	1.22 ± 0.04^{bc}	1.37 ± 0.04^{cd}	1.55 ± 0.08^{d}	<.05
GCCR	1.00 ± 0.09^{a}	1.40 ± 0.10^{b}	1.34 ± 0.07^{ab}	1.30 ± 0.08^{ab}	1.42 ± 0.09^{b}	<.05
TNF-α	1.00 ± 0.05^{a}	1.76 ± 0.09^{b}	1.57 ± 0.13^{b}	1.47 ± 0.14^{ab}	1.40 ± 0.25^{ab}	<.01
POMC	1.00 ± 0.08	0.99 ± 0.06	1.01 ± 0.06	0.98 ± 0.06	1.00 ± 0.08	NS
NPY	1.00 ± 0.04	1.02 ± 0.09	0.90 ± 0.06	0.88 ± 0.05	0.92 ± 0.08	NS
ObR	1.00 ± 0.11	0.92 ± 0.05	0.99 ± 0.05	1.07 ± 0.07	0.95 ± 0.05	NS
GHS-R	1.00 ± 0.07	0.98 ± 0.06	0.98 ± 0.08	1.02 ± 0.11	1.04 ± 0.09	NS
PPARγ	1.00 ± 0.36	1.07 ± 0.04	0.95 ± 0.05	0.99 ± 0.05	0.98 ± 0.07	NS
CPT1c	1.00 ± 0.09	0.92 ± 0.04	0.91 ± 0.01	0.97 ± 0.06	0.86 ± 0.04	NS
IRS-1	1.00 ± 0.04	0.99 ± 0.04	0.97 ± 0.02	1.06 ± 0.08	1.00 ± 0.06	NS

[1]Data are means ± SEM (n = 5-10). In each row, values without a common letter significantly differ, P < 0.05; NS, non-significant. Gene expression shown relative to the LFD control group set at 1.00.

IR, Insulin receptor; GCCR, Glucocorticoid receptor; TNF-α, Tumour necrosis factor alpha; POMC, Pro-opiomelancortin; NPY, Neuropeptide Y; ObR, Leptin receptor; GHS-R, Growth hormone secretatgogue receptor; PPARγ, Peroxisome proliferator activated receptor gamma; CPT1c, Carnitine palmitoyltransferase 1c; IRS-1, Insulin receptor substrate 1

Table 3.5 Gut microbiota composition as % of reads in mice fed a 45% kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1]

	HFD	20% WPI	30% WPI	40% WPI
Phylum				
Proteobacteria	0.36^{a}	0.63^{b}	0.34^{ab}	0.32^{a}
Actinobacteria	0.63^{a}	1.82^{b}	3.79^{b}	0.36^{c}
Deferribacteres	0.57^{a}	1.61^{b}	1.56^{ab}	2.03^{b}
Family				
Desulfovibrionaceae	0.12^{a}	0.31^{b}	0.21^{ab}	0.23^{ab}
Rikenellaceae	6.71^{ab}	7.54^{b}	3.9^{a}	6.4^{ab}
Bacteroidaceae	0.44^{a}	0.42^{a}	0.16^{b}	0.21^{b}
Lactobacillaceae	0.21^{a}	3.03^{b}	4.6^{b}	2.14^{b}
Bifidobacteriaceae	0.43^{a}	1.71^{b}	3.66^{b}	0.22^{c}
Deferribacteraceae	0.57^{a}	1.59^{b}	1.32^{ab}	2.03^{ab}
Peptostreptococcaceae	0.62^{a}	1.79^{a}	1.54^{a}	8.01^{b}
Succinivibrionaceae	0.13^{a}	0.15^{a}	0^{b}	0^{b}
Clostridiaceae	1.31^{a}	0^{b}	0^{b}	0^{b}
Veillonellaceae	0.02^{a}	0.12^{b}	0^{a}	0^{a}
Genus				
Anaerobiospirillum	0.13^{a}	0.15^{a}	0^{b}	0^{b}
Desulfovibrio	0.07^{a}	0.22^{b}	0.17^{ab}	0.15^{ab}
Alistipes	4.33^{ab}	4.41^{a}	2.24^{b}	3.76^{ab}
Rikenella	1.04^{ab}	0.49^{b}	0.68^{b}	1.08^{b}
Bacteroides	0.44^{a}	0.4^{a}	0.16^{b}	0.21^{ab}
Oscillibacter	0.24^{a}	0.67^{ab}	0.42^{ab}	0.52^{b}
Lactobacillus	0.2^{a}	3.03^{b}	4.6^{b}	2.39^{b}
Bifidobacterium	0.43^{a}	1.71^{b}	3.66^{b}	0.22^{c}
Mucispirillum	0.57^{a}	1.61^{b}	1.56^{ab}	1.92^{ab}
Coprococcus	0.11^{ab}	0.23^{b}	0.06^{a}	0.06^{ab}
Turicibacter	0.56^{a}	0.35^{a}	0.15^{ab}	0^{b}
Clostridium	1.3^{a}	0^{b}	0^{b}	0^{b}
Peptostreptococus	0.1^{a}	0.14^{a}	0.12^{a}	0.78^{b}

[1]Data are means ± SEM (n = 10). Statistically significant differences generated using the Kruskal-Wallis algorithm. In each row values without a common letter significantly differ, $P \leq 0.05$.

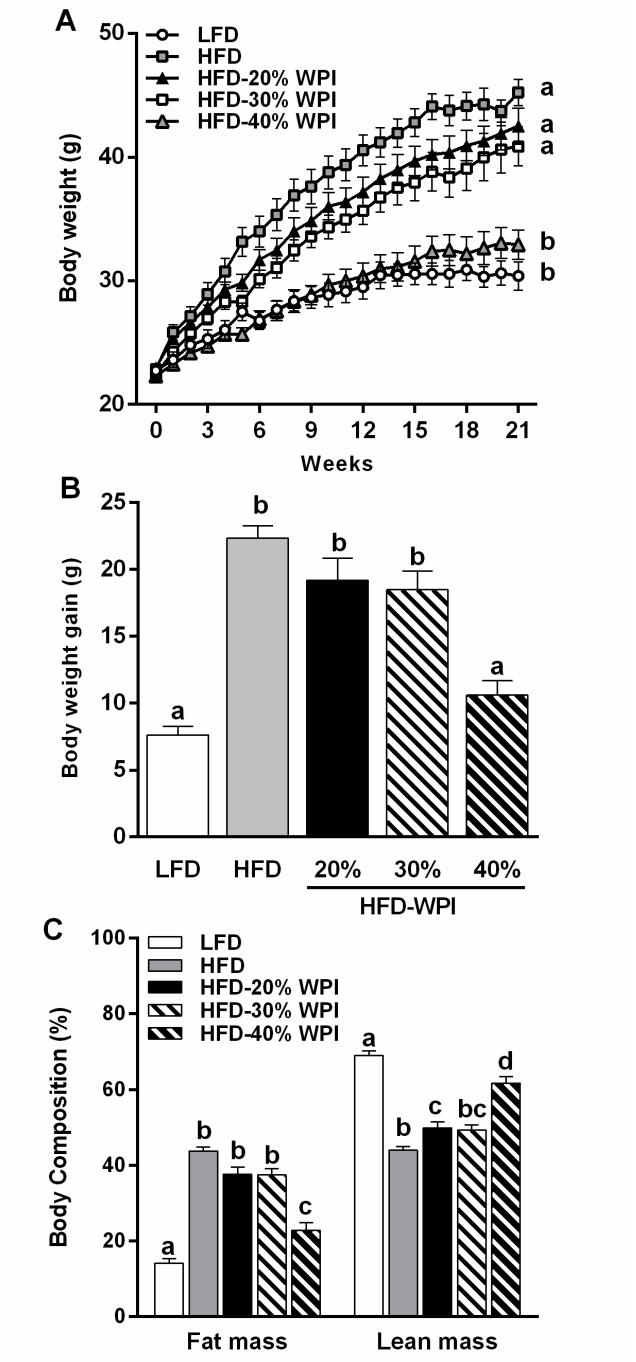


Figure. 3.1 Impact of whey protein isolate and protein to carbohydrate ratio on body weight and composition. (A) Shows the body weight trajectories of mice during 21 weeks of dietary treatment with a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Body weight gain (B) and body composition (C) of mice after 21 weeks on experimental diets are also shown. Data represent mean values ± SEM (n = 10 per group). Groups that do not share a common letter are significantly different at $P < 0.05$.

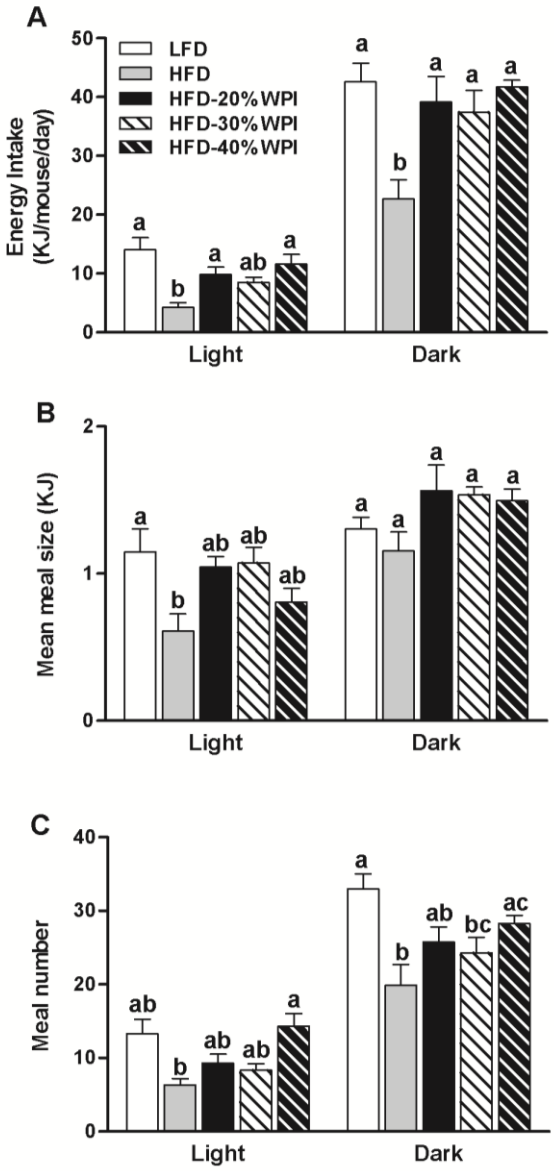


Figure. 3.2 Impact of whey protein isolate and protein to carbohydrate ratio on energy intake. Energy intake (A) and feeding behaviour (meal size & meal number) (B-C) was measured using TSE Phenomaster cages at weeks 17-20 for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data collected from individual mice at 9 minute intervals over a 24 hour period are shown as mean values ± SEM (n = 8-10 per group) for light and dark phases. In the light and dark phase, groups that do not share a common letter are significantly different at $P < 0.05$.

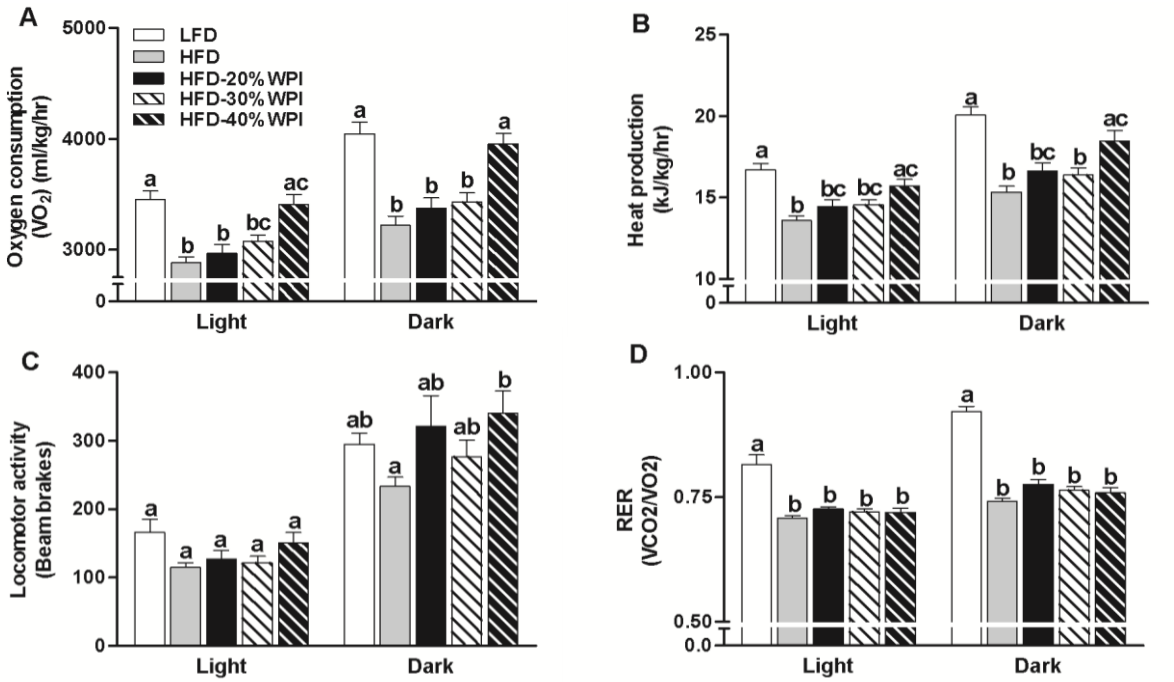


Figure 3.3 Impact of whey protein isolate and protein to carbohydrate ratio on metabolic activity. Metabolic activity was measured using TSE Phenomaster cages at 17-20 weeks for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data for (A) oxygen consumption (VO_2), (B) heat production, (C) locomotor activity and (D) respiratory exchange ratio (RER), collected from individual mice at 9 minute intervals over a 24 hour period, are shown as mean values ± SEM (n = 8-10 per group) for light and dark phases. In the light and dark phase, groups that do not share a common letter are significantly different at $P < 0.05$.

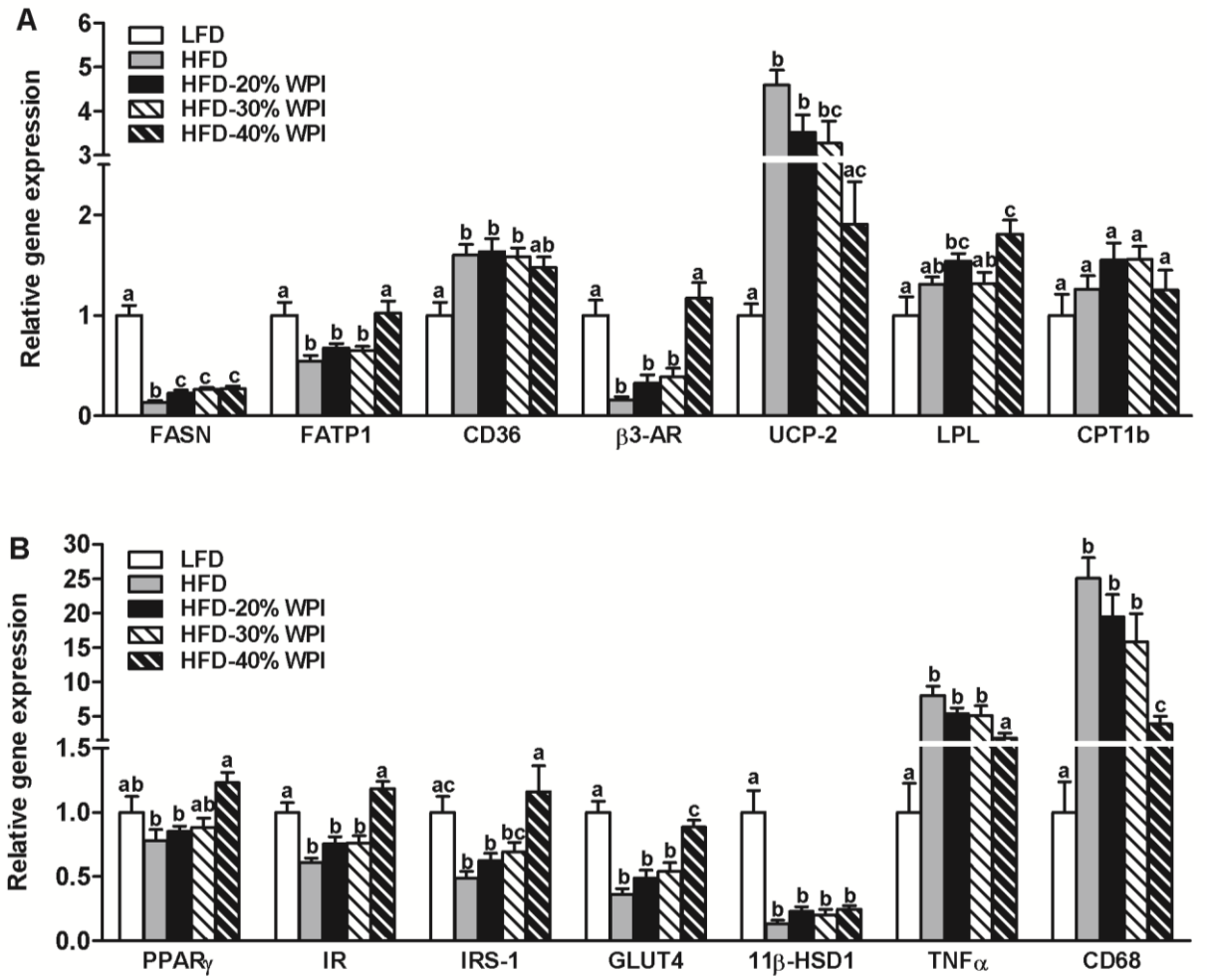


Figure 3.4 Impact of whey protein isolate and protein to carbohydrate ratio on adipose cellular activity. Epididymal adipose tissue gene expression was investigated in mice after 21 weeks on a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD), or HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Relative mRNA expression is shown for (A) fatty acid synthase (FASN), fatty acid transporter 1 (FATP1), cluster of differentiation 36 (CD36), β-3 adrenergenic receptor (β3-AR), uncoupling protein 2 (UCP-2), lipoprotein lipase (LPL) and carnitine palmitolytransferase 1b (CPT1b), and (B) for peroxisome proliferator activated receptor γ (PPARγ), insulin receptor (IR), insulin receptor substrate 1 (IRS-1), glucose transporter 4 (GLUT4), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), tumour necrosis factor α (TNF-α) and CD68. Data represent mean values ± SEM (n = 9-10 per group). Gene expression is shown relative to the LFD control group set at 1.00. Groups that do not share a common letter are significantly different at $P < 0.05$.

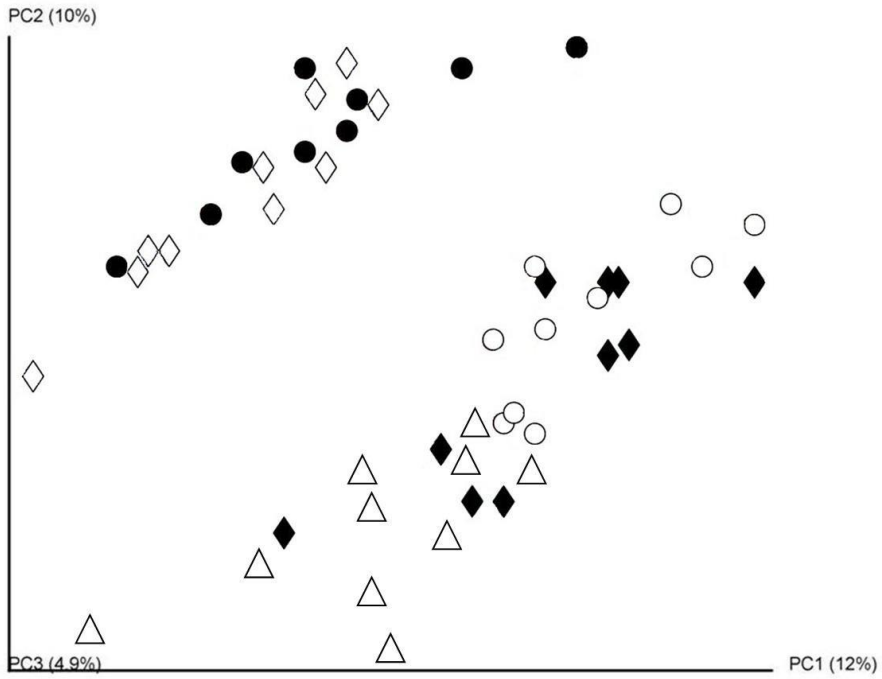


Figure 3.5 Impact of whey protein isolate and protein to carbohydrate ratio on the gut microbiota composition. Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of the 16S rRNA sequences, demonstrating where sequences cluster according to diet group. Data was generated from analysis of faecal samples collected from mice on a 10% kJ low fat diet (LFD, Δ), a 45% kJ high fat diet (HFD, ♦) or a HFD with 20% kJ whey protein isolate (HFD-20% WPI, ○), 30% kJ WPI (HFD-30% WPI, ◊) or 40% kJ WPI (HFD-40% WPI, ●)(n=10).

Chapter 3

SUPPLEMENTARY INFORMATION

Supplementary table 3.1 Composition of experimental diets[1]

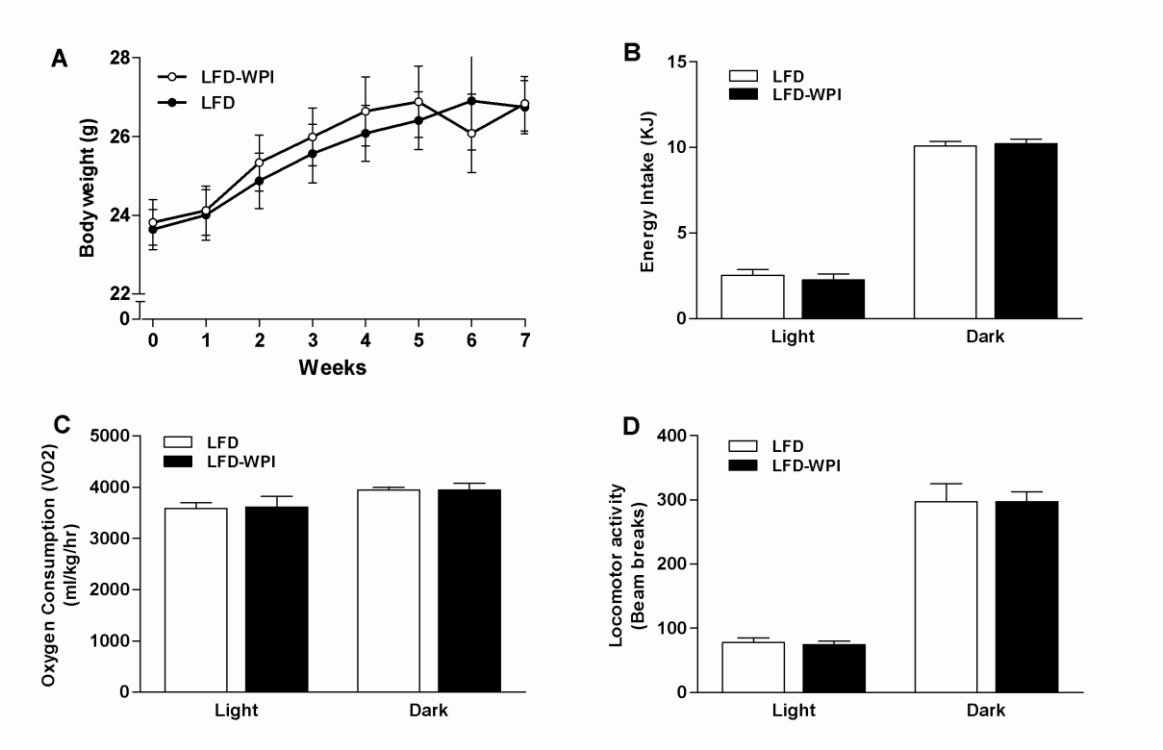
Contents (g)	Diets				
	LFD	HFD	HFD 20% WPI	HFD 30% WPI	HFD 40% WPI
Casein	200	200	0	0	0
Whey protein isolate	0	0	200	300	400
L-Cystine	3	3	3	4.5	6
Corn Starch	315	72.8	72.8	47	0
Maltodextrin 10	35	100	100	100	100
Sucrose	350	172.8	172.8	97.1	42.6
Cellulose, BW200	50	50	50	50	50
Soybean oil	25	25	25	25	25
Lard	20	177.5	177.5	177.5	177.5
Mineral mix S10026A	10	10	10	10	10
$CaHPO_4$	13	13	13	13	13
$CaCO_3$	5.5	5.5	5.5	5.5	5.5
$C_5H_6K_3O_7.1H_2O$	16.5	16.5	16.5	16.5	16.5
Vitamin mix V10001	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2
Energy (kcal/g)	3.8	4.7	4.7	4.7	4.7
Protein (% kcal)	20	20	20	30	40
Carbohydrate (% kcal)	70	35	35	25	15
Fat (% kcal)	10	45	45	45	45

[1] Diets formulated and produced by Research Diets Inc. (New Brunswick, NJ, USA)
LFD, low fat diet; HF, high fat diet; WPI, whey protein isolate.

Supplemenary table 3.2 Sequences mouse specific primers used in real-time PCR analysis

	Forward primer (5'-3')	Reverse primer (5'-3')
POMC	5'-gggcaagcgctcctactccatg-3'	5'-ctcgccttccagctccctcttg-3'
NPY	5'-ccctcgctctatctctgctcgtgtg-3'	5'-gtagtatctggccatgtcctctgc-3'
IR	5'-gatttccccaacgtgtcctctac-3'	5'-caatgcggtacccagtgaagtg-3'
IRS-1	5'-gcgcaggcaccatctcaacaacc-3'	5'-gcacgcacccggaaggaacc-3'
GLUT4	5'-ggcctgcccgaaagagtc-3'	5'-aggagctggagcaaaggac-3'
GLUT2	5'-tcctacttggcctatctgctgtgc-3'	5'-tgccctgacttcctcttccaac-3'
ObR	5'-gaccgccgaacacaaccgatgac-3'	5'-acacctagctggcgaaaaactgaag-3'
GCCR	5'-acctgacttccttgggggctatgaac-3'	5'-caggcagagtttgggaggtggtc-3'
TNFα	5'-tggcctccctctcatcag-3'	5'-acttggtggtttgctacgac-3'
CD36	5'-tgatactatgcccgcctctcc-3'	5'-tttcccacactccttttctcctcta-3'
β3-AR	5'-cgccttcaacccggtcatctactg-3'	5'-ggtggactctgcctggcttcaac-3'
PPARγ	5'-tcaggtttgggcggatgc-3'	5'-tcagcgggaaggactttatgtatg-3'
PPARα	5'-atgggggtgatcggaggctaatag-3'	5'-gggtggcaggaagggaacagac-3'
CPT1a	5'-agacttccaacgcatgacagcactg-3'	5'-ctcggccccgcaggtagatg-3'
CPT1b	5'-cgagaggggcggactgagactg-3'	5'-ggctaggcggtacatgttttggtg-3'
CPT1c	5'-caggtgcgggagtcggtgaag-3'	5'-cagcagagcgtgggcataagc-3'
FATP1	5'-ccggtgtggtggctgctcttctc-3'	5'-gctgccatctccccgccataaatg-3'
FATP5	5'- ccggcagcatggcgtaacag-3'	5'-acacatttgcccgaagtccattg-3'
FASN	5'-tccacctttaagttgccctg-3'	5'-tctgctctcgtcatgtcacc-3'
FABP1	5'-gaagcctcgttgccaccat-3'	5'-cgatttctgacacccccttgat-3'
LPL	5'-tgctcccaacaatataagactcc-3'	5'-aaggccaggtgtttcaatc-3'
11β-HSD1	5'-ccttggctgggaaaatgacc-3'	5'-ctatgaggccaaggacacagagag-3'
UCP-2	5'-ccatttcctgcaccccgatttacttcc-3'	5'-gctgggctggggatgaagatgaag-3'
GHS-R	5'-ccccgggacaccaacgagtg-3'	5'-aagaccggcaggaagaagaagacg-3'
Ghrelin	5'-cagaaagcccagcagagaaaggaatc-3'	5'-cggccatgctgctgatactgag-3'
CD68	5'-cacttcgggccatgtttctcttg-3'	5'-aggggctggtaggttgattgtcgtc-3'
β-actin	5'-agagggaaatcgtgcgtgac-3'	5'-caatagtgatgacctggcgt-3'
GAPDH	5'- ccattctcggccttgact-3'	5- tgaaggtcggtgtgaacg-3'
18-S	5'-aggaccgcggttctattttgttgg-3'	5'-atgctttcgctct-ggtccgtcttg-3'
YWHAZ	5'-cggagctgcgtgacatctgc-3'	5'-cctcggccaagtaacggtagtag-3'

POMC, Pro-opiomelancortin; NPY, Neuropeptide Y; IR, Insulin receptor; IRS-1, Insulin receptor substrate 1; GLUT4, Glucose transporter 4; ObR, Leptin receptor; GCCR, Glucocorticoid receptor; TNF-α, Tumour necrosis factor alpha; CD36, Cluster of differentiation 36; β-3 AR, Beta 3 adrenergic receptor; CPT1a, Carnitine palmitoyltransferase 1a PPARγ, Peroxisome proliferator-activated receptor γ;; FATP1, Fatty acid transporter 1; FASN, Fatty acid synthase; FABP1, Fatty acid binding protein 1; LPL, Lipoprotein lipase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; UCP-2, Uncoupling protein 2; GHS-R, Growth hormone secretagogue receptor.



Supplementary figure 3.1 Effect of a 10% kJ low fat diet with 20% kJ casein (LFD) or 20% kJ whey protein isolate (LFD-WPI) on (A) body weight, (B) energy intake (C) oxygen consumption (VO_2) and (D) locomotor activity, measured in individual mice at 9 minute intervals over a 24 hour period using TSE Phenomaster cages. Data are shown as mean values ± SEM (n = 8-10 per group) for light and dark phases.

Chapter 4

Whey protein isolate alters intestinal morphology and insulin and lipid-related gene expression in C57BL/6J mice

Submitted to *obesity* as:

McAllan L, Cryan, JF, Nilaweera KN (2014) Whey protein isolate alters intestinal morphology and insulin and lipid-related gene expression in C57BL/6J mice. *Obesity*.

4.1 ABSTRACT

Whey proteins have been shown to be acutely insulinotropic and to improve insulin sensitivity with prolonged intake. This study aimed to explore the underlying mechanisms. C57BL/6J mice were fed a 10% kJ fat and 20% kJ casein protein or whey protein isolate (WPI) diet for 7 (Study 1) or 15 weeks (Study 2), and energy balance and insulin signalling-related parameters were quantified. The 7 weeks of WPI intake had no effect on weight gain and cumulative energy intake, and only caused a trend towards an increase in plasma fasting insulin levels (P = 0.07), yet body fat was increased (P < 0.05) and insulin signalling-related gene expression decreased in the adipose tissue, skeletal muscle and liver. Moreover, lipolysis and lipid oxidation related gene expression was also decreased in the adipose tissue of these WPI fed mice. In contrast, 15 weeks of WPI intake reduced weight gain and cumulative energy intake (P < 0.05), with no change in insulin signalling-related gene expression. In both studies, WPI intake reduced the intestinal weight and length, while stomach weight was also reduced in the 15 week study (P < 0.05). The decreased insulin signalling and lipid metabolism-related gene expression coupled with the increased body fat in a background of plasma insulin levels that showed a trend towards an increase, suggested enhanced insulin signalling with WPI intake. This effect was largely reduced with prolonged WPI intake, presumably driven by the suppression of energy intake. Changes in gastrointestinal morphology, potentially similar to that caused by bariatric surgery, may underlie some of the WPI effects on insulin signalling-related gene expression and energy intake.

4.2 INTRODUCTION

Insulin is an important regulator of energy balance and glucose metabolism (Saltiel & Kahn, 2001). In regards to glucose homeostasis, insulin facilitates the uptake of glucose into both adipose tissue and skeletal muscle cells, for its storage as triacylglycerol (TAG) or utilisation in energy production via β-oxidation, respectively (Saltiel & Kahn, 2001). Insulin regulates this uptake of glucose in both tissues by promoting the expression and translocation of glucose transporter 4 (GLUT4) to the cell surface (Watson *et al*, 2004). In energy balance regulation, central insulin action induces a catabolic response, reducing both energy intake and body weight (Brief & Davis, 1984; Ikeda *et al*, 1986). Conversely, in the periphery, insulin has an anabolic response, promoting the storage of lipids by the stimulation of fatty acid uptake and synthesis, and via the inhibition of TAG breakdown and release as fatty acids and glycerol (Saltiel & Kahn, 2001).

Whey proteins are a by-product of cheese manufacture. These dietary proteins have gained considerable interest in recent years in relation to their health benefits (Luhovyy *et al*, 2007). Increasing evidence suggests that whey proteins exert a robust insulinotropic effect (Frid *et al*, 2005; Gaudel *et al*, 2013; Power *et al*, 2009), by both directly affecting its production and release, while also enhancing the translocation of GLUT4 to the plasma membrane (Morato *et al*, 2013a; Morato *et al*, 2013b). Additionally, whey proteins stimulate incretin hormone release, both glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), and increase their activity by suppressing dipeptidyl peptidase-4 (DPPIV) function, which together promotes glucose-dependent insulin section from pancreatic β-cells (Garber, 2011; Jakubowicz & Froy, 2013; Tulipano *et al*, 2011). We recently found that feeding whey protein isolate (WPI) to mice for

8 weeks normalises the high fat diet (HFD)-induced reduction in insulin receptor (IR) and GLUT4 gene expression in the epididymal adipose tissue (McAllan *et al*, 2013). These changes occurred in the absence of an altered plasma insulin, GLP-1 or glucose level, suggesting that WPI has a specific effect on insulin-related gene expression in this tissue. However, as body fat was also reduced by feeding the WPI in a high fat background, it is possible that the observed changes in gene expression may have been influenced by the altered fat mass in the animals.

Therefore, in this study we sought to obtain further evidence supporting the suggestion that WPI has a specific effect on insulin signalling by using mice fed a low fat diet (LFD) with either casein or WPI. This strategy allowed us to delineate WPI effects on insulin action without the confounding impact of the associated dietary protein-induced reductions in fat mass seen in HFD fed mice.

4.3 MATERIALS & METHODS

4.3.1 Animals & diets

Three week old C57BL/6J male mice (Harlan; Middlesex, UK) were housed as previously described (McAllan *et al*, 2013) and had *ad libitum* access to food and water throughout the trial. Mice were fed a diet containing 10% kJ fat, 70% kJ carbohydrate and 20% kJ casein protein (LF-CAS; #D12450; Research Diets, USA) or WPI (Alacen^{tm} 890 NZMP, Auckland, New Zealand) (LF-WPI). Mice were acclimatised to their environment for three weeks before experimental dietary treatment began. All procedures involving animals were approved by the University College Cork Animal Experimentation Ethics Committee and were licensed under the Cruelty to Animals Act 1876.

4.3.2 Experimental protocol

Two separate studies were conducted lasting for 7 weeks (Study 1) and 15 weeks (Study 2). In both studies, following acclimatisation, mice were separated into two weight matched dietary groups and provided either the LF-CAS or LF-WPI diet (n = 8). Body weight and energy intake were measured weekly. In addition, at week 6 (Study 1) and week 14 (Study 2), energy intake and metabolic activity were measured using the TSE Phenomaster system (Bad Homburg, Germany). Body composition was determined by NMR using the Bruker minispec LF50H (Bruker optics, Ettlingen, Germany) at the end of each study, after a 6h fast. Subsequently mice were anesthetised and their blood was collected. The mice were then sacrificed by cervical dislocation, and tissues of interest were excised, weighed and immediately snap frozen in liquid nitrogen (liver, subcutaneous adipose, epididymal adipose, gastrocnemius muscle, small intestine and colon). Plasma and tissue samples were stored at -80^{o}C.

4.3.3 Biochemical analysis

Blood collected into vacutinater EDTA tubes (BD; Franklin Lakes, NJ, USA) from anesthetised mice (ketamine; 65mg/kg, xylazine; 13mg/kg), was treated with Diprotin A and Aprotinin as described before (McAllan *et al*, 2014) to protect plasma peptides against proteolytic degradation. Plasma was isolated by centrifugation at 2000rpm and 4^{o}C for 15mins, and was analysed using colorimetric assay kits to determine plasma concentrations of glucose (Calibochem, Darmstadt, Germany) and TAG (LabAssay TAG, Wako Chemicals, Richmond, VI, USA). ELISA kits were used to determine plasma concentrations of GLP-1 (Millipore, St. Charles, MO, USA), leptin and insulin (Crystal Chem, Downers

Grove, IL, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) and hepatic triglyceride levels were measured as previously detailed (McAllan *et al*, 2014), while total faecal lipid content was determined by a modified version of that previously described (Chon *et al*, 2012; Newberry *et al*, 2006). Briefly, following an overnight incubation at room temperature in 1ml distilled water /100mg dry faecal weight, faecal samples were then vortexed thoroughly before the addition of 4 volumes of chloroform: methanol solution (2:1 v/v), after which 0.2 volumes of NaCl solution (0.58% in distilled water) were added and samples were centrifuged at 2000rpm and 4^{o}C for 20mins to separate the layers. The lower phase was collected into pre-weighed tubes, evaporated to dryness and re-weighed to measure lipid content gravimetrically.

4.3.4 Tissue gene expression

Total RNA was isolated from tissues of interest (epididymal adipose tissue, liver, skeletal muscle, duodenum and ileum) and purified using RNeasy mini kits (Qiagen, Hilden, Germany), with accompanied DNase treatment used to prevent genomic DNA contamination. Complementary DNA (cDNA) was synthesised from isolated RNA and subjected to Real-time qPCR analysis as described previously (McAllan *et al*, 2013). Primer sequences of target and reference genes are listed in supplementary table 1. Target gene mRNA expressions were calculated using $2^{-\Delta\Delta Cp}$, after normalising against housekeeping gene expression according to ΔΔCp= ΔCp target gene – ΔCp housekeeping gene. Housekeeping genes used were β-actin (adipose, liver, muscle, duodenum, and ileum), YWHAZ (liver and duodenum, ileum) and 18-S (adipose). The gene expression is shown relative to the LF-CAS group.

4.3.5 Statistical analysis

The data are presented as the mean ± SEM. Dietary treatment effects were analysed using unpaired T-tests. A two-way Repeated measures ANOVA with *Bonferroni* multiple comparisons was used to analyse bodyweight trajectories over both treatment periods. Mann-Whitney U tests were used to analyse non-parametric data. Statistical significant was established at $P \leq 0.05$, and data analyses were performed using Graphpad Prism (ver. 6) and Minitab (ver. 15).

4.4 RESULTS

4.4.1 Weight gain and body composition

Study 1: The figures 4.1(A & C) show that over the 7 week dietary period there was no difference in bodyweight or weight gain between the LF-WPI and LF-CAS groups. However, LF-WPI fed mice had significantly greater body fat mass (%) ($P < 0.05$) and a lower lean mass (%) ($P < 0.05$) than their LF-CAS counterparts (Fig. 4.1E). The weight and length of the small intestine was significantly lower in LF-WPI fed mice compared to the LF-CAS controls ($P < 0.001$) (Fig. 4.2A and B), and there was a trend towards an increased in epididymal fat weight with WPI intake (Fig. 4.2A; $P = 0.055$). This change in fat mass was also reflected in plasma leptin levels (Table 4.2). In a previous study, using grouped housed mice, we investigated the energy balance-related effects of WPI in a low fat background and obtained similar results as reported here (McAllan *et al*, 2014). The group housed mice fed WPI for 7 weeks gained similar weight as casein fed mice (2.71 ± 1.05g WPI vs. 2.73 ± 1.00g casein) and had a trend towards an increase in body fat mass (%)

(17.75 ± 1.44 WPI vs. 13.68 ± 0.45 casein; $P = 0.08$) and a significant decrease in lean mass (%) (65.97 ± 1.16 WPI vs. 69.38 ± 0.266 casein; $P < 0.05$).

Study 2: After 15 weeks of dietary treatment total body weight gain was significantly reduced in the LF-WPI group compared to the LF-CAS group (Fig. 4.1D; $P < 0.05$), although no significant difference in either body weight or body composition (fat or lean mass) was observed between the groups (Fig. 4.1B & F). This was reflected in plasma leptin levels, which showed no difference between the groups at the end of the study (Table 4.2). Again, the weight and length of the small intestine was reduced, but in addition the weight of the stomach was similarly reduced in the LF-WPI group (Fig 4.2C and D; $P < 0.05$).

4.4.2 Energy intake and metabolism related parameters

Study 1: Cumulative energy intake over the first 6 weeks did not significantly differ between LF-WPI and LF-CAS dietary groups (Fig. 4.1G), which was reflected in the 24h energy intake and meal pattern (meal number or meal size) as determined using TSE Phenomaster cages at week 6. (Table 4.1) The latter analysis did nonetheless reveal a significant increase in dark phase water intake in LF-WPI fed mice ($P < 0.05$) (Table 4.1). However, there was no change in oxygen consumption (VO_2), a marker of energy expenditure, or respiratory exchange ratio (RER), which predicts substrate utilisation, with WPI intake (Table 4.1). Moreover, WPI intake did not change faecal fat content (excretion), TAG levels in the plasma and/or liver that could correlate with the augmented fat deposition (Table 4.2).

Study 2: In contrast to study 1, cumulative energy intake was significantly reduced in WPI fed mice over the first 13 weeks (Fig. 4.1H; $P < 0.05$). The 24h energy

intake, meal number and meal size as determined by TSE phenomaster cages in week 14 (Table 4.1), showed a reduction in the WPI group, although this was not significant. As before, VO_2, RER and water intake did not differ between the diet groups (Table 4.1). Reductions in energy intake were not due to changes in satiety related hormone cholecystokinin (CCK) or proglucagon gene expression in the intestine (Table 4.5), which was corroborated with plasma GLP-1 levels (Table 4.2). Furthermore, WPI intake contrastingly reduced peptide YY (PYY) expression in the ileum (Table 4.5).

4.4.3 Insulin signalling and lipid metabolism related gene expression

Study 1: At week 7, plasma glucose and TAG levels were similar between the dietary groups, while fasting plasma insulin did show a trend towards an increase (P = 0.07) in WPI fed mice (Table 4.2). The expression of genes fundamental to insulin signalling and glucose transport in the adipose tissue, namely IR, insulin receptor substrate 1 (IRS-1) and GLUT4, were all significantly reduced in LF-WPI fed mice compared to LF-CAS fed controls at 7 weeks (Fig 4.3A). Similarly, genes involved in lipolysis and β-oxidation, namely β-3 adrenergic receptor (β-3 AR), hormone sensitive lipase (HSL) and carnitine palmitoyltransferase 1b (CPT1b), were also down-regulated in WPI fed mice at 7 weeks (Fig. 4.3A). Similar gene expression changes were observed in the skeletal muscle and liver. In both tissues, there was a reduction in the mRNA expression of IRS-1, while there was also a trend towards a decrease in IR expression in skeletal muscle (Table 4.3 & 4.4). In addition, hepatic levels of insulin-like growth factor 1 (IGF-1) were found to be significantly increased in LF-WPI fed mice (Table 4.4). Moreover, WPI also significantly reduced hepatic fatty acid synthase (FASN) and lipoprotein lipase

(LPL), and caused a trend towards a reduction in both fatty acid binding protein 1 (FABP1) and peroxisome proliferator-activated receptor gamma (PPARγ) (Table 4.4). In contrast, skeletal muscle expression of acetyl-CoA carboxylase (ACC) was significantly up-regulated in LF-WPI fed mice, while PPARγ mRNA expression also showed a trend towards an increase (Table 4.3; $P = 0.07$).

Study 2: While plasma insulin, glucose and TAG levels remained unchanged by WPI challenge, in contrast to study 1 there was no significant dietary effect on insulin signalling, lipolysis or β-oxidation gene expression in the epididymal adipose tissue, liver or skeletal muscle; although there was a trend towards a decrease in skeletal muscle IRS-1 expression in the WPI fed animals ($P = 0.06$) (Table 4.3). In contrast, the expressions of FASN, ACC and cluster of differentiation 36 (CD36) were significantly up-regulated in the epididymal adipose tissue of WPI fed mice at 15 weeks ($P < 0.05$), while LPL also showed a trend towards an increase ($P = 0.08$) (Fig. 4.3B). In the duodenum, there was a significant reduction in mRNA expression of IR and a trend towards a decrease in the expression of sodium-glucose co-transporter 1 (SGLT-1) in the WPI group (Table 4.5), while there was also an increase in GLUT2 expression in the ileum of these mice (Table 4.5). The LF-WPI diet also reduced fatty acid transporter 4 (FATP4) mRNA levels in the ileum (Table 4.5).

4.5 DISCUSSION

Our results show that 7 weeks of WPI intake does not affect weight gain or energy intake but does increase percentage body fat, while also reducing insulin signalling associated gene expression in multiple tissues, and lipolysis and β-oxidation-

associated gene expression in the epididymal adipose tissue. These changes occurred in a background of unaltered plasma glucose and GLP-1, with only a trend towards increased plasma insulin. In contrast, the 15 weeks of WPI intake, decreased weight gain and cumulative energy intake, and had no effect on insulin associated gene expression or on plasma hormones and metabolites, but did increase the expression of genes involved in lipid synthesis in the epididymal adipose tissue. As insulin facilitates cellular lipid storage and synthesis (Saltiel & Kahn, 2001), and suppresses energy intake (Brief & Davis, 1984; Ikeda *et al*, 1986), our data suggests that WPI has an effect on energy balance through its actions on insulin signalling. Importantly, in both studies WPI impacted on intestinal morphology, with significant reductions in both the weight and length of the small intestine, leading to the possibility that this could underlie how WPI affects insulin signalling and impacts on physiology.

Seven weeks of WPI intake facilitated whole body fat deposition, which was reflected by a trend towards an increased epididymal fat mass. Interestingly, this occurred without changing weight gain, presumably because their % lean mass was also decreased. The increased body fat cannot be explained by alterations to energy intake, metabolic activity, or faecal fat excretion as these did not differ between the two diet groups. As insulin promotes glucose and fatty acid uptake, augmenting lipid synthesis and storage, and suppresses intracellular lipolysis (Saltiel & Kahn, 2001), the observed changes may be related to a specific effect of WPI on insulin signalling. Indeed, both lipolytic and β-oxidation associated genes in the epididymal adipose tissue were suppressed in WPI fed mice. The concurrent reduction in mRNA expression of insulin and glucose related genes in the liver, adipose tissue and skeletal muscle demonstrated here, might reflect a negative

feedback response that is attempting to minimise further fat deposition. This is further supported by the finding that WPI intake increased hepatic IGF-1 mRNA expression, which has been shown to suppress further insulin production (Van Schravendijk *et al*, 1990; Zhang *et al*, 2007; Zhao *et al*, 1997) and is itself stimulated by insulin (Bereket *et al*, 1995; Phillips *et al*, 1998). Interestingly, given evidence that whey protein intake both promotes GLUT4 translocation to the cell surface (Morato *et al*, 2013a; Morato *et al*, 2013b) and normalises insulin signalling associated gene expression in the epididymal adipose tissue of mice fed a HFD (McAllan *et al*, 2013; Pilvi *et al*, 2009), the trend towards an increase in plasma insulin in WPI fed mice, coupled with the observed down-regulation of insulin-related gene expression in LF-WPI fed mice most likely represents a homeostatic response to increased WPI-induced insulin signalling, rather than a direct suppressive effect of WPI on insulin related gene expression. The difference in the results between these previous studies and the present work on how WPI influences the expression of genes related to insulin and glucose homeostasis may be related to the dietary composition in which WPI was presented to the animals (LFD vs. HFD).

Extending the WPI intake to 15 weeks reduced body weight gain, presumably because of the gradual decrease in cumulative energy intake, since neither metabolic rate, substrate utilisation nor faecal fat excretion differed between WPI and casein groups. Despite the change in energy intake and the fact that whey proteins are known to stimulate incretin hormone production (Gunnarsson *et al*, 2006), the levels of anorexigenic hormones GLP-1 and leptin in the plasma did not change in WPI fed mice, as neither did the intestinal gene expression of incretin hormones gastric inhibitory peptide (GIP) or proglucagon,

or the satiation factor CCK. Moreover, mRNA levels of the satiety hormone PYY were significantly decreased in the ileum of LF-WPI fed mice, albeit a similar effect of WPI has been reported on colonic expression of PYY mRNA previously (Xiao *et al*, 2005). Notably, whilst plasma insulin levels did not change, the insulin regulated FASN and ACC gene expression increased significantly in the epididymal adipose tissue, raising the possibility that insulin signalling may be enhanced in the WPI fed mice even after 15 weeks of dietary challenge, and that the reduction in cumulative energy intake may be, at least in part, a consequence of its central effects. Additionally, the reduction in stomach weight may also have contributed to the latter physiological change in a manner similar to bariatric surgery, which as part of the intervention reduces stomach size to achieve a significant decrease in food intake.

Our data raises the key question that if WPI intake potentially influences insulin signalling in the 7 and 15 week studies, as suggested by the data, then how do WPI fed mice maintain their fasting glucose concentrations at a comparable level to casein fed animals? The observed down-regulation of the insulin and glucose-related gene expression across multiple tissues in the 7 week study may offer an explanation to this question, since this potential negative feedback mechanism could avoid excess insulin-dependent glucose transport and uptake into cells. Extending the feeding trial to 15 weeks caused a reduction in cumulative energy intake, with normalisation of plasma insulin and glucose related gene expression in tissues, presumably to maintain glucose homeostasis. The latter suggestion is further supported by the augmented ileum GLUT2 mRNA expression of WPI fed mice. In future studies glucose and/or insulin tolerance tests should

also be included to establish direct evidence of whether any alterations to insulin sensitivity or glucose clearance occur with WPI intake.

At present we don't know how WPI could potentially increase insulin signalling and glucose transport without substantially changing plasma insulin or GLP-1 as demonstrated here, and as shown by others in young male subjects (Akhavan *et al*, 2014) and in Wistar rats (Morato *et al*, 2013a) independently of effects on plasma insulin. Interestingly, one common denominator of our two studies here was the change in intestinal morphology, where both the weight and length of the tissue was reduced. It is noteworthy in this regard that surgical reduction of the intestinal length, as that achieved by attaching the distal small intestine to the stomach so that nutrients bypass the proximal part of the small intestine, causes enhanced insulin sensitivity that is independent of changes to energy intake or plasma levels of insulin or GLP-1 (Rubino *et al*, 2006; Salinari *et al*, 2013b). It has been proposed that the underlying cause may be due to a reduction in the production of yet unknown negative regulatory factors that impede insulin action, arising from the reduced exposure of the proximal intestinal cells to nutrient passage following by-pass surgery. In fact, duodenal-jejunal secreted proteins isolated from diabetic *db/db* mice, when injected intravenously, were found to impair insulin signalling in wild type mice (Salinari *et al*, 2013a). In the same way, WPI-induced shortening of the intestine, may have caused a surgery-like effect on the proximal small intestine with a presumed reduction in cell number in this region reducing production of the proposed factor. Whether or not the whey protein effect on the GI tract truly mimics gastric by-pass surgery and its potential health related outcomes remains to be determined.

In conclusion, the results here show that WPI intake in a low-fat diet background reduces body weight gain after prolonged (15 week) intake by suppressing energy intake. In the short-term (7 week) WPI appears to facilitate greater fat deposition, possibly via its effects on insulin signalling. In fact, we propose the associated reductions in insulin signalling-related gene expression across multiple tissues to be a negative feedback response to minimise additional insulin-induced fat deposition. In spite of a reduction in body weight gain at 15 weeks prolonged WPI intake still appeared to enable a retention of body fat, which corresponded with enhanced lipogenic potential in the adipose tissue, arising possibly because of the above mentioned WPI-induced effects on insulin action, which could also be correlated to the reduction in energy intake. Our data also shows that WPI has a specific effect on intestinal morphology, whereby WPI decreased intestinal length irrespective of the duration of WPI feeding, and this may be linked to how WPI increases insulin signalling in multiple tissues and brings about changes in energy balance and body composition in mice. However further investigation is required to uncover the clinical significance of this alteration of the intestinal environment.

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4.7 AUTHOR CONTRIBUTIONS

LM, KNN & JFC conceived and designed the research; LM preformed the research, analysed the data and wrote the paper; KNN and JFC corrected the manuscript.

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Chapter 4

TABLES & FIGURES

Table 4.1
TSE phenomaster metabolic and energy intake measurements of mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) diet for 6 or 14 weeks.

		6 weeks		14 weeks	
	Phase	LF-CAS	LF-WPI	LF-CAS	LF-WPI
Energy intake (kJ/mouse)	Light	12.54 ± 1.54	10.41 ± 1.67	12.17 ± 1.46	10.40 ± 2.42
	Dark	40.61 ± 2.47	42.26 ± 2.43	43.06 ± 2.10	32.86 ±3.91
Meal number	Light	12.63 ± 1.22	9.5 ± 1.55	13.0 ± 1.1	20.3 ± 5.0
	Dark	32.5 ± 1.75	30.25 ± 1.98	31.9 ± 2.9	25.4 ± 3.0
Meal size (kJ)	Light	1.00 ± 0.09	1.14 ± 0.10	0.99 ± 0.16	0.80 ± 0.12
	Dark	1.28 ± 0.11	1.43 ± 0.10	1.46 ± 0.20	1.15 ± 0.19
Water intake (ml/mouse)	Light	0.49 ± 0.06	0.50 ± 0.07	0.61 ± 0.10	0.82 ± 0.22
	Dark	2.28 ± 0.22[a]	3.03 ± 0.15[b]	2.83 ± 0.32	3.65 ± 0.69
VO_2 (ml/hr/kg)	Light	3370.7 ± 78.7	3224.2 ± 45.4	3837.7 ± 132.9	3772.3 ± 69.9
	Dark	4181.6 ± 58.8	4125.3 ± 99.1	4351.0 ± 125.1	4417.4 ± 50.9
RER (VCO_2 / VO_2)	Light	0.85 ± 0.01	0.85 ± 0.01	0.83 ± 0.01	0.82 ± 0.03
	Dark	0.96 ± 0.01	0.97 ± 0.01	0.98 ± 0.01	0.92 ± 0.02

Data are means ± SEM (n = 8). For each trial length, values in a row without a common letter differ significantly, $P \leq 0.05$; NS, non-significant.

VO_2, oxygen consumption; VCO_2, Carbon dioxide consumption; RER, Respiratory exchange ratio.

Table 4.2
Plasma parameters, liver TAG levels and faecal lipid content in mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 7 or 15 weeks.

	7 weeks		15 weeks	
	LF-CAS	LF-WPI	LF-CAS	LF-WPI
Plasma TAG (mg/dL)	28.71 ± 4.07	39.95 ± 5.08	36.18 ± 1.42	33.68 ± 2.59
Plasma leptin (ng/ml)	2.05 ± 0.26[a]	3.30 ± 0.49[b]	6.65 ± 1.53	5.80 ± 1.38
Plasma GLP-1 (pM)	16.14 ± 1.25	20.64 ± 2.34	24.70 ± 3.50	23.59 ± 1.50
Plasma glucose (mmol/L)	13.13 ± 0.95	11.67 ± 0.94	12.18 ± 1.57	13.11± 0.84
Plasma insulin (ng/ml)	0.12 ± 0.01	0.16 ± 0.02	0.21 ± 0.01	0.22 ± 0.01
HOMA-IR	1.76 ± 0.21	2.04 ± 0.17	3.07 ± 0.39	3.12 ± 0.16
Liver TAG (mg/g Tissue)	32.88 ± 1.07	29.97 ± 1.63	54.62 ± 2.08	53.50 ± 2.32
Faecal lipid content (%)	14.80 ± 1.69	13.88 ± 2.37	7.80 ± 0.58	9.13 ± 1.41

Data are means ± SEM (n = 8). For each trial length, values in a row without a common letter differ significantly, $P \leq 0.05$.

TAG, triacylglycerol; GLP-1, Glucagon-like peptide 1; HOMA-IR, homeostasis model assessment of insulin resistance.

Table 4.3
Relative skeletal muscle gene expression in mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 7 or 15 weeks.

	7 Weeks			15 Weeks		
	LF-CAS	LF-WPI	*P* value	LF-CAS	LF-WPI	*P* value
IR	1.00 ± 0.14	0.71 ± 0.06	0.09	1.00 ± 0.19	1.01 ± 0.08	NS
GLUT4	1.00 ± 0.10	1.04 ± 0.06	NS	1.00 ± 0.10	0.82 ± 0.05	NS
IRS-1	1.00 ± 0.08	0.78 ± 0.04	<.05	1.00 ± 0.08	0.81 ± 0.04	0.06
CPT1b	1.00 ± 0.08	0.87 ± 0.07	NS	1.00 ± 0.13	0.85 ± 0.06	NS
UCP-3	1.00 ± 0.09	1.14 ± 0.09	NS	1.00 ± 0.17	0.94 ± 0.09	NS
PPARα	1.00 ± 0.17	1.05 ± 0.18	NS	1.00 ± 0.17	0.93 ± 0.19	NS
PPARγ	1.00 ± 0.12	1.72 ± 0.32	0.07	1.00 ± 0.26	0.89 ± 0.21	NS
LPL	1.00 ± 0.16	1.48 ± 0.29	NS	1.00 ± 0.14	0.87 ± 0.16	NS
M-GS	1.00 ± 0.10	1.05 ± 0.07	NS	1.00 ± 0.10	0.83 ± 0.05	NS
ACC	1.00 ± 0.10	2.95 ± 0.82	<.05	1.00 ± 0.43	0.62 ± 0.21	NS
CD36	1.00 ± 0.28	0.62 ± 0.10	NS	1.00 ± 0.14	1.05 ± 0.15	NS

Data are means ± SEM (n = 7-8). For each dietary trial length, values in a row without a common letter differ significantly, $P < 0.05$; NS, non-significant. Gene expressions are shown relative to the LF-CAS group which is set at 1.00.

IR, Insulin receptor; GLUT4, Glucose transporter 4; IRS-1, Insulin receptor substrate 1; CPT1b, Carnitine palmitoyltransferase 1b; UCP-3, Uncoupling protein 3; PPARα, Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ; LPL, Lipoprotein lipase; M-GS, Muscle glycogen synthase; ACC, Acetyl-CoA carboxylase; CD36, Cluster of differentiation 36.

Table 4.4
Relative liver gene expression in mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 7 or 15 weeks.

	7 Weeks			15 Weeks		
	LF-CAS	LF-WPI	*P* value	LF-CAS	LF-WPI	*P* value
GLUT2	1.00 ± 0.04	1.14 ± 0.08	NS	1.00 ± 0.12	1.03 ± 0.09	NS
IRS-1	1.00 ± 0.04	0.81 ± 0.07	<.05	1.00 ± 0.12	0.93 ± 0.06	NS
IGF-1	1.00 ± 0.05	1.21 ± 0.07	<.05	1.00 ± 0.09	0.95 ± 0.07	NS
CPT1a	1.00 ± 0.12	1.12 ± 0.07	NS	1.00 ± 0.18	1.08 ± 0.12	NS
UCP-2	1.00 ± 0.09	0.83 ± 0.04	NS	1.00 ± 0.07	1.07 ± 0.08	NS
PPARα	1.00 ± 0.10	1.06 ± 0.05	NS	1.00 ± 0.14	1.06 ± 0.10	NS
PPARγ	1.00 ± 0.23	0.48 ± 0.15	0.09	1.00 ± 0.17	0.76 ± 0.15	NS
L-GS	1.00 ± 0.11	1.03 ± 0.10	NS	1.00 ± 0.16	0.93 ± 0.13	NS
FASN	1.00 ± 0.10	0.54 ±0.12	<.05	1.00 ± 0.29	0.96 ± 0.12	NS
ACC	1.00 ± 0.08	0.84 ± 0.07	NS	1.00 ± 0.14	1.10 ± 0.14	NS
LPL	1.00 ± 0.10	0.36 ± 0.07	<.001	1.00 ± 0.09	0.96 ± 0.07	NS
FABP1	1.00 ± 0.05	0.68 ± 0.16	0.08	1.00 ± 0.13	0.82 ± 0.07	NS
CD36	1.00 ± 0.21	0.71 ± 0.29	NS	1.00 ± 0.20	0.92 ± 0.07	NS

Data are means ± SEM (n = 7-8). For each dietary trial length, values in a row without a common letter differ significantly, $P < 0.05$; NS, non-significant. Gene expressions are shown relative to the LF-CAS group which is set at 1.00.

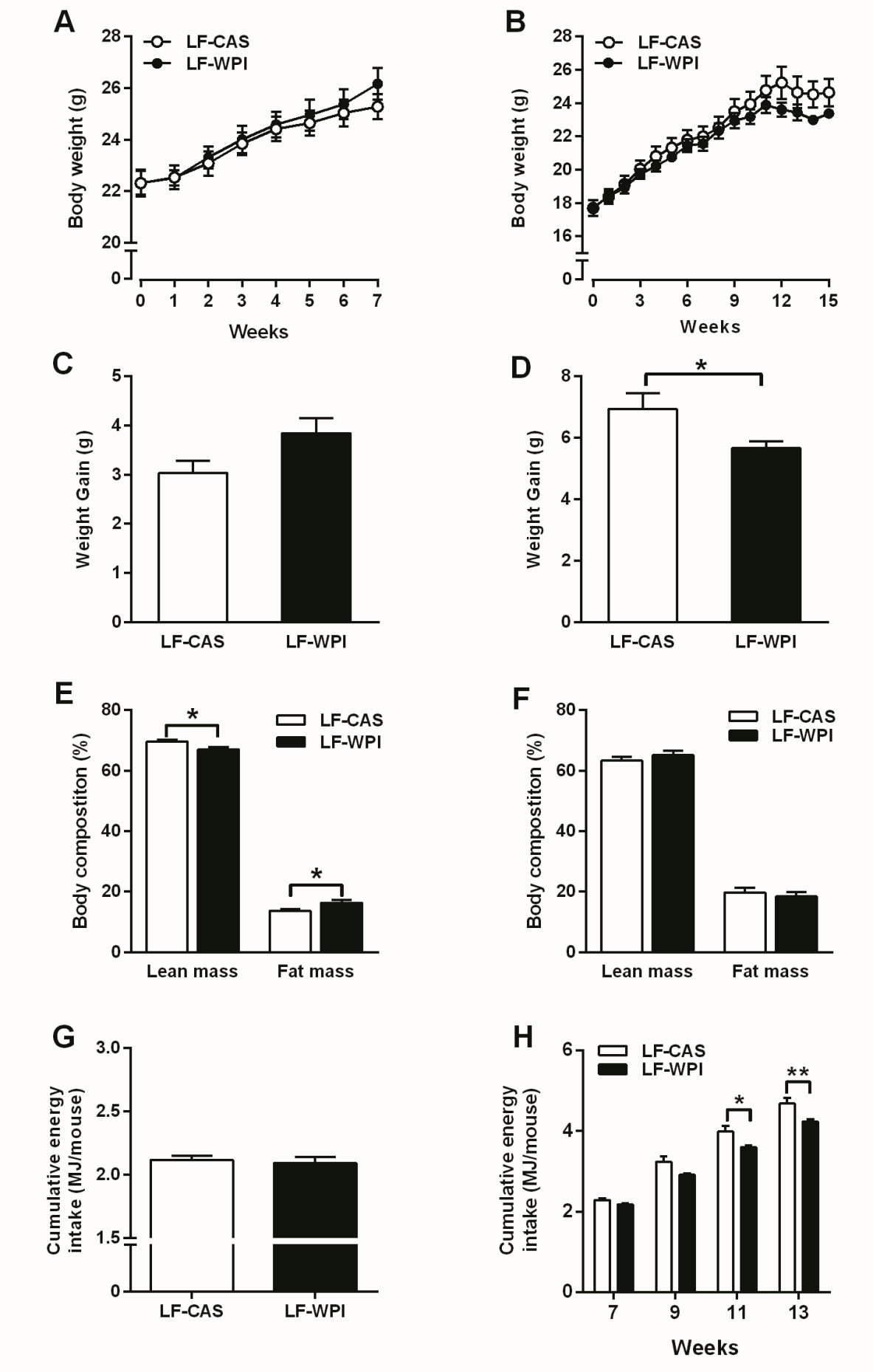
GLUT2, Glucose transporter 2; IRS-1, Insulin receptor substrate 1; IGF-1, Insulin-like growth factor 1; CPT1a, Carnitine palmitoyltransferase 1a; UCP-2, Uncoupling protein 2; PPARα, Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ; L-GS, Liver glycogen synthase; FASN, Fatty acid synthase; ACC, Acetyl-CoA carboxylase; LPL, Lipoprotein lipase; FABP1, Fatty acid binding protein 1; CD36, Cluster of differentiation 36.

Table 4.5
Relative intestinal (duodenal & ileual) expression in mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 15 weeks.

	LF-CAS	LF-WPI	*P* value
Duodenum			
IR	1.00 ± 0.12	0.68 ± 0.09	0.05
GLUT2	1.00 ± 0.09	0.94 ± 0.06	NS
IRS-1	1.00 ± 0.09	0.84 ± 0.07	NS
SGLT-1	1.00 ± 0.07	0.83 ± 0.05	0.07
CCK	1.00 ± 0.05	0.76 ± 0.15	NS
GIP	1.00 ± 0.10	0.65 ± 0.15	NS
PPARα	1.00 ± 0.11	0.81 ± 0.10	NS
FATP4	1.00 ± 0.06	1.13 ± 0.13	NS
FABP1	1.00 ± 0.12	0.81 ± 0.09	NS
CD36	1.00 ± 0.30	0.56 ± 0.12	NS
DGAT	1.00 ± 0.12	0.81 ± 0.09	NS
Occludin	1.00 ± 0.11	0.80 ± 0.07	NS
Ileum			
IR	1.00 ± 0.04	1.00 ± 0.07	NS
GLUT2	1.00 ± 0.21	1.89 ± 0.31	<.05
IRS-1	1.00 ± 0.06	1.13 ± 0.17	NS
SGLT-1	1.00 ± 0.09	0.99 ± 0.10	NS
Proglucagon	1.00 ± 0.07	1.13 ± 0.17	NS
PYY	1.00 ± 0.11	0.60 ± 0.06	<.01
PPARα	1.00 ± 0.11	0.99 ± 0.12	NS
FATP4	1.00 ± 0.08	0.77 ± 0.04	<.05
FABP1	1.00 ± 0.45	1.87 ± 0.56	NS
CD36	1.00 ± 0.19	1.56 ± 0.60	NS
DGAT	1.00 ± 0.06	1.08 ± 0.10	NS
Occludin	1.00 ± 0.05	0.85 ± 0.09	NS

Data are means ± SEM (n = 6-8). $P < 0.05$ differ significantly; NS, non-significant. Gene expressions are shown relative to the LF-CAS group which is set at 1.00.

IR, Insulin receptor, GLUT2, Glucose transporter 2; IRS-1, Insulin receptor substrate 1; SGLT-1, Sodium-glucose co-transporter 1; CCK, Cholecystokinin; GIP, Glucose-dependent insulinotrophic peptide; PYY, Peptide YY; PPARα, Peroxisome proliferator-activated receptor α; FATP4, Fatty acid transport protein 4; FABP1, Fatty acid binding protein 1, CD36, Cluster of differentiation 36; DGAT, Diglyceride acyltransferase.


A
Body weight (g)
LF-CAS
LF-WPI
Weeks
B
Body weight (g)
LF-CAS
LF-WPI
Weeks
C
Weight Gain (g)
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LF-WPI
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Weight Gain (g)
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LF-CAS
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Body compostiton (%)
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LF-CAS
LF-WPI
Lean mass
Fat mass
F
Body compostiton (%)
LF-CAS
LF-WPI
Lean mass
Fat mass
G
Cumulative energy intake (MJ/mouse)
LF-CAS
LF-WPI
H
Cumulative energy intake (MJ/mouse)
LF-CAS
LF-WPI
*
**
Weeks

Figure 4.1 Body weight, body composition and energy intake of C57BL/6J mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 7 weeks (Study 1; A, C, E, G) or 15 weeks (Study 2; B, D, F & H). The body weight trajectories over the 7 week (A) and 15 week (B) dietary treatment periods. Total weight gain after 7 weeks (C) and 15 weeks (D) of dietary treatment. Body composition (lean and fat mass %) after 7 weeks (E) and 15 weeks (F) of dietary treatment. Cumulative energy intake over the first 6 weeks (G) and 13 weeks (H) of dietary treatment. Data represent means ± SEM (n = 8). * $P < 0.05$, ** $P < 0.01$.

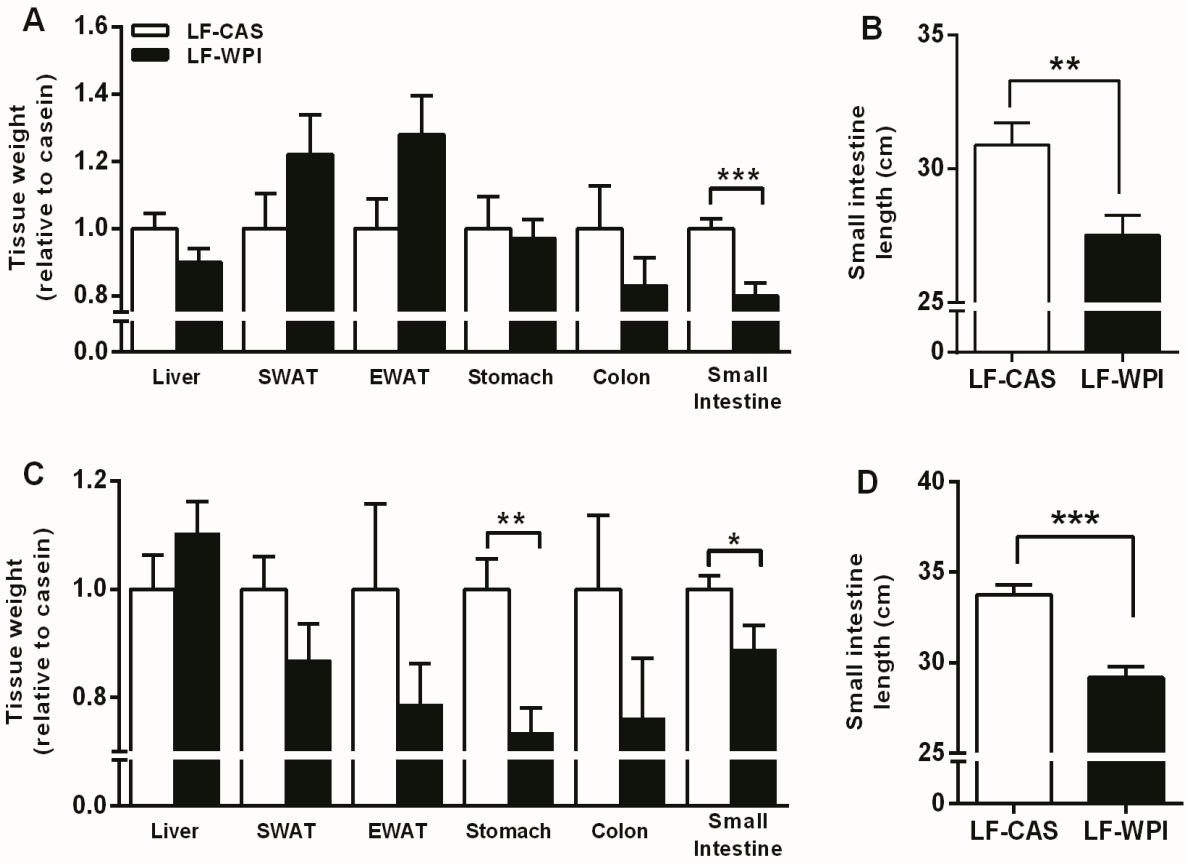

Figure 4.2 Tissue weights and intestinal length in C57BL/6J mice fed a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for 7 weeks (Study 1; A-B) and 15 weeks (Study 2; C-D). Relative tissue weights after 7 weeks (A) and 15 weeks (C) of dietary treatment. Intestinal lengths after 7 weeks (B) and 15 weeks (D) of dietary treatment. Tissue weights are shown relative to casein fed animals, which were set as 1.00 for both studies. Data represent means ± SEM (n = 8). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$. SWAT, subcutaneous white adipose tissue; EWAT, epididymal white adipose tissue.

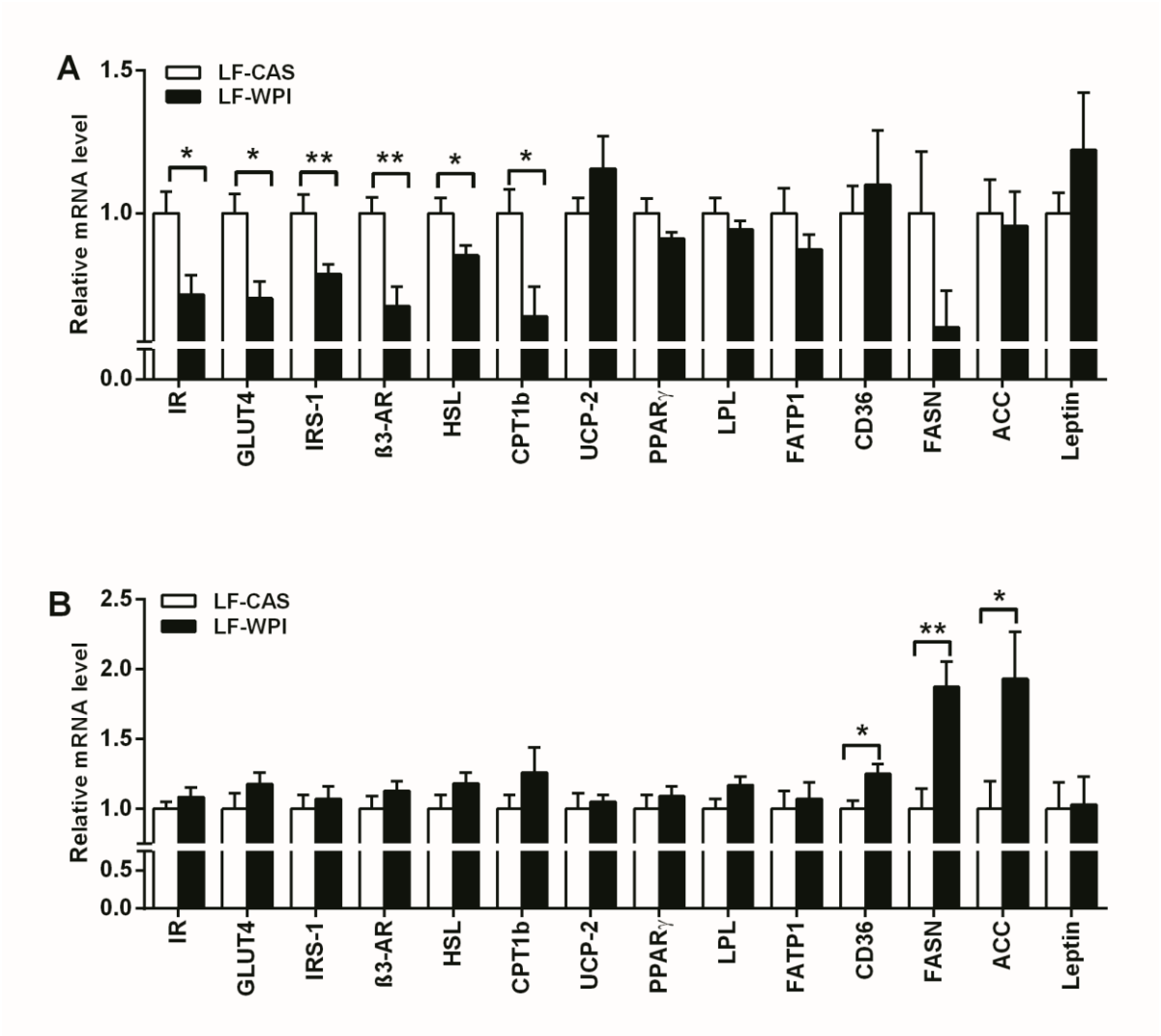

Figure 4.3 The effects of feeding C57BL/6J mice a low fat diet with casein protein (LF-CAS) or whey protein isolate (LF-WPI) for (A) 7 weeks (Study 1) or (B) 15 weeks (Study 2) upon their epididymal adipose tissue mRNA expression of insulin receptor (IR), glucose transporter 4, (GLUT4), insulin substrate 1 (IRS-1), βeta-3 adrenergic receptor (β3-AR), hormone sensitive lipase (HSL) carnitine palmitoyltransferase 1b (CPT1b), uncoupling protein 2 (UCP-2), peroxisome proliferator-activated receptor γ (PPARγ), lipoprotein lipase (LPL), fatty acid transporter 1 (FATP1), cluster of differentiation 36 (CD36), fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC) and leptin. Gene expressions are shown relative to the LF-CAS group which is set at 1.00. Data represent means ± SEM (n = 8). * $P < 0.05$, ** $P < 0.01$.

Chapter 4

SUPPLEMENTARY INFORMATION

Supplementary table 4.1

Sequences of mouse specific primers used in real-time PCR analysis

	Forward primer (5'-3')	Reverse primer (5'-3')
ACC	5-tgttgagacgctggtttgtagaa-3'	5'-ggtccttattattgtcccagacgta-3'
β3-AR	5'-cgccttcaacccggtcatctactg-3'	5'-ggtggactctgcctggcttcaac-3'
CD36	5'-tgatactatgcccgcctctcc-3'	5'-tttcccacactcctttctcctcta-3'
CPT1a	5'-agacttccaacgcatgacagcactg-3'	5'-ctcggccccgcaggtagatg-3'
CPT1b	5'-cgagaggggcggactgagactg-3'	5'-ggctaggcggtacatgttttggtg-3'
CCK	5'-gtgccgaggactacgaatacc-3'	5'-tctgggagtcactgaaggaaacac-3'
DGAT	5'-cacaggtgccgtcttgggttatc-3'	5'-cagacttggggtgtggctcagga-3'
FABP1	5'-gaagcctcgttgccaccat-3'	5'-cgatttctgacacccccttgat-3'
FASN	5'-tccacctttaagttgccctg-3'	5'-tctgctctcgtcatgtcacc-3'
FATP1	5'-ccggtgtggtggctgctcttctc-3'	5'-gctgccatctccccgccataaatg-3'
FATP4	5'-tggcgtttcatccgggtcttcatc-3'	5'-gcaaacagcaggggcaccgtcttc-3'
GIP	5'-tctctgttgctggtgctcctgttc-3'	5'-gctctcctgtgcctctttgtcctc-3'
GLUT2	5'-tcctacttggcctatctgctgtgc-3'	5'-tgccctgacttcctcttccaac-3'
GLUT4	5'-ggcctgcccgaaagagtc-3'	5'-aggagctggagcaaaggac-3'
HSL	5'-ctattcagggacagaggcag-3'	5'-cgatgtggtcttttggggc-3'
IGF-1	5'-gtgtggaccgaggggcttttac-3'	5'-ggggcacagtacatctccagtctc-3'
IR	5'-gatttccccaacgtgtcctctac-3'	5'-caatgcggtacccagtgaagtg-3'
IRS-1	5'-gcgcaggcaccatctcaacaacc-3'	5'-gcacgcacccggaaggaacc-3'
Leptin	5'-ccccgcaccgctggaagtac-3'	5'-atgtgccctgaaatgcggtatg-3'
LPL	5'-tgctcccaacaatataagactcc-3'	5'-aaggccaggtgtttcaatc-3'
L-GS	5'-gagttgctggctcaccctgtag-3'	5'-agcggcctctgagcattcttc-3'
M-GS	5'-tacgaccgcgcggccaatgacg-3'	5'-cacagaccaacgccagccaacc-3'
Occludin	5'-atgtccggccgatgctctc-3'	5'-tttggctgctcttgggtctgtat-3'
PPARα	5'-atgggggtgatcggaggctaatag-3'	5'-gggtggcaggaagggaacagac-3'
PPARγ	5'-tcaggtttgggcggatgc-3'	5'-tcagcgggaaggactttatgtatg-3'
Peptide YY	5'-ggacgcctaccctgccaaacca-3'	5'-agtgccctcttcttaaaccaaaca-3'
Pro-glucagon	5'-agggacctttaccagtgatgtga-3'	5'-acgagatgttgtgaagatggttgt-3'
SGLT-1	5'-gagccccgcggttactgc-3'	5'-cctgcggctgctcctgtg-3'
UCP-2	5'-ccatttcctgcaccccgatttacttcc-3'	5'-gctgggctggggatgaagatgaag-3'
UCP-3	5'-acaggcccacacggtccagaacc-3'	5'-cccatcaggtcagtgcaaaacagagg-3'
β-actin	5'-agagggaaatcgtgcgtgac-3'	5'-caatagtgatgacctggcgt-3'
18-S	5'-aggaccgcggttctattttgttgg-3'	5'-atgctttcgctct-ggtccgtcttg-3'

Acetyl-CoA carboxylase; β3-AR, Beta-3 adrenergic receptor; CD36, Cluster of differentiation 36; CPT1a, Carnitine palmitoyltransferase 1a; CCK, Cholecystokinin; DGAT, Diglyceride acyltransferase; FABP1, Fatty acid binding protein 1; FASN, Fatty acid synthase; FATP1, Fatty acid transport protein 1; GIP,

Glucose-dependent insulinotropic peptide; GLUT4, Glucose transporter 4; HSL, Hormone sensitive lipase; IGF-1, Insulin-like growth factor; IR, Insulin receptor; IRS-1; Insulin receptor substrate 1; LPL, Lipoprotein lipase; L-GS, Liver glycogen synthase; M-GS, Muscle glycogen synthase; PPARγ, Peroxisome proliferator-activated receptor γ; SGLT-1, Sodium-glucose co-transporter 1; UCP-2, Uncoupling protein 2.

Chapter 5

Lysophosphatidic acid receptor 5 is a novel candidate in the regulation of 3T3-L1 pre-adipocyte growth and differentiation

To be submitted to *Lipids in Health and Disease* as:

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5.1 ABSTRACT

Lysophosphatidic acid receptor 5 (LPA_5) may have roles in the regulation of energy balance, although the supporting evidence is inconclusive. In the present study we sought to determine whether LPA_5 could regulate adipocyte activity linked to lipid metabolism. To that end, LPA_5 mRNA expression in mouse epididymal adipose tissue in response to energetic challenges was investigated, as well as the receptor's influence on 3T3-L1 pre-adipocyte proliferation and differentiation. LPA_5 mRNA expression was found to be increased in the epididymal adipose tissue of high fat diet fed mice and obese *ob/ob* mice compared to appropriate controls. Differentiation of wild type 3T3-L1 cells also increased LPA_5 receptor mRNA expression. To understand the cellular role of the receptor, LPA_5 mRNA was over-expressed in 3T3-L1 pre-adipocytes. This manipulation provided a growth advantage to the 3T3-L1 pre-adipocytes, and it also increased the mRNA expression of genes previously shown to be involved in cellular growth, namely β-actin and LPA_1. Upon stimulation with an adipogenic cocktail, cellular differentiation was partially suppressed in 3T3-L1 pre-adipocytes over-expressing the receptor, as indicated by decreased mRNA expression of peroxisome proliferator activated receptor-γ, fatty acid synthase, glucose transporter 4 and β3-adrenergic receptor, and by increased LPA_1 mRNA expression. Correspondingly, lipid accumulation as measured by Oil red O lipid staining was significantly reduced in these differentiating cells over-expressing the receptor. These data suggest an important role for LPA_5 in pre-adipocyte growth and differentiation.

5.2 INTRODUCTION

Lysophosphatidic acid (LPA) is a bioactive glycerophospholipid that regulates a wide variety of cellular responses including platelet aggregation, cell proliferation and neurite retraction (Choi *et al*, 2010; Moolenaar, 1999). LPA has been detected in several biological fluids including the extracellular fluid in the adipose tissue (Choi *et al*, 2010; Valet *et al*, 1998) and is primarily produced by the hydrolysis of phospholipids resulting from the actions of a secreted lysophospholipase D enzyme, called autotaxin (Nakanaga *et al*, 2010). LPA mediates its effects through several G-protein coupled receptors (GPRs) (Choi *et al*, 2010). To date, six characterised LPA receptors (LPA_{1-6}) have been identified, and these can be divided into two subfamilies; LPA_{1-3} belong to the endothelial cell differentiation (Edg) subfamily, while the structurally distinct LPA_{4-6} make up the non-Edg subfamily (Yanagida & Ishii, 2011). The receptors show a wide tissue distribution, although the level of expression of each of the LPA receptors varies between tissues (Choi *et al*, 2010). This, along with gene deletion studies (Choi *et al*, 2008), suggests that distinct receptors could mediate specific actions of LPA including the recently identified roles in the regulation of energy balance.

It has been shown that LPA increases proliferation of 3T3-F442A pre-adipocytes by activation of LPA_1 (Pages *et al*, 2001; Valet *et al*, 1998), and suppresses their differentiation into lipid laden mature adipocytes by decreasing the expression of peroxisome proliferator activated receptor (PPAR)-γ2 (Simon *et al*, 2005). Moreover, mice generated with the targeted deletion of LPA_1 show increased adiposity compared to wild-type mice (Simon *et al*, 2005). These data suggest important role(s) for LPA in the regulation of adipogenesis, the process by which pre-adipocytes attain a mature cell fate (Fajas, 2003). Recently, a potential

role for LPA_5 (GPR93/92) in the regulation of food intake has been suggested based on the evidence that LPA_5 activation in intestinal STC-1 enteroendocrine cells augments the production and the release of the satiation hormone cholecystokinin (CCK) (Choi *et al*, 2007a; Choi *et al*, 2007b). Moreover, the stimulation of CCK gene expression and protein secretion upon cell stimulation with protein hydrolysate was observed to be enhanced in these cells over-expressing the LPA_5 receptor, indicating a potential mechanism by which dietary protein could regulate food intake (Choi *et al*, 2007b).

While the above data supports a role for LPA_5 in the regulation of energy balance, here, we aimed to obtain further evidence of whether the receptor does indeed have a role in energy balance regulation. To this end, we focussed on the adipose tissue and assessed whether LPA_5 could regulate the activity of these cells. We provide *in vivo* and *in vitro* evidence supporting a role for the receptor in the regulation of pre-adipocyte proliferation and differentiation.

5.3 MATERIALS & METHODS

5.3.1 Materials

The pCI-neo plasmid containing the coding sequence of the rat LPA_5 gene (Choi *et al*, 2007a), was kindly provided by Professor G. W. Aponte (University of California, Berkeley). All reagents were purchased from Sigma-Aldrich unless otherwise indicated.

5.3.2 Animals

Experiments involving mice were licensed under the Cruelty to Animals Act 1876 and received ethical approval from the University College Cork Animal Ethics

Review Committee. Male 3-4 week old C57BL/6J mice (Harlan, UK) were group housed in cages (n = 5 per cage) maintained at 21 ± 1°C and humidity 55 ± 10%, with a 12 h light/dark cycle (06:00-18:00). Mice had *ad libitum* access to food and water, unless otherwise stated. Two experiments were conducted after the initial four week acclimatisation period. In the first, mice were either fasted or had *ad libitum* access to a low fat diet (LFD) (10% kJ as fat) (Research Diets, NJ, USA) for a 24 hour period (n = 5). In the second experiment, mice were provided with either the LFD or a high fat diet (HFD) (45% kJ as fat) (Research Diets, NJ, USA) for 14 weeks (n = 5). All mice were sacrificed by cervical dislocation. The epididymal adipose fat pads were harvested, and snap frozen in liquid nitrogen and stored at -80°C. In addition, epididymal adipose tissue samples corresponding to lean (+/?) and obese (*ob/ob*) mice (n = 8) from a previous study (Murphy *et al*, 2010) were also used to investigate the expression of genes of interest.

5.3.3 Cell culture

3T3-L1 pre-adipocyte cells were purchased from European Cell Culture Collection. The 3T3-L1 pre-adipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) penicillin/streptomycin maintained at 37°C in a 5% (v/v) CO_2 incubator. The media was changed every 2 days, and the cells were grown to approx. 80 % confluency, at which point they were harvested with trypsin, and either stored at -80°C for subsequent analysis of gene expression (undifferentiated) or re-seeded into new plate(s).

To generate the 3T3-L1 clones over-expressing LPA_5 (3T3-LPA5), 3T3-L1 cells in supplemented DMEM were seeded at $1x10^5$ cells/ml into 35-mm cell

culture dishes. After 24 hours, the media was replaced with unsupplemented DMEM and the cells were transfected with 2μg of the pCI-neo plasmid containing the LPA_5 DNA using the FuGENE HD transfection reagent (Clone 1), or the X-treme GENE HP transfection reagent (Roche, Germany) (Clone 2) according to the manufactures' instructions. After 3 hours, supplemented DMEM was added to the dish to allow cell growth. Parallel experiments were also performed to transfect 3T3-L1 cells with the pCI-neo plasmid (Promega, USA) without an insert DNA (3T3-NEO). The cells stably transfected with the plasmids of interest were selected using Geneticin (G418) (Promega, USA) at 500 μg/ml. All subsequent experiments involving stably transfected 3T3-LPA5 and 3T3-NEO cells contained G418 in the media. In experiments where cellular activity was compared between 3T3-LPA5 and 3T3-NEO cells, the same passage number in both cell lines was used.

To differentiate 3T3-L1 cell lines, the corresponding pre-adipocytes were seeded into either 35mm cell culture dishes at $2.5x10^5$ cells/ml or 96 well plates at $1.5x10^5$ cells/ml. Two days post-confluency, the cells were stimulated with 5μg/ml of bovine insulin, 0.4μg/ml dexamethasone and 0.5mM 3-iso-butyl-1-methylxanthine in supplemented DMEM. After 3 days, the adipogenic media was replaced with DMEM containing 5μg/ml bovine insulin. This step was performed every 2^{nd} day thereafter until 10 days had passed since differentiation was initiated, at which point the cells were harvested for analysis of gene expression (35mm dishes) or stained with Oil red O for lipid quantification (96 well plates).

5.3.4 Cell growth assay

The xCELLigence system (Roche and ACEA biosciences) was used to measure cell growth in clone 1 of 3T3-NEO and 3T3-LPA5 pre-adipocytes. This system detects

electronic impedance resulting from changes in number/morphology of cells grown in E-plates (Roche). The relative changes are represented as cell index (CI), where CI = $(Z_i - Z_0)/15$ (Z_i: individual time point impedance, Z_0: Background impedance). Experiments to assess the impact of LPA_5 over-expression on cell growth were conducted using cells seeded at 25 cells/µl in supplemented DMEM at 200 µl per E-plate well. The CI for 3T3-NEO and 3T3-LPA5 pre-adipocytes was normalised to a single time point to account for any discrepancies in the starting cell number and was compared every hour over a 64 hour period.

5.3.5 Oil red O staining and lipid quantification

To stain differentiated 3T3-LPA5 and 3T3-NEO adipocytes, cells were washed twice with PBS before being fixed in 3.7% (w/v) paraformaldehyde for 1 hour. Cells were then washed again twice with PBS before being stained with 0.2% (w/v) Oil red O working solution for 20 minutes, after which time the cells were washed with distilled water until all excess Oil red O dye solution was removed. The working solution was a 3:2 ratio of Oil red O stock solution (0.35g Oil red O dye dissolved in 100ml isopropanol) to distilled water, which was filtered through a 0.2µm membrane before use. The Oil red O dye was extracted from stained adipocytes by treating them with equal volumes of 100% isopropanol, and the lipid content was quantified by measuring the absorbance of the solution at 520nm.

5.3.6 RNA extraction and Real-time PCR analysis

Total RNA was isolated from frozen cell pellets (3T3-L1, 3T3-LPA5 and 3T3-NEO) and from mouse epididymal adipose tissue into diethylpyrocarbonate (DEPC)

treated distilled water using the RNeasy mini kit (Qiagen, Germany) according to the manufacturers' instructions. To degrade any potential genomic DNA contamination, on-column DNase treatment (Qiagen, Germany) was performed during RNA isolation. Complementary DNA (cDNA) was synthesised from equal quantities (either 500ng or 1µg) of total RNA using 2.5ng/µl random hexamer primers (Bioline, UK), 0.5mM dNTP (Promega, USA), 2U/µl RNase inhibitor (Promega, USA) and the first strand synthesis system containing the Super Script II reverse transcriptase enzyme (Invitrogen, UK), according to the manufacturers' instructions. A parallel reaction without the inclusion of the reverse transcriptase enzyme was also performed as a negative control. The amplification of the cDNA was performed in the Lightcycler 480 system (Roche) using 0.25µM primers (Eurofins MWG operon), 1µl cDNA and the Lightcycler 480 SYBR Green I Master kit (Roche), according to manufacturers' instructions. Real-time PCR conditions were: 95^{o}C for 10 min followed by 50 cycles at 95^{o}C for 10s, 60^{o}C for 5s, and 72^{o}C for 15s. Primers used are listed in table 1. Automated sequencing was performed to verify PCR product sequences. Melting curve analysis also allowed validation of the authenticity of the real-time PCR products. Data obtained as C_p values were normalised to the expression of either GAPDH (adipose tissue) or 18S (3T3-adipocytes) according to $\Delta\Delta C_p = \Delta C_p$ target gene $- \Delta C_p$ housekeeping gene. The gene expression was expressed as $2^{-\Delta\Delta Cp}$ and was shown relative to the expression of the control group.

5.3.7 Statistical analysis

Data are presented as means ± SEM, with results for the 3T3 cell line work being the mean of 3-4 independent experiments. Differences between experimental

groups were analysed by Students T-test or One way ANOVA with *Tukey* post-hoc multiple comparison analysis. Repeated measures two-way ANOVA with *Bonferroni* post-hoc multiple comparisons was used to analyse the cell growth measured using the xCELLigence system. Non-parametric data was analysed by Mann-Whitney tests. Significance was accepted at $P < 0.05$, and the statistical analysis was performed using GraphPad Prism version 6 (San Diego, CA, USA).

5.4 RESULTS

5.4.1 LPA_5 expression was increased in the epididymal adipose tissue of obese mice and in differentiated pre-adipocytes

Feeding a HFD for 14 weeks increased the body weight of mice (35.82 g ± 0.79 in HFD group vs. 26.06 g ± 1.70 in LFD group; $P < 0.05$) (n = 5). HFD feeding and *ob* gene mutation induced weight gain was associated with increased LPA_5 mRNA expression in the epididymal adipose tissue (Fig. 5.1A and C). Fasting for 24 hours, decreased the body weight of mice (23.59 g ± 0.63 in fasted vs. 27.80 g ± 0.39 in *ad libitum* fed; $P < 0.05$) (n = 5), but did not affect the mRNA expressions of LPA_5 (Fig. 5.1E). The mRNA expression of LPA_1 did not respond to any of the energetic challenges (Fig. 5.1B, D and F). The expression of adipogenesis associated genes PPARγ and fatty acid synthase (FASN) were also investigated. The energetic challenges did not significantly alter PPARγ mRNA expression (1.00 ± 0.18 in LFD vs. 0.89 ± 0.13 in HFD; 1.00 ± 0.12 in +/? vs. 1.20 ± 0.15 in *ob/ob*; 1.00 ± 0.37 *in ad libitum* vs. 1.81 ± 0.61 in fasted). FASN mRNA expression was decreased in HFD fed mice (1.00 ± 0.24 in LFD vs. 0.25 ± 0.04 in HFD; $P < 0.05$) and in obese *ob/ob* mice (1.00 ± 0.09 in +/? vs. 0.28 ± 0.03 in *ob/ob*; $P < 0.001$) with no significant change

observed in fasted mice compared to their respective controls (1.00 ± 0.57 in *ad libitum* vs. 0.45 ± 0.15 in fasted).

To obtain further insight into how the observed LPA_5 gene expression changes in response to energetic challenges may relate to adipocyte function and lipid metabolism in the adipose tissue, we compared the expression between differentiated (Diff) and undifferentiated (Undiff) 3T3-L1 cells grown *in vitro*. Cellular differentiation increased mRNA expression of LPA_5 (Fig. 5.2A). Differentiation also decreased LPA_1 mRNA expression (Fig. 5.2B) and increased the mRNA expression of PPARγ (Fig. 5.2C; $P < 0.001$) and FASN (Fig. 5.2D; $P < 0.001$), as previously shown for 3T3-F442 cells (Schmid *et al*, 2005; Simon *et al*, 2005).

5.4.2 LPA_5 influenced 3T3-L1 pre-adipocyte cellular activity

To further define the role of the receptor in adipogenesis, we first focused on the pre-adipocyte stage and assessed whether increasing the endogenous mRNA (which is low compared to differentiated stage) may alter cellular activity. To this end, two stable 3T3-L1 clones over-expressing the receptor mRNA (3T3-LPA5) were created (Fig. 5.3A-B). The mRNA expressions for both LPA_1 (Fig. 5.3C-D) and β-actin (Fig. 5.3E-F) in both clones of 3T3-LPA5 cells were found to be increased compared to 3T3-NEO controls ($P < 0.05$). Investigation of cellular growth using xCELLigence technology revealed growth in clone 1 of the 3T3-LPA5 cells was increased compared to its respective clone of 3T3-NEO control cells (Fig. 5.4A), which became statistically significant at 52 hours ($P < 0.05$) and continued to increase in significance until termination of the experiment ($P < 0.001$).

5.4.3 LPA_5 influenced the cellular activity of differentiating pre-adipocytes

Differentiation of 3T3-LPA5 (clone 1) cells decreased LPA_5 mRNA expression (Fig. 5.5A), but increased LPA_1 mRNA (Fig. 5.5B). In addition, the mRNA expressions of several other adipogenesis-associated genes, namely PPARγ, fatty acid synthase (FASN), β3-adrenergic receptor (β3-AR) and glucose transporter 4 (GLUT4) (Fig. 5.5C-F), were also decreased in these cells over-expressing LPA_5. However, the mRNA expressions of β-actin (3T3-NEO, 1.00 ± 0.14 vs. 3T3-LPA5, 0.79 ± 0.09) and Wnt10b (3T3-NEO, 1.00 ± 0.08 vs. 3T3-LPA5, 1.08 ± 0.12) (a ligand of the Wnt signalling pathway important for pre-adipocyte differentiation) were not significantly different between these differentiated 3T3-LPA5 and 3T3-NEO cells. To determine if the decreased adipogenesis-associated gene expression was also associated with an expected reduction in lipid accumulation, Oil red O staining and quantification was performed on cells at day 10 of differentiation. Data showed a significant reduction in relative lipid content in differentiating 3T3-LPA5 (clone 1) cells compared to 3T3-NEO (clone 1) controls ($P < 0.001$; Fig. 5.6A).

5.5 DISCUSSION

In the present study we show evidence that LPA_5 could regulate cellular activity linked to pre-adipocyte growth and differentiation, which suggests an important role for the receptor in the regulation of adipogenesis.

Development of adiposity in HFD fed and obese *ob/ob* mice is accompanied by cellular hyperplasia and hypertrophy (Faust *et al*, 1978; Hausman *et al*, 2001; Johnson *et al*, 1978; Lemonnier, 1972; Marques *et al*, 1998), suggesting a role for adipogenesis in the development of the phenotype. In contrast, food-deprivation, which decreases adiposity, does not change cell number (Miller *et al*, 1983). As LPA_5 expression was increased in the epididymal adipose tissue of HFD fed and

obese *ob/ob* mice, but was not altered in 24h fasted mice, one could argue that the expression level of LPA_5 in the obese mice could be linked to some change associated with the development of adiposity, including pre-adipocyte proliferation and/or differentiation. In further support of this suggestion, we observed an increased LPA_5 mRNA expression in differentiating 3T3-L1 cells.

To understand the cellular role of the receptor in the pre-adipocyte stage, we increased the endogenous levels of LPA_5 mRNA, which is low compared to the differentiated state, by creating a stable pre-adipocyte 3T3-L1 cell line over-expressing the LPA_5 mRNA (3T3-LPA5). Previous studies have shown that (1) LPA_5 is expressed in RH7777 rat hepatoma cells and that its expression correlated with the (un)methylated status of the promoter (Okabe *et al*, 2011), (2) LPA_5 activation of Rho/Rho-kinase signalling pathway causes changes in neurite retraction (Lee *et al*, 2006) and (3) this Rho-kinase signalling pathway initiates actin fibre formation (Arnsdorf *et al*, 2009), the disruption of which impedes cell growth and migration (Bunnell *et al*, 2011). Consistent with this notion of LPA_5 signalling through the above pathway, we observed an increased β-actin expression in 3T3-L1 cells over-expressing the receptor. Moreover, the investigation of cellular growth using xCELLigence technology revealed the growth of 3T3-LPA5 cells to be increased compared to its 3T3-NEO control cells. Thus the data are consistent with the suggestion that LPA_5 activation in 3T3-L1 cells provides a growth advantage to the cells.

LPA is known to promote pre-adipocyte proliferation via LPA_1, as knock-down of LPA_1 expression in 3T3-F442A pre-adipocytes suppresses the ability of LPA to induce cell proliferation (Pages *et al*, 2001). Our investigation found increased levels of LPA_1 mRNA in pre-adipocytes over-expressing LPA_5, suggesting

a functional relationship between LPA_5 and LPA_1 that may be important for cellular growth. Thus, given the important role of LPA_1 in pre-adipocyte cell proliferation and its augmented expression in 3T3-LPA5 cells that display enhanced cellular growth compared to 3T3-NEO control cells, it could be argued that LPA_5 and LPA_1 may mediate distinct roles of LPA in pre-adipocyte growth or that the two receptors may transduce signals of different ligands that are important for regulating pre-adipocyte growth. The contrasting LPA_5 and LPA_1 responses to HF feeding and *ob* gene mutation, and differentiation of 3T3-L1 cells, further suggest that the two receptors may have distinct activation and signalling mechanisms.

It has been established (and also shown in the present study for at least some of the genes) that PPARγ, GLUT4, FASN and β3-AR mRNA expressions are increased during cellular differentiation with PPARγ increasing early, while the others show a late stage increase (Gregoire *et al*, 1998). We found that 3T3-L1 pre-adipocytes over-expressing LPA_5, when stimulated with an adipogenic cocktail, show a marked decrease in the expression of PPARγ, GLUT4, FASN and β3-AR mRNA compared to 3T3-NEO controls. Given the temporal variation in mRNA expression of these genes during differentiation as described above, it is likely that the observed effects in 3T3-LPA5 cells were due to LPA_5 action during early stages of differentiation. Correspondingly, lipid accumulation as measured by Oil red O lipid staining was significantly reduced in these differentiating cells over-expressing the receptor. The data therefore suggests that the receptor impedes cellular differentiation. Indeed, pre-adipocyte differentiation into mature fat cells is characterised by the appearance of lipid droplets and is associated with a down-regulation of LPA_1 mRNA expression (Pages *et al*, 2001; Simon *et al*, 2005), thus

the elevated LPA_1 mRNA expression and reduced lipid accumulation in these differentiating cells over-expressing LPA_5 (3T3-LPA5) further strengthens the suggestion that activation of this receptor impedes cellular differentiation.

The differentiation process reduced LPA_5 mRNA in 3T3-LPA5 cells, which was surprising given that at their pre-adipocyte stage the cells were over-expressing the receptor via a stably transfected plasmid construct containing the coding sequence for LPA_5. Since the construct was integrated into the genome, one could envisage that receptor signalling may have impeded expression of specific genes (PPARγ, FASN, GLUT4, β3-AR) important for cellular differentiation, but in doing so may also have created a cellular environment that could have prevented the subsequent expression of its own mRNA, which is naturally found to increase during differentiation. An alternative possibility is that LPA_5 mRNA expression may be coupled to receptor activation, such that over-activation of the receptor, as that may be occurring in differentiating 3T3-LPA5 cells, could possibly have caused receptor internalisation (Lee *et al*, 2006) and down-regulation of its mRNA expression. This negative feedback mechanism may be more apparent in the differentiating 3T3-LPA5 cells because the cells were attempting to over-express the receptor in a background of elevating endogenous LPA_5 mRNA during differentiation.

Adipogenesis is known to be regulated by a diverse array of receptors, of which β3-AR is one example. While this receptor negatively regulates adipogenesis (Li *et al*, 2010), its mRNA increases during adipogenesis (Monjo *et al*, 2005), presumably to regulate this process. In a similar manner, the increased expression of LPA_5 in adipogenesis may represent a mechanism that could regulate the extent to which pre-adipocytes may undergo growth and differentiation. Disruption of the

receptor activation or signalling, as that may be occurring during HFD feeding and by leptin deficiency (due to *ob* gene mutation), may lead to the development of the obese phenotype.

In summary, we have shown that LPA_5 expression is increased in the epididymal adipose tissue in response to HFD feeding and *ob* gene mutation, as well as in differentiated 3T3-L1 cells. Over-expression of the LPA_5 receptor in 3T3-L1 pre-adipocytes provided a growth advantage to the cells, with concurrent increases in the LPA_1 and β-actin expression. In contrast, the manipulation impeded the cellular differentiation process, which was reflected by the down-regulation of adipogenesis-related gene expression (PPARγ, FASN, GLUT4, β3-AR) and by reduced lipid accumulation in these cells. Whilst further work is required to establish whether LPA_5 has a role in adipogenesis *in vivo*, our data does suggest it to be a new regulatory component in adipogenesis and provides the foundation for future work investigating the effect of LPA_5 in adipocyte/adipose tissue, including whether it may be involved in mediating the direct effect of dietary proteins on adipose tissue function.

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5.7 AUTHOR CONTRIBUTIONS

The authors' contributions are as follows: LM, EFM, JFC and KNN, conceived and designed the research; LM preformed the majority of the research, including all the cell culture work and the food deprivation mouse study; EFM preformed the LFD vs. HFD mouse trial and provided the lean (+/?) and *ob/ob* epididymal adipose tissue samples; LM, analysed the data and wrote the manuscript; JFC & KNN corrected the manuscript. The authors declare that there are no conflicts of interest.

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Chapter 5

TABLES & FIGURES

Table 5.1 Sequences of primers used for Real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
LPA_5	5'-tgccaattcttcagccaacac-3'	5'-tagtaggagaggcgcaggggaagtg-3'
LPA_1	5'-cagcgcaacgagaaccctaatgg-3'	5'-ccggctggcttccttctaaacc-3'
PPARγ	5'-tcaggtttgggcggatgc-3'	5'-tcagcgggaaggactttatgtatg-3'
FASN	5'-tccacctttaagttgccctg-3'	5'-tctgctctcgtcatgtcacc-3'
GLUT4	5'-ggcctgcccgaaagagtc-3'	5'-aggagctggagcaaaggac-3'
β-3 AR	5'-cgccttcaacccggtcatctactg-3'	5'-ggtggactctgcctggcttcaac-3'
β-actin	5'-agagggaaatcgtgcgtgac-3'	5'-caatagtgatgacctggcgt-3'
WNT10b	5'-acgtgggaatggggtggctgtaac-3'	5'-tgttgttgtggatccgcattctcg-3'
GAPDH	5'-tgaaggtcggtgtgaacg-3'	5'-ccattctcggccttgact-3'
18-S	5'-aggaccgcggttctattttgttgg-3'	5'-atgctttcgctctggtccgtcttg-3'

LPA_5, Lysophosphatidic acid receptor 5; LPA_1, Lysophosphatidic acid receptor 1; PPARγ, Peroxisome proliferator-activated receptor γ; FASN, Fatty acid synthase; GLUT4, Glucose transporter 4; β-3 AR, Beta-3 adrenergic receptor; WNT10b, Wingless-type MMTV integration site family, member 10B; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

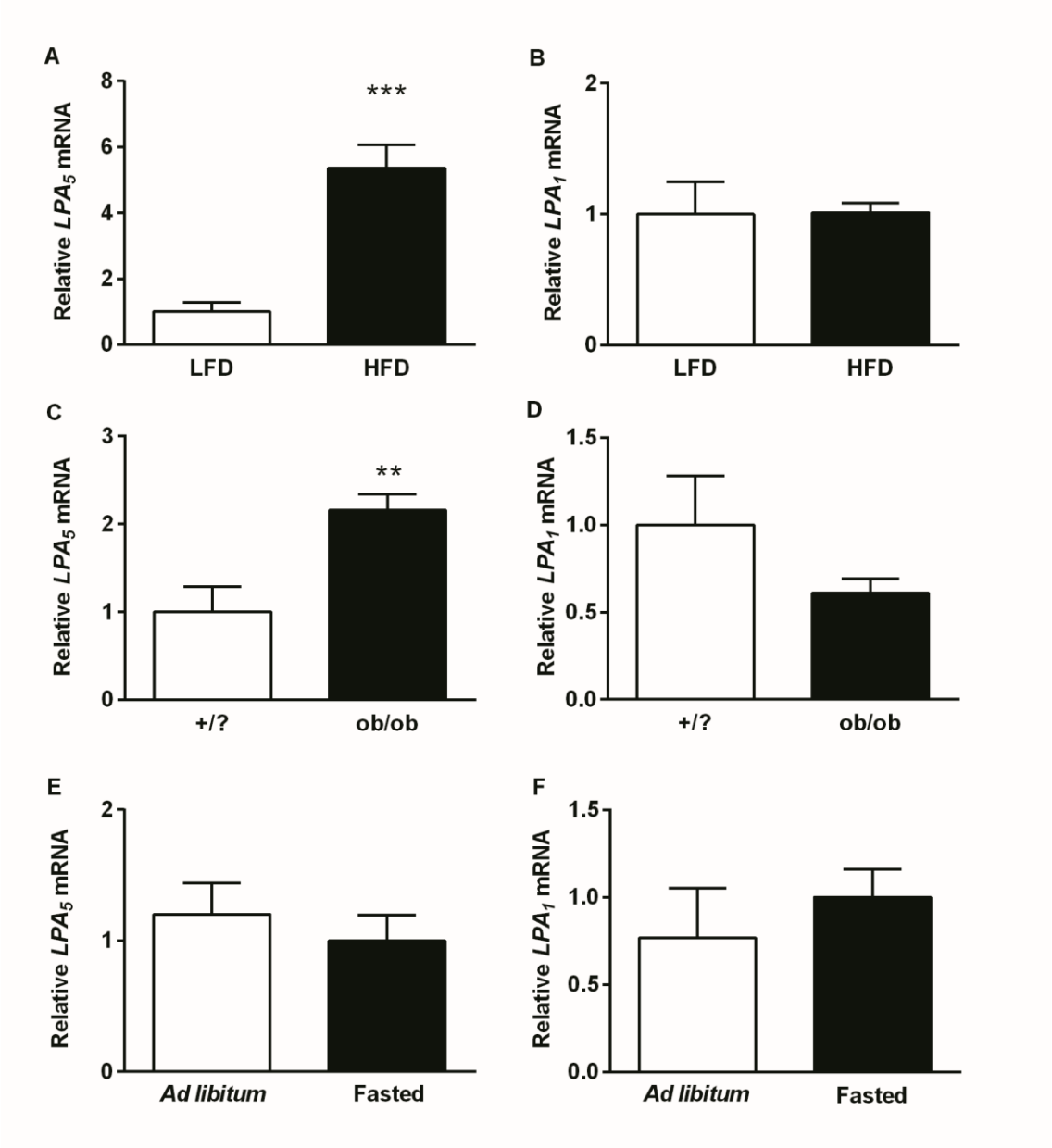


Figure 5.1 The effect of energetic challenges on epididymal adipose tissue gene expression. The mRNA expression of lysophosphatidic acid receptor 5 (LPA_5) (A, C and E) and lysophosphatidic acid receptor 1 (LPA_1) (B, D and F) in mice fed a low fat diet (LFD) vs. high fat diet (HFD) (A and B) for fourteen weeks (n = 5), in lean (+/?) vs. obese *ob/ob* mice (C and D) (n = 8), and in *ad libitum* fed vs. 24 hour fasted mice (E and F) (n = 4-5). The mRNA expressions were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are shown relative to the respective controls. Data represent means ± SEM. ** $P < 0.01$, ***$P < 0.001$.

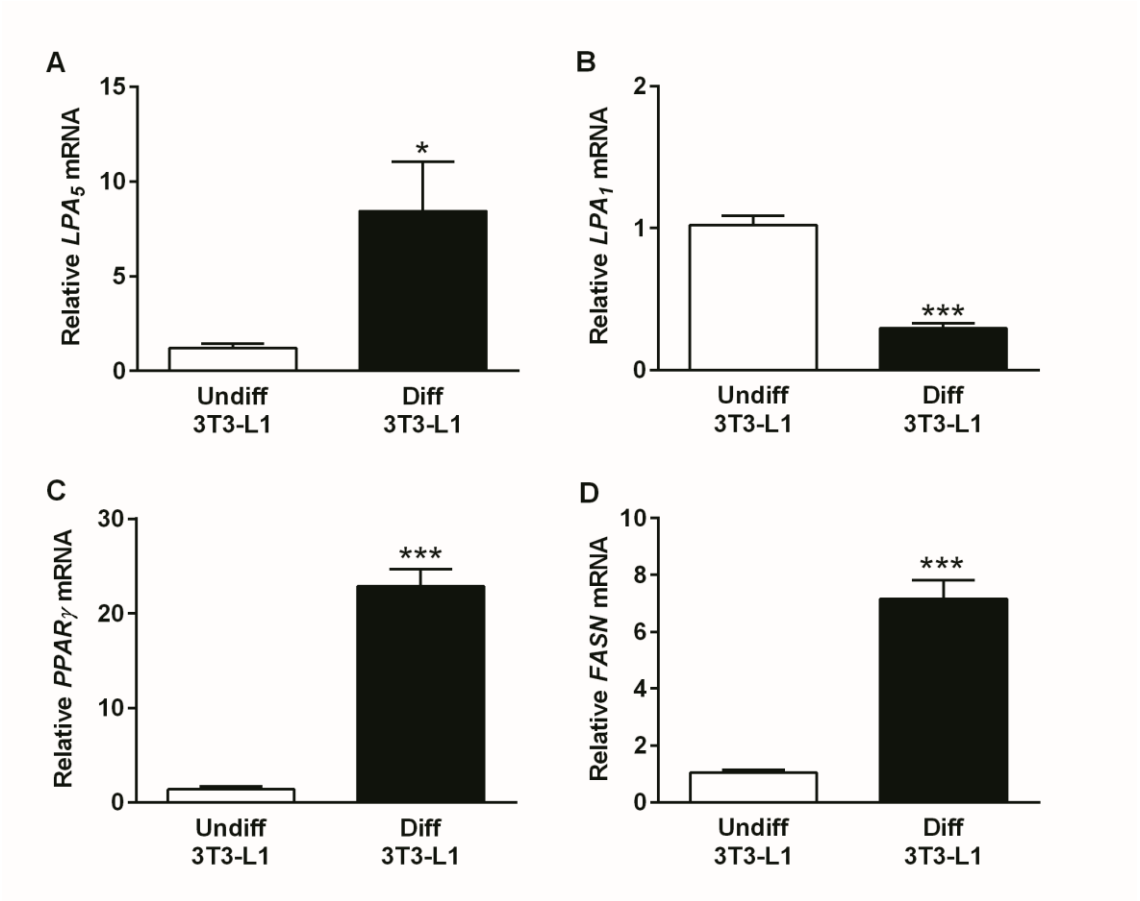


Figure 5.2 The effect of differentiation on 3T3-L1 pre-adipocyte mRNA expressions of (A) lysophosphatidic acid receptor 5 (LPA_5), (B) LPA_1, (C) peroxisome proliferator-activated receptor (PPAR)-γ and (D) fatty acid synthase (FASN) were investigated by comparing their expression in undifferentiated (Undiff 3T3-L1) vs. differentiated (Diff 3T3-L1) wild-type 3T3-L1 cells. The mRNA expression was normalised to 18S expression and are shown relative to the undifferentiated group. Data represent means ± SEM of 3 independent experiments (n = 4). *$P < 0.05$, ***$P < 0.001$.

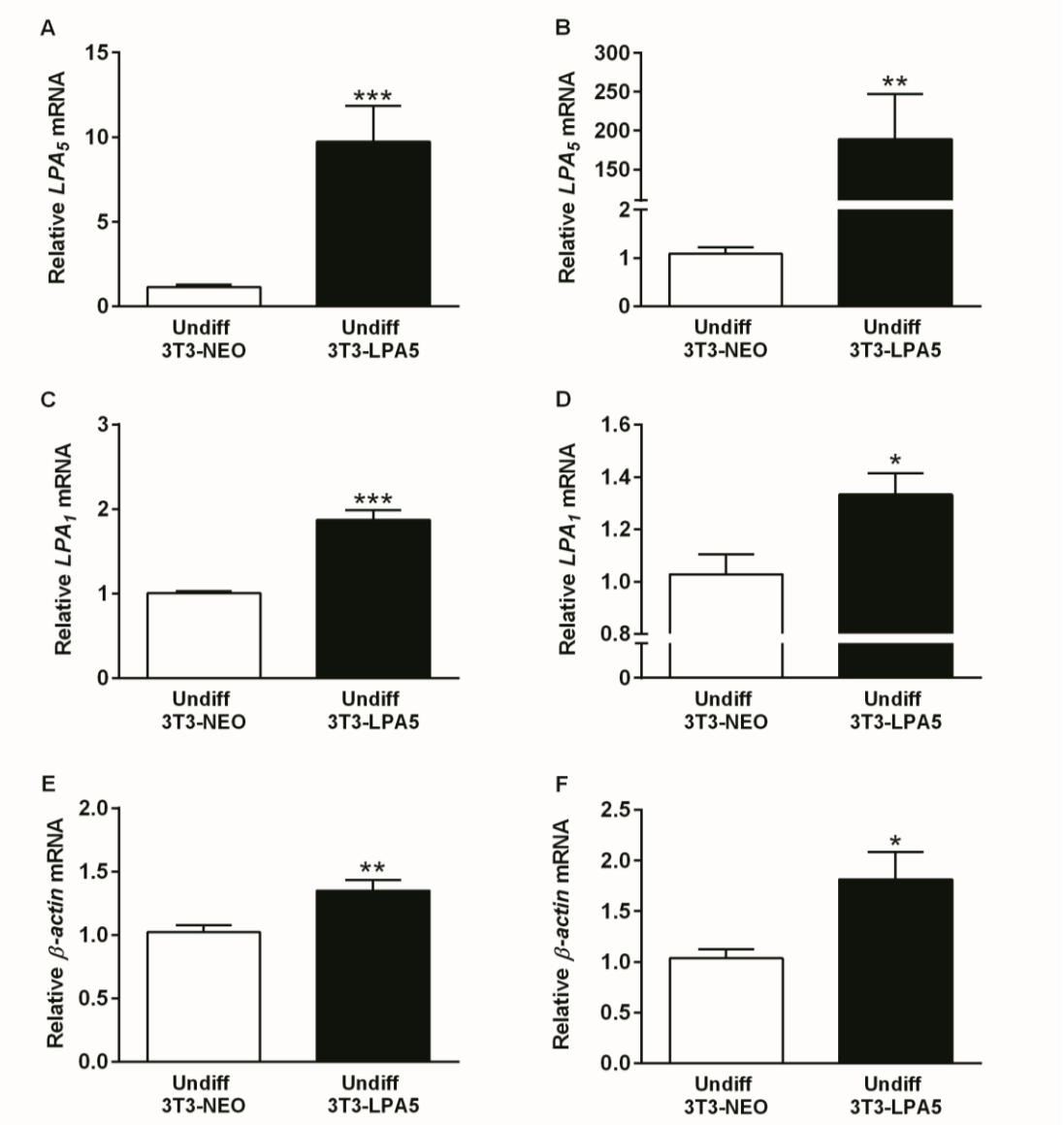


Figure 5.3 The effect of lysophosphatidic acid receptor 5 (LPA_5) over-expression on 3T3-L1 pre-adipocyte mRNA expression. The mRNA expressions of LPA_5 (A-B), LPA_1 (C-D) and β-actin (E-F) in two independent clones of undifferentiated 3T3-L1 pre-adipocytes stably transfected with either the pCI-neo plasmid containing LPA_5 DNA (Undiff 3T3-LPA5) or the pCI-neo vector without an insert DNA (Undiff 3T3-NEO). The gene expressions for clone 1 shown in (A, C, E), and for clone 2 in (B, D, F). The mRNA expressions were normalised to 18S expression. Data represent means ± SEM of 3-4 independent experiments, (n = 4). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

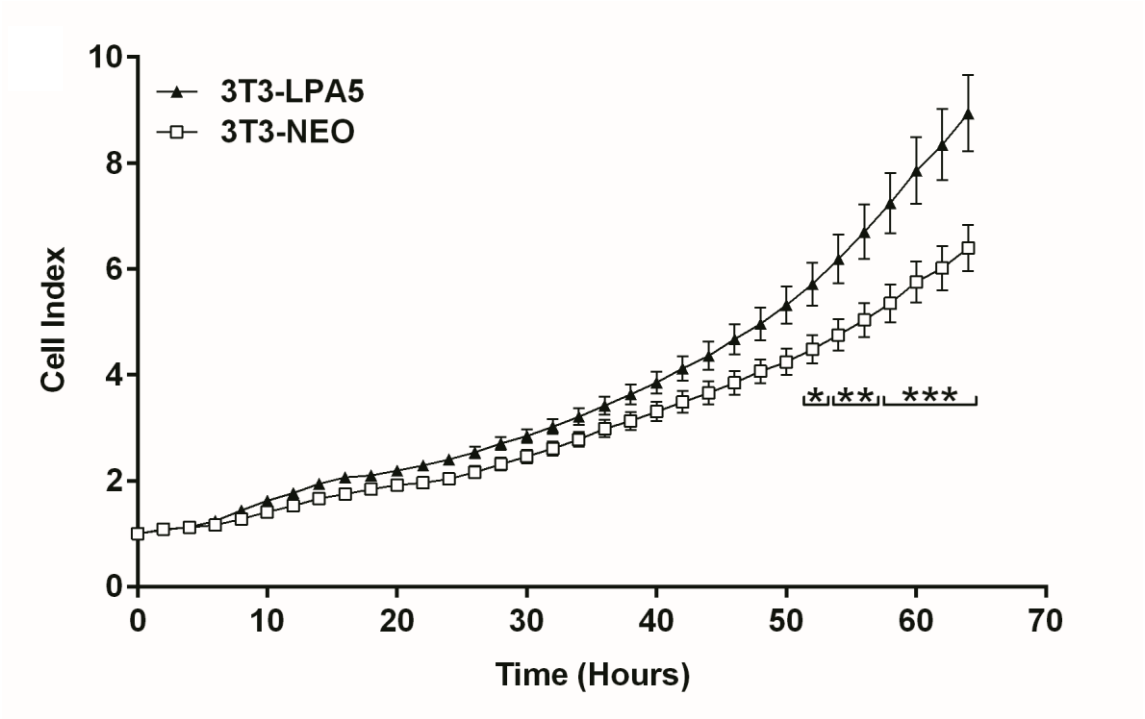


Figure 5.4 The effect of lysophosphatidic acid receptor 5 (LPA_5) over-expression on 3T3-L1 pre-adipocyte growth. Cell growth is shown for undifferentiated 3T3-L1 pre-adipocytes stably transfected with either the pCI-neo plasmid containing lysophosphatidic acid receptor 5 (LPA_5) DNA (3T3-LPA5) or the pCI-neo vector without an insert DNA (3T3-NEO) over a 64 hr time frame. The relative changes in cell growth/morphology are represented as cell index (CI), where $CI = (Z_i - Z_0)/15$ (Z_i: individual time point impedance, Z_0: Background impedance). Data represent means ± SEM of 3 independent experiments (n = 8). *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$.

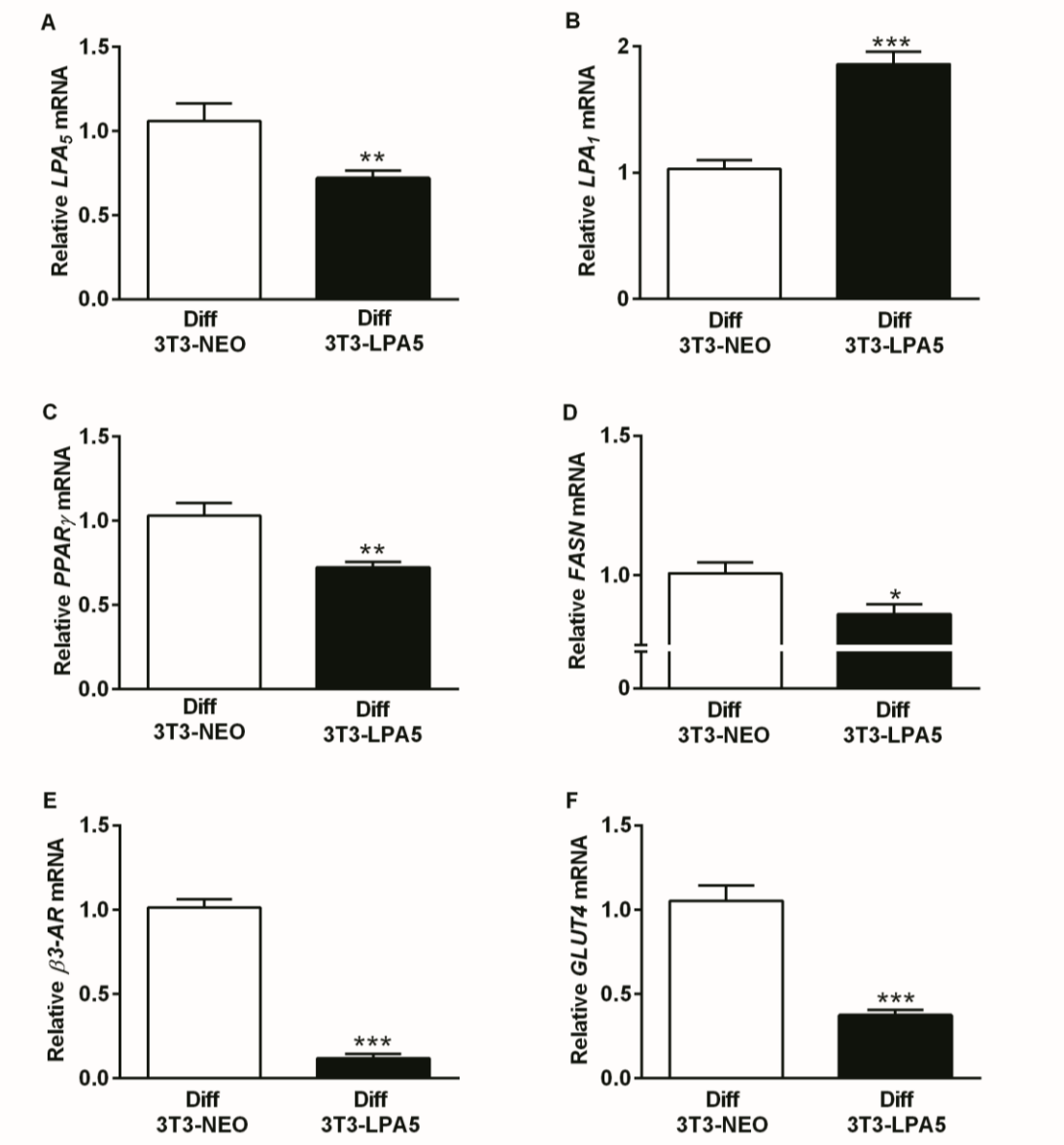


Figure 5.5 The effect of differentiation on gene expression in 3T3-L1 pre-adipocytes over-expressing lysophosphatidic acid receptor 5 (LPA_5). The mRNA expression for (A) LPA_5, (B) LPA_1, (C) peroxisome proliferator-activated receptor (PPAR)-γ, (D) fatty acid synthase (FASN), (E) β3 adrenergic receptor (β3-AR) and (F) glucose transporter 4 (GLUT4) at day 10 of differentiation treatment in 3T3-L1 pre-adipocytes stably transfected with either the pCI-neo plasmid containing LPA_5 DNA (Diff 3T3-LPA5) or the pCI-neo vector without an insert DNA (Diff 3T3-NEO). The mRNA expression was normalised to 18S expression. Data represent means ± SEM of 3 independent experiments (n = 4). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

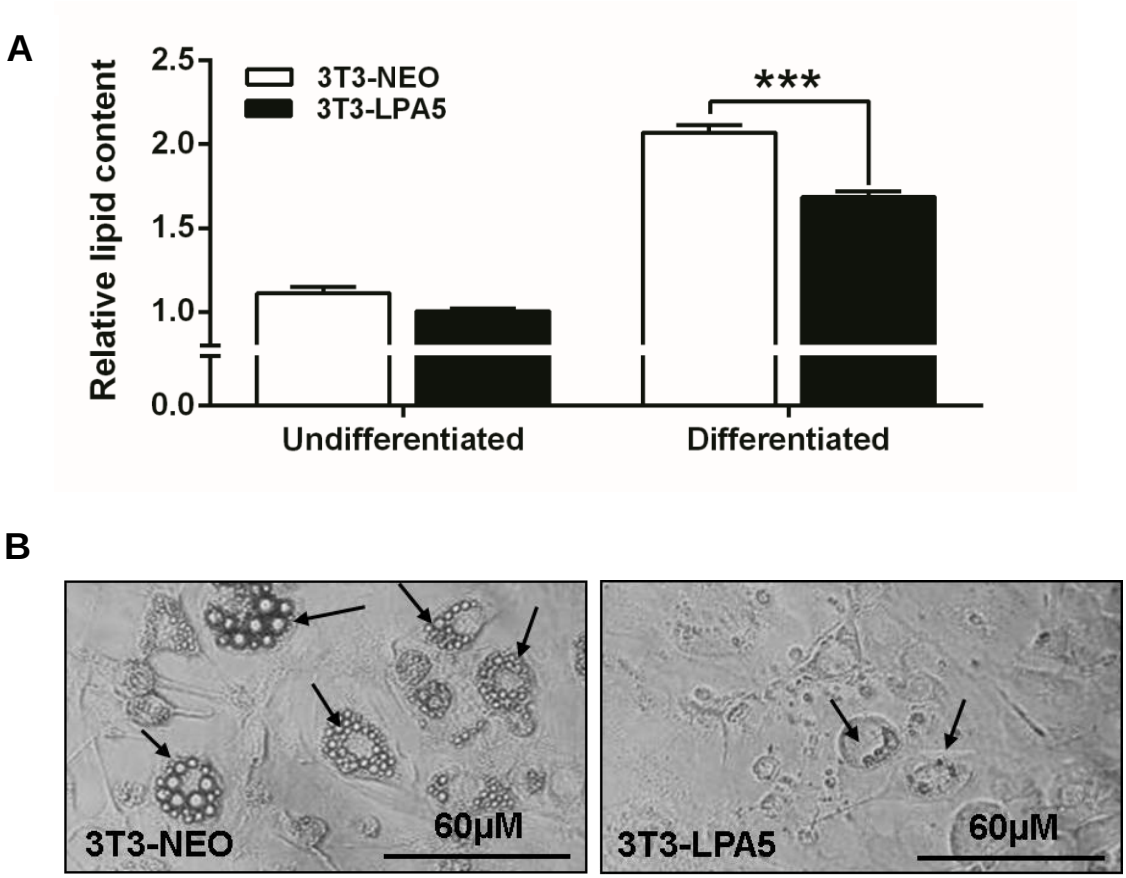


Figure 5.6 The effect of differentiation on lipid accumulation in 3T3-L1 pre-adipocytes over-expressing lysophosphatidic acid receptor 5 (LPA_5). (A) Relative lipid content as measured by Oil Red O staining and quantification in undifferentiated and differentiating pre-adipocytes stably transfected with either the pCI-neo plasmid containing LPA_5 DNA (3T3-LPA5) or the pCI-neo vector without an insert DNA (3T3-NEO). (B) Photo micrographs show the lipid vacuoles at day 10 in differentiated 3T3-NEO and 3T3-LPA5 pre-adipocytes. Data represent means ± SEM of 3 independent experiments (n = 6-10). ***$P < 0.001$.

Chapter *6*

General Discussion

6.1 INTRODUCTION

WPI has been increasingly demonstrated to provide a beneficial effect on weight management (Pal & Radavelli-Bagatini, 2013), including a specific ability to reduce weight gain and adiposity in HFD-fed rodents (Pichon *et al*, 2008; Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2011; Tranberg *et al*, 2013). Interestingly, a recurring theme of these studies has been the observation that the reduction in adiposity associated with WPI intake occurs independently of any significant changes to energy intake. Indeed, this outcome has been consistently demonstrated in HFD rodent studies of varying lengths, including those lasting for 11, 14 and 21 weeks (Pilvi *et al*, 2007; Shertzer *et al*, 2011; Shi *et al*, 2012). This project was therefore conducted to investigate the hypothesis that WPI directly impacts on adiposity by influencing tissue lipid metabolism. In support of this hypothesis, we show that WPI reduces body fat and causes alterations to lipid metabolism and insulin signalling-related gene expression in the adipose tissue (Table 6.1). These effects of WPI may be due to the direct action of WPI, or its derivatives, on adipose tissue lipid metabolism, which may potentially be mediated through the dietary protein responsive receptor LPA_5, given that it's over expression in 3T3-L1 adipocytes resulted in a significant reduction in lipid accumulation during cellular differentiation. In addition to the direct effects, evidence also implicates indirect routes by which WPI could influence adiposity, specifically by deposition of lean mass, central regulatory pathways, changes in the gut microbiota and alterations to gastrointestinal morphology (Figure 6.1).

Table 6.1-Overview of key outcomes of WPI dietary inclusion*

	High fat diet		Low fat diet	
Physiological response	**Short-term**	**Long-term**	**Short-term**	**Long-term**
Energy intake	↑ (Metabolic) ↔ (Home)	↑ (Metabolic) ↔ (Home)	↔(Metabolic) ↔ (Home)	↔(Metabolic) ↓ (Home)
Energy expenditure	↔	↔	↔	↔
Fat mass	↓	trend ↓	↑	↔
Lean mass	↑	↑	↓	↔
Liver TAG	↓	↓	↔	↔
Plasma glucose	↔	↔	↔	↔
Plasma Insulin	↔	↔	↑	↔
Plasma TAG	↑	↓	↔	↔
Gut microbiota changes	N/A	↑ *Bifidobacteria* ↑ *Lactobacillus* ↓ *Clostridium*	N/A	N/A
Hypothalamic gene expression	↓ IR, ObR, CPT1b, TNF-α.	↑ IR	N/A	N/A
EAT insulin signalling gene	↑ GLUT 4, IR	↔	↓ GLUT 4, IR, IRS-1	↔
EAT lipid metabolism genes	↑ trend β3-AR ↑ trend FASN	↑ FASN	↓ β3-AR, HSL, CPT1b	↑ ACC, FASN, CD36

*The noted changes are relative to casein fed mice

TAG; Triacylglycerol; EAT, Epididymal white adipose tissue; Metabolic, metabolic cage; Home, home cage; N/A, not available. ↑ = increased compared to casein; ↓ = decreased compared to casein; ↔ = no difference to casein

IR, Insulin receptor; ObR, leptin receptor; CPT1b, carnitine palmitoyltransferase 1b; TNF-α, Tumour necrosis factor α; GLUT4, glucose transporter 4; IRS-1, Insulin receptor substrate 1; FASN, fatty acid synthase; HSL, hormone sensitive lipase; CD36, cluster of differentiation; β3-AR, β3-adrenergic receptor; ACC, acetyl CoA carboxylase

6.2 DISCUSSION

The hypothesis that WPI directly impacts on adiposity by influencing tissue lipid metabolism was investigated using HFD and LFD fed C57BL/6J mice. The results of the current study show that WPI impedes the progression of obesity, although differential effects on weight management are seen dependent on macronutrient composition of the WPI enriched diet and on the duration of feeding (Fig 6.1). These data will be of significant importance to the Irish food industry to develop Functional Foods that improve the health status of individuals. The data also shows how the use of WPI in the lean state may have detrimental implications in terms of body fat accumulation, at least in the short term, in the absence of an exercise regime. Furthermore, the contrasting results related to WPI in the literature, for example in terms of energy intake between this study and those of others (Bendtsen *et al*, 2013; Hall *et al*, 2003; Luhovyy *et al*, 2007; Pal & Ellis, 2010; Pichon *et al*, 2008), raises the need to obtain an in depth understanding of the functions of constitute proteins in WPI to assess if their variability within distinct WPI's, obtained by different extraction methods and conditions, may underlie the above mentioned variability and importantly, may also allow for the development of WPI's with enhanced bioactivity by manipulation of such methods and conditions.

Whilst strict dietary interventions can cause up to 10% weight loss in obese individuals, maintaining the lost weight has been a challenge after cessation of the dietary intervention (Anderson *et al*, 2001; Crujeiras *et al*, 2010; Haufe *et al*, 2013; Ryttig & Rossner, 1995; Sarwer *et al*, 2009; Wang *et al*, 2008). This is because the weight rebound effect often seen following weight loss makes obesity reversal difficult to achieve (Ryan *et al*, 2012), and thus prevention, rather than treatment,

appears to be a much more attractive solution for combating the obesity epidemic. In this regard, this study clearly demonstrates the potential of WPI to prevent the progression of obesity, with data here demonstrating its ability to suppress HFD-induced body fat accumulation even in response to prolonged high fat dietary exposure, and in the absence of a negative energy balance (Table 6.1). Moreover, by showing WPI effects on mice from early stages of development, the data here may offer an insight into how WPI could be utilised in the prevention of childhood obesity in humans and thereby open up a potential avenue to tackle the obesity epidemic.

Our results show a specific WPI effect in terms of promoting the development of lean mass at the expense of fat accumulation in HFD fed mice, which is seen at both 8 and 21 week dietary challenge. These data are indicative of enhanced muscle protein synthesis, which is supported by previous findings showing a specific ability of WPI to enhance muscle protein synthesis compared to other protein sources (Norton *et al*, 2012; Pennings *et al*, 2011; Tang *et al*, 2009). These data could be exploited to create WPI enriched Functional Foods targeting elderly people suffering from sarcopenic obesity (Pennings *et al*, 2011; Pennings *et al*, 2012), which arises due to age-related muscle loss, and accompanying increased fat mass. Moreover, it suggests WPI supplements or enriched Functional Foods could also be utilised, as demonstrated in Frestedt *et al,* (2008), to maintain lean body mass content during weight loss in energy restriction dietary interventions.

Another group of malnourished individuals that could benefit from WPI intake are patients that undergo bariatric surgery. This surgery is the only long-term solution for obesity, but this treatment causes both fat and muscle loss. Thus,

as described above for people undergoing dietary restriction, these proteins could potentially be given to patients that had undergone bariatric surgery as a means to help prevent muscle loss and preserve their lean mass content. Notably, WPI effects on the gastrointestinal tract shown here, where the dietary proteins reduced stomach size and intestinal length in LFD fed mice, are similar to that seen in bariatric surgery. Whilst we did not investigate if these changes could underlie the reduced weight gain in HFD fed mice with WPI intake, the data nonetheless poses the possibility to create Functional Foods with WPI that could bring about a surgery like effect ("nutritional surgery"). However, direct evidence of whether whey protein effects on GIT morphology truly mimic those associated with gastric bypass surgery is required. This nonetheless is an exciting new area for future investigation, as it may also help to establish health claims for WPI incorporated Functional Foods, and may even eventually replace bariatric surgery, which is known to carry substantial health risks including death. Therefore, the data here has opened up key unanswered questions that require investigation; (1) Does WPI similarly influence intestinal morphology in the HFD background, and if so, is this altered by the HFD duration? (2) Do alterations to the gastrointestinal environment underlie some of the beneficial effects of WPI in HFD fed mice, including insulin signalling? and (3) What connection is there between intestinal morphology and gut microbiota changes observed with WPI intake?

Surgical reduction in the length of the intestine so that nutrients by-pass the proximal small intestine has been shown to enhance insulin sensitivity independently of changes to plasma levels of insulin, GLP-1, energy intake or body weight (Rubino *et al*, 2006; Salinari *et al*, 2013b). It has been suggested that absence of nutrient passage in the proximal intestine, as that arises from gastric

bypass surgery, reduces negative regulatory factors that impede insulin action, and that this underlies the improved insulin action following surgery (Salinari *et al*, 2013a). Notably, we observed that mice fed a low fat and high carbohydrate enriched WPI diet exhibit a reduced intestinal length and show increased fat accumulation, presumably due to a whey induced insulin driven anabolic effect which when combined with a high carbohydrate content leads to enhanced glucose disposal as lipid in tissues. Whether the latter effect on fat accumulation is due to a WPI induced surgery-like effect on the production of intestinal-derived negative regulatory factors of insulin action, remains to be determined. Thus, overall the data suggests that in a lean context, intake of Functional Foods enriched with WPI should be consumed in combination with low carbohydrate diets or with a proper exercise regime to maximise the beneficial effects of protein intake and to reduce the potential anabolic effects on lipid storage. These findings are of particular importance to the sport industry and to athletes, and also highlight that when developing Functional Foods it is important to evaluate how different dietary backgrounds influence their action and whether any have health implications arise from their consumption in these different dietary backgrounds.

Health claims are a key aspect of Functional Food development, and for this it is important to establish mechanisms of action. With regard to WPI, our data shows that WPI prevention of HFD-induced weight gain and fat mass was accompanied by increased deposition of lean mass, an energy consuming physiological process, and by altered gut microbiota composition, and hypothalamic gene expression changes linked to adipose catabolic output. However, because these changes were correlative, we weren't able to definitely address the issue of how HFD-WPI fed mice consuming equivalent or more energy

than HFD controls were not storing this energy as body fat, particularly in the absence of a change in energy expenditure. Therefore, the work here has opened up the challenge to understand how this energy deficit arises? Interestingly, a recent study, which followed on from our short-term HFD study, showed an increased urinary metabolite loss with WPI intake that may help explain how some of this energy is being lost (Lillefosse *et al*, 2014). Thus, an overall impact of this work may be to promote a greater research focus on alternate modes of energy dissipation with WPI intake, which may uncover novel targets and associated pathways for therapeutic intervention.

Although others have demonstrated the potential of WPI to impact on tissue lipid metabolism (Pilvi *et al*, 2007; Pilvi *et al*, 2008; Shi *et al*, 2011; Tauriainen *et al*, 2011), this project made an significant contribution to the field by further exploring these WPI effects on lipid metabolism in an effort to better understand the associated mechanisms. Firstly, the data showing a specific ability of WPI to suppress hepatic lipid accumulation, as demonstrated similarly elsewhere (Hamad *et al*, 2011; Shertzer *et al*, 2011; Shi *et al*, 2011), supports the suggestion of WPI being a potentially attractive option in the prevention and/or treatment of NAFLD, however the mechanism(s) involved will need to be uncovered to support these health claims. As WPI prevented HFD-induced reductions in epididymal β3-AR gene expression, the data also suggests an adipose tissue lipolysis potential of these dietary proteins, which may contribute to its ability to suppress fat mass gains. Interestingly, recent data has shown WPI to impact on the subcutaneous adipose tissue mass to greater extent than the epididymal adipose tissue (Tranberg *et al*, 2013). Thus, a possible limitation of the current work was the decision to only characterise WPI induced changes in the epididymal adipose

deposit, and therefore future investigations should investigate the impact of WPI on both adipose tissue deposits.

The study here was also the first to explore whether WPI may indirectly influence adiposity through central signalling pathways, by measuring hypothalamic gene expression in HFD fed mice consuming WPI. In the short-term the inclusion of WPI suppressed HFD-induced increases in the expression of insulin receptor, leptin receptor, CPT1b, TNF-α and PPARγ, potentially representing a lower threshold of leptin and insulin action in the hypothalamus (Ryan *et al*, 2012), in association with reductions in both hypothalamic lipid accumulation and inflammation (Gao *et al*, 2009; Posey *et al*, 2009; Ryan *et al*, 2011). Indeed, an increased catabolic outflow from the CNS, via the SNS, to the adipose tissue, induces lipolysis and causes a reduction in TAG storage by catecholamine mediated activation of β3-AR receptors. This pathway may help explain why adipose tissue β3-AR expression was maintained in the WPI fed mice, and in turn why their adiposity was reduced. The involvement of β3-AR in WPI mediated reductions in adiposity could be further explored by investigating the impact of WPI in adipose tissue β-adrenergic receptor knock-out mice.

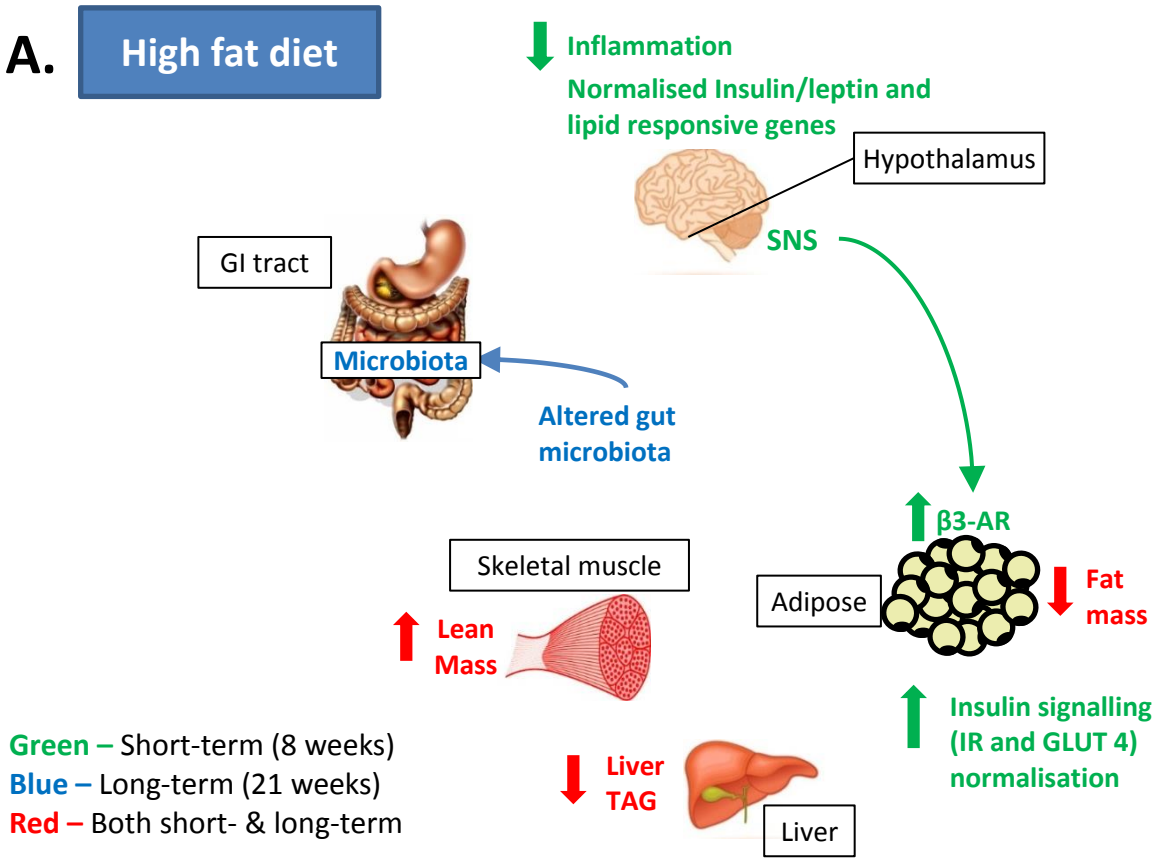
The ability of WPI to influence the gastrointestinal environment shown here through specific alterations to the gut microbiota composition in long-term HFD fed mice provides an exciting new avenue of WPI research. Specific changes in gut microbiota at the phylogenic level were that, in comparison to HFD fed casein controls, WPI consumption specifically increased *Lactobacillus* and *Bifidobacterium* levels, and reduced *Clostridium* levels to such an extent that they were undetectable. This profile is similar to that reported by Sprong *et al,* (2010) and thus may potentially represent a compositional shift beneficial to the

reduction of HFD-induced obesity. However, the true impact that WPI effects on the gut microbiota composition have in terms of the physiological outcomes observed with WPI intake, requires further investigation, possibly with the use of germ free mice. Furthermore, whether or not the impact of WPI on gut microbiota relates to dietary protein-induced alterations in GIT morphology and improvements in gut barrier integrity (i.e. reduced permeability)(Benjamin *et al*, 2012), are potential new avenues of research that if investigated may provide a major breakthrough in the field of obesity prevention.

Understanding and then enhancing the bioavailability of food-derived bioactive components (e.g. bioactive peptides) to influence target tissues within the body will be increasingly more important in Functional Food research and design (Udenigwe & Aluko, 2012). WPI is no exception in this, and future work will set to uncover ways to maximise WPI bioactive uptake across the intestinal wall in sufficient quantities to cause its intended effect in target tissues within the body. Techniques that can influence digestion and absorption of WPI bioactive components, such as pre-hydrolysation, encapsulation and using treatments such as pressure and temperature to alter isolate physiochemical properties, could be utilised to enhance the bioactivity of WPI. Indeed, in a recent clinical trial involving prehypertensive patients, WPI encapsulated in lycopene micelles, which is a carotenoid resistant to human digestive enzymes, improved its effects on cardiovascular related parameters compared to WPI intake alone (Petyaev *et al*, 2012). In relation to investigating bioavailability, identifying targets in the body for WPI bioactive components to act upon is also an important area of future research for Functional Food development and could also lead to therapeutic targets for obesity treatment in general. In this regard the work here identified, $LPA_{5,}$ as a

potential target; however future work is needed to further evaluate its role in adipocyte metabolism and to access if indeed it could mediate the effects of WPI derived bioactives on adipose tissue.

Although the benefits of WPI intake are clearly evident, and the total dietary protein content used in our studies was around the recommended dietary level for humans, a drawback of the current research in terms of translating this work for human consumption is the nature of the dietary challenges used, since WPI was the only source of proteins in the diet, which is unlikely to represent the human situation. Therefore, future studies should investigate whether lower quantities of WPI may still prove effective in preventing weight gain and obesity, and thus be more directly applicable to the human context, and also investigate whether diets containing other protein sources as well as WPI still allow maintenance of the whey proteins effects. Notably, a recent mouse trial demonstrated that replacing 5% of the dietary protein content in a HFD with a novel WPI leads to a significant reduction in HFD-induced obesity (Shi *et al*, 2011), suggesting that small quantities of WPI may still be effective. Also, whilst the current study utilised one source of WPI, there are a wide variety of isolates derived from different extraction methods and conditions which are likely to provide WPI's with different bioactivities. This would relate to the differences in amounts of individual whey proteins present in the isolates. This wide variation in available WPI's used in the literature does create problems when attempting to compare findings across studies and uncovering the true potential for use in weight management. Thus, a greater understanding of the protein quality within distinct WPI's and how each individual whey protein influences energy balance is required, so that a protein mix that provides the greatest beneficial effect for humans can be created.


A.
High fat diet
Inflammation
Normalised Insulin/leptin and lipid responsive genes
Hypothalamus
SNS
GI tract
Microbiota
Altered gut microbiota
β3-AR
Skeletal muscle
Adipose
Fat mass
Lean Mass
Insulin signalling (IR and GLUT 4) normalisation
Liver TAG
Liver
Green – Short-term (8 weeks)
Blue – Long-term (21 weeks)
Red – Both short- & long-term

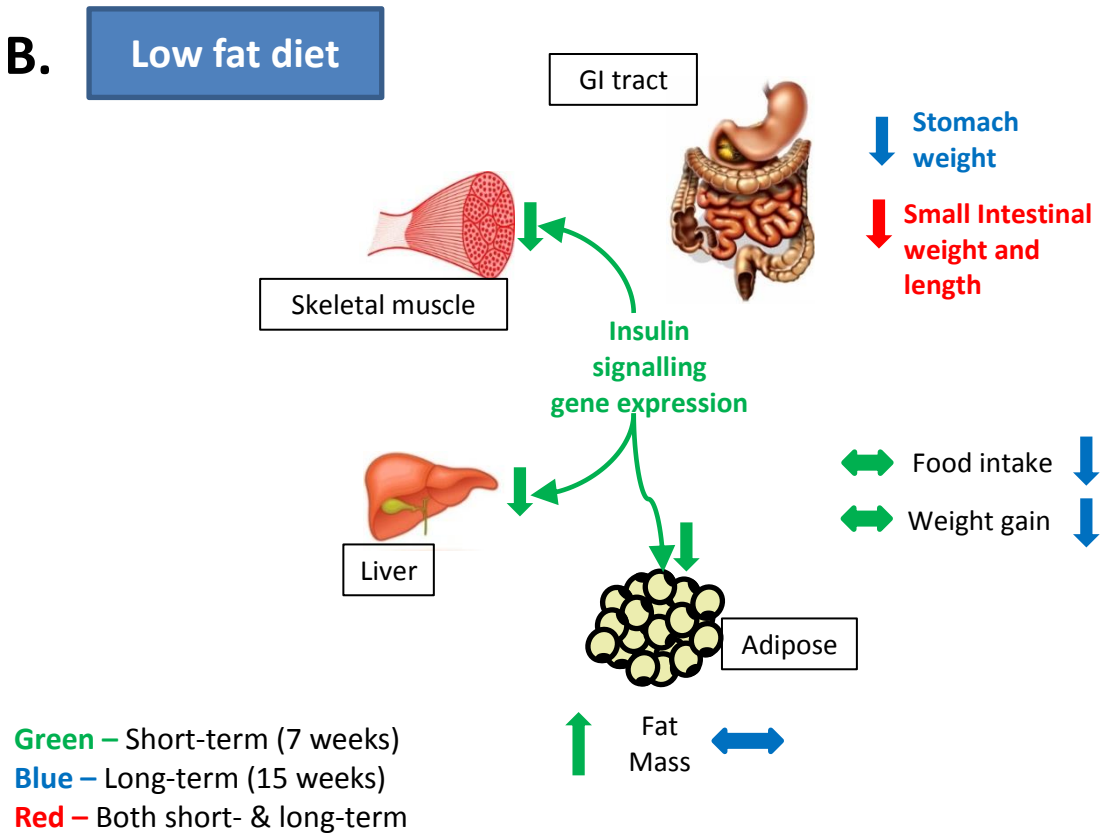

B.
Low fat diet
GI tract
Stomach weight
Small Intestinal weight and length
Skeletal muscle
Insulin signalling gene expression
Food intake
Weight gain
Liver
Adipose
Fat Mass
Green – Short-term (7 weeks)
Blue – Long-term (15 weeks)
Red – Both short- & long-term

Figure 6.1 The contribution of this study to the field of body weight regulation control by whey protein isolate (WPI). Overall, differential impacts of WPI were evident depending on the macronutrient background in which they were provided and the duration of feeding. The effects of WPI in a high fat diet (HFD) and a low fat diet (LFD) background are demonstrated in (A) and (B) respectively. Effects in green show outcomes observed only in the short-term (8 weeks in HFD; 7 weeks in LFD), blue in the long-term only (21 weeks in HFD; 15 weeks in LFD) and red shows outcomes observed in both the short-and long-term.

β3-AR, Beta-3 adrenergic receptor; GI tract, gastrointestinal tract; GLUT4, glucose transporter 4; IR, Insulin receptor; SNS, sympathetic nervous system; TAG, triacylglycerol.

6.3 CONCLUSION

The data presented here revealed that the ability of WPI to influence lipid metabolism differed according to macronutrient content in which it is provided and also with the duration of the experimental treatment period. In fact, WPI inclusion into a HFD suppressed the diet-associated increases in fat mass and liver lipid accumulation in the short-term, while these WPI associated effects were diminished somewhat with prolonged HFD intake. In contrast, in a LFD background, the inclusion of WPI augmented body fat content in the short-term, while in the long-term it had no effect. Additionally, the data presented here provides support for direct and indirect actions of WPI on adiposity by influencing lipid metabolism in the tissue. Notably, our investigation into the functionality of

the LPA_5 receptor, which is responsive to protein hydrolysate, identified it as a novel candidate in regulation of pre-adipocyte growth and differentiation, which suggests this receptor may mediate the potential direct dietary protein (or its derivative) effects on adipose tissue metabolism. In addition to the direct effects of WPI, evidence presented here also implicates indirect routes of WPI action, namely by lean mass deposition, modifications of hypothalamic regulatory pathways, gut microbiota changes and alterations to gastrointestinal morphology, which need to be investigated further to uncover the extent of their contribution to the WPI-induced effects observed here. Moreover, the novel effects of WPI on the GIT environment (morphology and gut microbiota) found here, as well as the effects on central energy balance regulation (hypothalamic gene expression), potentially offer exciting new avenues of whey protein research. The work here also highlights the great potential of WPI to be utilised as a Functional Food in obesity prevention, particularly in relation to childhood obesity, while also demonstrating its potential to be applied in preventing lean mass loss in vulnerable groups such as the elderly and post-operative bariatric surgery patients. Overall, the work presented here may help to focus future studies exploring the weight management effects of WPI and its derived bioactives.

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Appendix A

Replacement of dietary saturated fat with monounsaturated fat protects against obesity-induced adipose tissue inflammation and hyperinsulinemia

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Introduction: Recent evidence indicates NLRP3-mediated processing of pro- to active IL-1β accentuates adipose tissue inflammation promoting insulin resistance (IR). Furthermore saturated fatty acids (SFA) but not monounsaturated fatty acids (MUFA) can prime pro-IL-1β production. In this study we hypothesized that replacement of SFA for MUFA in high-fat diets (HFD) would attenuate adipose inflammation through reduced priming of NLRP3 inflammasome and improve metabolic health despite development of obesity.

Methods: C57BL/6 mice were placed on a chow or HFD (45% palm oil/45% olive oil) for 24 weeks. Glucose (GTT) and insulin (ITT) tolerance tests were performed. Energy expenditure was monitored using TSE Phenomaster. Insulin secretion response to glucose challenge was quantified by ELISA. Macrophage recruitment into adipose tissue was measured by flow cytometry. IL-1β secretion from cultured adipocytes and stromal vascular fraction (SVF) stimulated ±ATP (100μm) was quantified by ELISA. Insulin-induced phosphorylated AKT in adipose tissue was determined by immunoblot analysis.

Results: A slight reduction in body-weight was observed in MUFA-fed mice compared to SFA-fed mice concomitant with increased VO2 and heat production. MUFA-fed mice were protected against obesity induced hyperinsulinemia despite exhibiting comparable glucose homeostasis to SFA-fed mice by GTT and ITT. M1 adipose tissue macrophage number was lower in MUFA-fed mice compared to SFA-fed mice. IL-1β cytokine secretion was lower from SVF of MUFA-fed mice with a corresponding improvement in adipose tissue insulin sensitivity.

Conclusion: Dietary intake of MUFA protects against obesity-induced hyperinsulinemia coincident with reduced adipose tissue NLRP3 activation and IL-1β release.

Appendix B

Metabolic Syndrome

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Review Article Open Access

Bioactivity in Whey Proteins Influencing Energy Balance

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Abstract

Obesity develops due to energy (food) intake exceeding energy expenditure. Nutrients that reduce the positive energy balance are thus being considered as therapies to combat obesity. Here, we review the literature related to the physiological, cellular and endocrine effects of intake of whey proteins, namely α-lactalbumin, β-lactoglobulin, glycomacropeptide and lactoferrin. Moreover, we discuss how dietary composition and obesity may influence whey protein effects on the above parameters. Evidence suggests that intake of whey proteins causes a decrease in energy intake, increase in energy expenditure, influence insulin sensitivity and glucose homeostasis and alter lipid metabolism in the adipose, liver and muscle. These physiological changes are accompanied by alterations in the plasma levels of energy balance related hormones (cholecystokinin, ghrelin, insulin and glucagon-like peptide-1) and the expression of catabolic and anabolic genes in the above tissue in the direction to cause a negative energy balance.

Keywords: Nutrition; Diet; Metabolic syndrome; Human; Rodent

Abbreviation: CCK: Cholecystokinin; FAS: Fatty Acid Synthase; GLP-1: Glucagon-Like Peptide-1; TAG: Triacylglycerol

Introduction

Obesity is a major health problem in the world because it increases the risk of development of several clinical conditions including cardiovascular disease, stroke, hypertension and type 2 diabetes [1]. The weight gain occurs due to storage of energy consumed in excess of daily requirement, as triacylglycerol (TAG) in the adipose tissue. The resulting increase in the mass of the adipose tissue causes the gain of weight, and it may even lead to the development of obesity [2]. The prevalence of obesity and associated co-morbidities has reached epidemic proportions globally. Hence, it is no surprise that there is a growing interest to identify therapies in particular those involving nutrients that could reduce weight gain and thus the development of obesity.

Whey is the milk serum that remains after precipitation of casein during cheese production, and it contains proteins, vitamins, minerals and trace amounts of fat. Whey associated proteins include α-lactalbumin, β-lactoglobulin, glycomacropeptide and lactoferrin [3]. There is accumulating evidence suggesting that whey protein intake influences the balance between energy (food) intake and energy expenditure, insulin sensitivity and glucose homeostasis as well as lipid metabolism in tissues in particular in the adipose tissue. Here, we review data from *in vivo* (human and rodent) and *in vitro* studies related to the above effects of α-lactalbumin, β-lactoglobulin, glycomacropeptide and lactoferrin and briefly discuss in the last section the actions of recently identified minor whey proteins. Furthermore, we discuss how dietary composition and obesity could influence the actions of these dietary proteins. Because authors of some of the studies mentioned below have not stated whether the whey proteins were provided as a concentrate or isolate, we have used the term "whey proteins" where this classification has not been specified.

Food (Energy) Intake

Studies conducted in humans have shown that intake of 20 to 50 g of whey proteins reduces short term *ad libitum* food intake. Akhavan et al. showed that whey protein concentrate (20-40 g) dose dependently reduces subsequent (30 min) *ad libitum* pizza meal energy intake in lean subjects, with 40g having the greatest impact compared to the water control [4]. This effect extends up to 2 h in lean subjects [5]. In comparison to casein, whey proteins (48 g) delay the desire to eat a subsequent meal by up to 180 mins in lean subjects [6], suggesting that the whey proteins induce satiety in comparison to casein intake. The results of rodent studies with regard to the effects of whey proteins on food (energy) intake are largely in agreement with that observed in humans. The suggestion that whey proteins induce satiety has been further confirmed in mice by showing that whey protein isolate providing 30% energy increased intermeal interval (satiety) compared to soy protein during the 7 day period of the study [7]. Similar findings have been reported from a long term study lasting 10 weeks [8]. These data are consistent with the findings from human studies that whey proteins reduce food intake by inducing satiety in the lean state. However, a functional relationship between whey proteins and food intake has not been consistently reported. For instance, although Hall et al. showed that whey proteins reduce food intake compared to casein in humans [6], this response was not observed in rats given a whey protein isolate in comparison to casein diet (300 g/kg diet) for 7 weeks, despite the fact that the rats on the whey protein isolate diet showed a significantly reduced weight gain compared to the rats on the casein diet [9]. It is possible that physiological and neuroendocrine differences that exist between the species (humans vs. rats) may have given rise to the inconsistent effects of whey protein and casein on food intake. There is evidence that macronutrient composition in the diet could also impact upon the effect of whey proteins on food intake. Veldhorst et al. showed that a diet containing proteins, carbohydrates and fat providing 10, 55 and 35% energy, respectively, reduces hunger in humans if whey

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proteins were included in comparison to either soy and casein [10]. Similar results have been obtained from a study conducted in rats, which showed that whey protein concentrate with energy content of 55% reduced cumulative energy intake compared to whole milk protein if the former protein had been supplied in a diet with either 35% and 10% energy from carbohydrates and lipids, respectively, or in a diet with 45% energy solely from lipids [11]. No differences in cumulative energy intake were observed if the proteins were provided in diets with 15% and 30% energy from carbohydrates and fats, respectively. These data highlights the importance of the carbohydrate composition in the diet on whey protein effects on food intake. In addition to dietary composition, weight gain also seems to influence the effect of whey proteins on short term food intake. This is revealed by the finding that intake of whey protein isolate (50 g) reduced subsequent pizza intake in normal weight but not in obese subjects [12]. Similar findings have been reported by another human study [13] and in high fat diet induced obese mice [14]. In the latter case, diet induced obese mice that drank water supplemented with whey protein isolate at 100 g/L for 11 weeks show similar energy intake to obese mice on un-supplemented water, despite the former group showing a significant decrease in body weight [14]. Thus, in addition to protein source, quality and time of consumption mentioned by Anderson et al. [5], both macronutrient composition in the diet and state of energy balance (lean vs. obese), should be considered as important factors influencing the whey protein effects on food intake.

A number of studies have attempted to identify the individual whey proteins present in the concentrate or isolate that might be causing the reduction in food intake in humans and rodents. Because glycomacropeptide has been shown to stimulate the production of the satiety hormone cholecystokinin (CCK) [15], a role for this whey protein in the regulation of food intake has been suggested. The findings of Veldhorst et al. support the latter suggestion, since it was shown that ingestion of a breakfast diet with whey protein providing 10% energy reduced lunchtime energy intake in lean subjects more than intake of a whey protein associated breakfast diet without glycomacropeptide [16]. This suggests that glycomacropeptide may reduce energy intake possibly by inducing satiety, as previously reported. This finding is not in agreement with data from several other studies performed on lean subjects with diets containing whey protein without glycomacropeptide (providing 44% energy) [17] and whey protein diets with added glycomacropeptide (21% w/w) [18]. To explore the possibility that the discrepancies in the data on the effects of glycomacropeptide on food intake might be due to the variations in the degree of protein glycosylation, Keogh et al. provided 50 g of minimal glycosylated or glycosylated glycomacropeptide as well glycomacropeptide depleted whey protein concentrate to obese and overweight subjects and found that their subsequent lunch time meal intake was not affected by the dietary challenges [19]. The data suggest that the degree of glycosylation studied in the glycomacropeptide is not critical for the actions of this whey protein on food intake. As the above study was performed on obese or over-weight individuals that as described above appear to be less sensitive to whey protein effects on food intake, it would be interesting to find out whether the outcome on food intake would be different with lean individuals. However, taking in to account the data from human studies described above, and data from a rat study which revealed that glycomacropeptide does not impact on food intake [9], it could be argued that this whey protein does not have a significant effect on this physiological process. Utilising a similar approach as in their previous studies, Veldhorst et al. showed that breakfast diets with α-lactalbumin providing 10% or 25% energy reduces lunch time energy intake in lean subjects compared to a breakfast diet with casein, soy or whey without glycomacropeptide [20]. This data is in agreement with the results of another study [21], which together suggest an important role for α-lactalbumin in suppression of food intake in lean humans. In addition to this whey protein, β-lactoglobulin has also been shown to influence energy intake. The study by Pichon et al. that showed data highlighting the importance of dietary composition on whey protein effects on energy intake, also tested the effects of β-lactoglobulin on energy intake in rats and found that β-lactoglobulin reduced energy intake compared to whole milk proteins [11]. In summary, consumption of whey proteins as an isolate or concentrate appears to reduce food (energy) intake in humans and rodents by inducing satiety compared to casein, soy or carbohydrates. This effect is influenced by the macronutrients in the whey protein diet and by development of obesity. In the former case, a high carbohydrate composition appears to favour the actions of whey proteins. Of the whey proteins that have been studied, data suggests an important role(s) for α-lactalbumin and β-lactoglobulin in the regulation of food (energy) intake, with no significant regulatory role for glycomacropeptide in this physiological process.

Lipid Metabolism

In over-weight or obese humans and rodents, whey proteins have been shown to improve lipid metabolism, particularly elevated plasma, adipose and hepatic TAG levels. Recently Pal et al. demonstrated that intake of whey protein isolate (27 g) twice daily for 12 weeks causes a reduction in fasting TAG levels in over-weight and obese subjects compared to casein intake [22]. A similar effect has also been observed in obese rats with intake of whey protein concentrate at 32% (wt/wt) compared to 8% (wt/wt) [23]. Even at a reduced whey protein isolate content (24% wt/wt providing 18% energy), these dietary proteins reduce high fat (providing 60% energy) diet induced weight gain and body fat in mice compared to casein intake [24]. The reduction in body weight and fat content observed by the latter study was not due to a difference in the intestinal fat absorption in the two groups. The authors thus investigated whether whey protein isolate may affect adipocyte lipid metabolism [25]. By providing the same high fat diets containing either whey protein isolate or casein for 21 weeks, it was shown that the adipocyte cross sectional area was reduced in mice fed with whey proteins compared to casein. This suggests that whey proteins influence lipid metabolism in adipocytes much more than casein, although the extent of the reduction in adipocyte size may have been influenced by the fact the diets contain calcium, which is known to influence lipid metabolism in this tissue [26]. Given that the liver is also important for regulation of lipid metabolism, the same authors assessed in a separate study how the same dietary challenge may impact upon the lipid metabolism in the liver of high fat diet-induced obese mice subjected to a 7 week calorie restriction [27]. Compared to calorie restricted obese mice on the casein diet, calorie restricted obese mice on the whey protein isolate diet had reduced TAG levels in the liver. The specificity with which whey protein isolate influences hepatic lipid content has been further demonstrated (in the absence of calorie restriction) by the study by Shertzer et al. [14]. In the studies mentioned above, except for the latter, the observed effects of whey protein on lipid metabolism were shown relative to casein. Interestingly, in contrast, soy proteins at 24% (wt/wt) in the diet of rats had a similar effect on body weight and abdominal fat as whey proteins [8]. This data could be interpreted to suggest that whey protein effects on lipid metabolism are detectable only when casein is used as the control protein, or that the lack of an effect on lipid metabolism in comparison to soy might be due to the fact that the above study assessed dietary protein effects on lean rats as oppose to obese rodents, which are known to have a higher body

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weight and body fat content and thus, are likely to be more susceptible to whey protein effects on lipid metabolism. A number of studies have attempted to determine how each individual whey protein affects lipid metabolism. Two human studies show date related to the actions of lactoferrin and glycomacropeptide. By providing overweight human subjects with enteric coated lactoferrin (100 mg/d) or placebo tablets for a period of 8 weeks, it has been found that lactoferrin reduces body weight, visceral fat mass as well as hip circumference [28]. In contrast, ingestion of glycomacropeptide-enriched whey protein diet (with 15 g of proteins) twice daily replacing two daily meals for 6 months or once daily for further 6 months, had no significant effect on body weight or plasma TAG concentrations compared to skim milk powder diet [29]. It would be interesting to further define whether the outcome would have been different if a higher glycomacropeptide content was used, given also that data from a rodent study described below suggests an important role for this whey protein in the regulation of lipid metabolism. It has been shown that rats ingesting whey protein isolate diet supplemented with glycomacropeptide at 100 and 200 g/kg reduces carcass fat compared to in rats on a whey protein isolate diet or casein diet [9]. Because there were no differences in food intake between the groups, the data suggests a food intake-independent effect of glycomacropeptide on lipid metabolism (fat content) in these animals. With the view to assessing how other whey proteins, namely α-lactalbumin, β-lactoglobulin and lactoferrin may influence body weight and body fat content in rodents, Pilvi et al. subjected high fat diet induced obese mice to a 70% energy restricted diet containing whey protein isolate or each of the whey proteins mentioned above (18% energy) for a period of 7 weeks to induce weight loss [30], and subsequently allowed *ad libitum* access to the diets for further 7 weeks to allow weight gain. Results suggest that α- lactalbumin is the most beneficial whey protein in terms of causing fat loss when provided as part of an energy restricted diet or fed *ad libitum*. Although lactoferrin caused the most weight loss and had a similar effect on percentage fat content to α-lactalbumin in the initial 7 weeks, during the *ad libitum* feeding period however, lactoferrin caused the most gain in total fat content. In contrast, the human study mentioned above found that lactoferrin reduces body weight and fat content [28]. In further support for a role for lactoferrin in reducing adiposity, it has been shown *in vitro* that this whey protein specifically inhibits adipogenesis and lipid accumulation in adipocytes [31,32]. Although Pilvi et al. study showed that β-lactoglobulin was the least effective whey protein for inducing weight loss and for preventing weight gain [30], Pichon et al. found that β-lactoglobulin (55% energy) causes the least weight gain when supplemented in a diet containing only fat providing 45% of energy in comparison to a diet containing 35% and 10% energy from carbohydrates and fat or in a diet with 15% and 30% energy from the same macronutrients, respectively [11]. The data again highlights the importance of macronutrient composition on whey proteins effects on energy balance. Note here that the macronutrient composition providing the maximum whey protein effects on body weight and body fat content appear to differ from the composition that seemed to provide a greater reduction in food intake mentioned previously; in both cases however, a diet with carbohydrate and fat providing 35% and 10% energy respectively appear to be effective to bring about both changes in food intake and body weight/lipid metabolism. In summary, whey protein isolate appears to reduce body weight and lipid metabolism both in obese humans and in rodents in comparison to casein intake, with specific effects observed in the adipocytes and in the liver, although these effects appear to be influenced by macronutrient composition in the diet. Available data suggest an important role(s) for α-lactalbumin, β-lactoglobulin, lactoferrin and glycomacropeptide in the regulation of lipid metabolism in humans and/or in rodents.

Insulin Sensitivity and Glucose Homeostasis

In healthy and overweight humans and rodents whey protein intake has been shown to cause an acute insulinotropic response. This has been shown by supplementation of whey proteins into glucose drinks or test meals, which augments postprandial insulin release, resulting in an enhancement of glucose disposal in both healthy [33,34] and type-2 diabetic [35] subjects. Similar effects have been seen in anesthetised mice, where gastic gavage of whey protein (75 mg) and glucose (75 mg) together augmented the insulin response 3-fold and increased glucose disposal by 31% in comparison to glucose alone [36]. In addition to acute responses, prolonged whey protein intake also improves insulin sensitivity in the obese state in both rodents [14,23,27,37,38] and humans [22]. Of the whey proteins that have been tested, both α-lactalbumin and glycomacropeptide have been found to increase the postprandial insulin release (in comparison to casein protein intake) with glycomacropeptide having a greater effect than whey protein isolate or α-lactalbumin [16]. Long-term glycomacropeptide intake has also been shown to improve fasting blood insulin levels in both humans [29] and rats [9]. Similarly a high protein diet (55% kcal) with β-lactoglobulin as it source of protein was shown by Pichon et al. [11] to reduce insulin resistance and improve fasting blood insulin levels in rats to a greater extent than that of comparable high whey protein concentrate diet. The actions of α-lactalbumin and β-lactoglobulin on insulin sensitivity and glucose homeostasis may be due to the bioactive peptides in the proteins, since several dipeptides from α-lactalbumin and β-lactoglobulin have been found to increase glucose uptake in L6 myotubes and isolated skeletal muscles *in vitro* [39]. A role for lactoferrin in the regulation of glucose homeostasis has also been suggested based on the findings that circulating levels of lactoferrin correlate negatively with hyperglycemia and positively with insulin sensitivity [40], and that intake of a lactoferrin rich whey protein isolate supplemented high fat diet improves glucose tolerance in mice in comparison to high fat diet containing casein [38]. In summary, whey protein intake appears to stimulate insulin release and could improve glucose tolerance and insulin sensitivity long term, even when accompanied by high fat feeding. The available data also suggest that this effect may be a common feature of α-lactalbumin, β-lactoglobulin, lactoferrin and glycomacropeptide, with the latter being more potent than α-lactalbumin.

Energy Expenditure

Whey protein (18 g) intake prior to a bout of heavy resistance training increases post-training resting energy expenditure in humans compared to carbohydrate intake [41]. Interestingly, this effect diminishes if the whey protein meal was ingested after the resistance exercise, even if the protein content in the diet was at 30 g [42]. The data suggest that the timing of the dietary challenge is crucial for detecting whey protein induced changes in resting energy expenditure. Intake of whey proteins also appears to increase thermogenesis, possibly because of its higher thermic effect compared to soy or casein [33]. This may possibly be due to the whey protein induced increased protein metabolism in tissues [43]. To our knowledge, of the whey proteins associated with regulation of energy balance, only α-lactalbumin has been shown to influence energy expenditure. The study by Hursel et al. which reported an effect of α-lactalbumin (41% energy from the protein) ingestion on lunchtime meal intake in the healthy humans, also found that this whey protein significantly increases diet-induced thermogenesis compared to intake of whole milk protein rich diet [21]. Whether α-lactalbumin, or any other whey protein, could influence energy expenditure in obese humans remains to be determined, although data from a rodent study clearly suggests

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such an effect; it has been shown that diet-induced obese mice drinking water supplemented with whey proteins (100 g/L) have increased O_2° consumption compared to obese mice drinking unsupplemented water [14]. In summary, compared to carbohydrates, soy and casein, intake of whey proteins appears to increase energy expenditure in lean and obese states by influencing thermogenesis and resting energy expenditure. In the latter case, the timing of the dietary challenge might be important, in particular when associated with exercise, for obtaining an effective change in this parameter linked to energy expenditure. It is tempting to suggest that the effect of whey proteins on energy expenditure might be due to the actions of α-lactalbumin present in the whey protein isolate or concentrate, however, this may be premature given that there are no data showing whether other whey proteins could influence energy expenditure to the same extent.

Cellular Activity and Endocrine System

It is well established that the hormones CCK, ghrelin, insulin and glucagon-like peptide-1 (GLP-1) play important roles in energy balance regulation by inhibiting (CCK, insulin and GLP-1) and stimulating (ghrelin) food (energy) intake and/or by inducing a catabolic (GLP-1) and anabolic (ghrelin and insulin) responses on lipid metabolism in tissues [44,45]. Given the effects of whey proteins on food intake and body weight, it is no surprise that whey proteins modulate these hormones. A study by Bowen et al. showed that whey protein induced decrease in energy intake in humans, is accompanied by an increased plasma level of CCK and GLP-1 and reduces levels of ghrelin [46]. These changes were noted as early as 15 mins and continued up to 180 mins after the dietary challenge, suggesting potential acute and chronic effects of whey proteins on hormonal levels. In the case of insulin, whey proteins appear to acutely increase hormone levels, but over a time, there is a notable decline. Similar effects on hormone levels have been observed in studies conducted with only lean [6] and obese subjects [13], suggesting that whey proteins modulate hormone levels independent of weight gain and that the changes are consistent with an attempt to increase catabolism. Similar data have been obtained from rodent studies. Zhou et al. showed that the reduction in food intake in lean rats on a diet supplemented with 24% (wt/wt) whey protein concentrate, is accompanied by an increased GLP-1 level in the plasma [8]. Given that both α-lactalbumin and β-lactoglobulin have been shown to influence food (energy) intake, it would be interesting to find out whether these proteins are responsible for the observed changes in hormone levels detected in human and rodent studies.

An advantage of conducting rodent studies in comparison to human studies is that in the former case, it is possible to dissect and analyse tissues of interest to identify specific changes in expression of energy balance related genes. To our knowledge, such investigations have not been conducted to assess the impact of whey proteins on gene expression in centres of the brain important for regulation of energy balance. In contrast, there is data to suggest that whey proteins influence gene expression related to lipid metabolism in the adipocytes and in the liver cells. The study by Pilvi et al. mentioned above performed a detailed microarray analysis of gene expression in the (reduced) adipocytes of obese mice that had ingested whey proteins [25]. This analysis revealed an increased expression of several genes involved in insulin signalling pathway in the whey protein group compared to the control casein group. The microarray data also revealed an increased expression of genes for leptin and β_3-adrenergic receptor. Since the mass of the adipose tissue and leptin gene expression has previously been shown to be closely linked [47], the significance of the increased expression of leptin gene in adipocytes with reduced cross sectional area remains to be determined. In contrast, a potential functional relationship could exist between the reduction in the adipose tissue and increased β_3-adrenergic receptor expression, given that the receptor activation is known to increase hormone sensitive lipase-mediated hydrolysis of fat, increase fat oxidation and induce uncoupling protein-mediated thermogenesis [48,49], all of which are likely to reduce mass of the adipose tissue. This also indicates a potential mechanism by which whey proteins could reduce adiposity. With regard to the rat liver, whey proteins have been found to reduce activity of several lipogenic enzymes including fatty acid synthase (FAS) compared to casein intake [50]. In contrast, in the muscle, FAS expression and activity was increased in response to whey protein challenge [50], possibly to reduce hepatic production of lipids and to promote synthesis of lipids in the muscle so that these could be oxidised in the mitochondria in the muscle cells to generate energy in the form of adenosine 5' triphosphate. With regard to the above mentioned changes in the cellular activity observed in the adipose, muscle and liver, it is important to further define how they arise by investigating the impact of the whey proteins implicated in lipid metabolism, namely α-lactalbumin, β-lactoglobulin, lactoferrin and glycomacropeptide. In summary, in comparison to casein and carbohydrates (glucose and fructose), whey proteins appear to drive endocrine and cellular changes consistent with a catabolic effect. This is achieved by up-regulation of the production of catabolic hormones (CCK, GLP-1 and insulin), by reduction in the production of anabolic hormone ghrelin and by modulation of the expression or activity of lipogenic, lipolytic and fat oxidation related genes in the liver, adipose and in the muscle. The decrease in insulin levels observed over time with whey protein intake might be a mechanism to reduce anabolic effects of this hormone on adipocytes [45].

Minor Whey Proteins

In addition to the above mentioned whey proteins, many other lower-abundance proteins have been found within the whey fraction. Due to recent advances in milk proteomics [51,52], this list of minor whey proteins is increasing. In fact, a recent proteomic investigation of the whey fraction has found 293 unique gene products, 176 of which were newly identified in whey [52]. Although the potential energy balance related roles of these minor whey proteins have yet to be investigated, it is interesting that some of these proteins such as lipoprotein lipase, perilipin-2 and fatty acid binding proteins 3 and 5, have defined roles in lipid metabolism and storage. Recently, a study assessed the impact of a novel whey protein isolate rich in lactoperoxidase on high fat diet induced obesity [38] and found that this diet dose-dependently reduced bodyweight, fat mass gain, hepatic lipid accumulation and improved glucose tolerance [38]. These findings again raise the energy balance related impact of minor whey proteins.

Summary and Conclusions

Overall the data suggest that whey proteins (α-lactalbumin and β-lactoglobulin) decrease food intake, possibly by altering the plasma levels of hormones (CCK, GLP-1, ghrelin and insulin) important for energy balance regulation. In addition, whey proteins (α-lactalbumin, β-lactoglobulin, lactoferrin and glycomacropeptide) also alter lipid metabolism. This may be achieved by (1) decreasing FAS gene expression and hence TAG production in the liver, (2) by increasing lipogenesis in the muscle possibly for oxidation, and (3) by increasing β_3-adrenergic receptor expression in the adipocytes, possibly to decrease FAS expression in this tissue. Whey protein intake (α-lactalbumin, β-lactoglobulin, lactoferrin and glycomacropeptide) improves insulin sensitivity and glucose tolerance, preventing high fat diet induced insulin resistance. With regard to energy expenditure, whey proteins (α-lactalbumin) increases this energy balance related parameter

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possibly by increasing protein anabolism in the tissues. As the macro- and micro-nutrient (calcium) composition in the diet and obesity all influence the mechanisms involved in the regulation of energy balance, one could envisage that any changes in these mechanisms could greatly impact upon the ability of whey proteins to influence the balance between energy intake and energy expenditure. A better understanding of how specific whey proteins influence energy balance may help in the formulation of dietary interventions that could prevent or reduce obesity.

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Appendix C

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Whey protein isolate counteracts the effects of a high-fat diet on energy intake and hypothalamic and adipose tissue expression of energy balance-related genes

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Abstract

The intake of whey protein isolate (WPI) is known to reduce high-fat diet (HFD)-induced body-weight gain and adiposity. However, the molecular mechanisms are not fully understood. To this end, we fed C57BL/6J mice for 8 weeks with diets containing 10 % energy as fat (low-fat diet, LFD) or 45 % energy as fat (HFD) enriched with either 20 % energy as casein (LFD and HFD) or WPI (high-fat WPI). Metabolic parameters and the hypothalamic and epididymal adipose tissue expression of energy balance-related genes were investigated. The HFD increased fat mass and plasma leptin levels and decreased the dark-phase energy intake, meal number, RER, and metabolic (VO_2 and heat) and locomotor activities compared with the LFD. The HFD increased the hypothalamic tissue mRNA expression of the leptin receptor, insulin receptor (*INSR*) and carnitine palmitoyltransferase 1b (*CPT1b*). The HFD also reduced the adipose tissue mRNA expression of *GLUT4* and *INSR*. In contrast, WPI reduced fat mass, normalised dark-phase energy intake and increased meal size in HFD-fed mice. The dietary protein did not have an impact on plasma leptin, insulin, glucose or glucagon-like peptide 1 levels, but increased plasma TAG levels in HFD-fed mice. At a cellular level, WPI significantly reduced the HFD-associated increase in the hypothalamic tissue mRNA expression of the leptin receptor, *INSR* and *CPT1b*. Also, WPI prevented the HFD-induced reduction in the adipose tissue mRNA expression of *INSR* and *GLUT4*. In comparison with casein, the effects of WPI on energy intake and hypothalamic and adipose tissue gene expression may thus represent a state of reduced susceptibility to weight gain on a HFD.

Key words: Obesity: Energy balance: Whey proteins: Gene expression: Mice

Obesity develops when energy consumed in excess of daily metabolic requirements is stored as TAG in the adipose tissue. The prevalence of obesity and associated conditions has reached epidemic proportions worldwide[1,2], and therefore there is a growing interest to identify nutritional factors that could influence energy balance and thus could reduce the susceptibility to develop obesity, such as that caused by a high-fat diet (HFD)[3–5].

Whey protein isolate (WPI) is a mixture of milk proteins obtained after precipitation of casein during cheese production, and it contains β-lactoglobulin, α-lactalbumin, bovine serum albumin, glycomacropeptide, Ig, lactoferrin and other minor proteins. WPI has received a lot of attention recently because of its associated health benefits[6]. Prolonged intake of whey proteins has been found to improve insulin sensitivity and glucose homeostasis[7–10]. Also, using healthy lean subjects or rodents, a number of studies have reported a short-term reduction in energy intake and an increase in thermogenesis following WPI intake in comparison with other dietary proteins including casein[7,11–13].

Abbreviations: FQ, food quotient; HFD, high-fat diet; HF-WPI, high-fat diet with whey protein isolate; INSR, insulin receptor; LFD, low-fat diet; WPI, whey protein isolate.

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Although there are discrepancies[14–17], it is largely recognised that WPI intake influences the balance between energy intake and energy expenditure in the lean state[18,19]. In contrast, the effects of WPI on energy intake and energy expenditure in the HFD-induced state are less well known, although WPI has consistently been shown to reduce HFD-induced body-weight gain and adiposity[10,20–23]. However, three studies have reported no effects on energy intake in mice fed a HFD with WPI for 11[10], 14[23] or 21 weeks[21] in comparison with the HFD-fed controls. In the study by Shertzer *et al.*[10], WPI was found to increase metabolic activity, while Pilvi *et al.*[21] found no changes in metabolic activity in mice fed a HFD with WPI compared with those fed a control diet. It is possible that these discrepancies arise partly due to the difference in the duration of HF feeding, with prolonged feeding-induced neuroendocrine changes having a greater impact on the ability of WPI to regulate energy balance. Thus, one could argue that a shorter duration of HF feeding (in comparison with the above-mentioned studies) may reveal how WPI affects physiological and cellular parameters important for energy balance regulation.

The present study sought to investigate the short-term (8 weeks) impact of WPI in conjunction with a HFD, specifically focusing on how the dietary protein influences HFD-induced (1) body-weight gain and body composition, (2) energy intake (total intake, meal number and meal size) and parameters related to energy expenditure (VO_2, heat production and locomotor activity), (3) plasma levels of hormones and metabolites and (4) the hypothalamic and epididymal adipose tissue expression of genes involved in leptin and insulin signalling, inflammation and lipid metabolism.

Materials and methods

Animals, diets and reagents

Experiments involving mice were licensed under the Cruelty to Animals Act 1876 and received ethical approval from the University College Cork Animal Experimentation Ethics Committee (no. 2011/005). Male C57BL/6J mice (Harlan), 3–4 weeks old, were maintained at 20–22°C and 45–65% humidity, with a 12 h light–12 h dark cycle (06.00–18.00 hours). The animals had *ad libitum* access to food and water throughout the trials and all diets were purchased from Research diets. The experimental diets were a low-fat diet (LFD; 10% energy as fat and 20% energy as casein; no. D12450), a HFD (45% energy as fat and 20% energy as casein; no. D12451) and a HFD with WPI (HF-WPI) (Alacen™ 895; NZMP) in place of casein (45% energy as fat and 20% energy as WPI). All reagents were purchased from Sigma unless stated otherwise.

Experimental protocol

The dietary challenges were performed on group-housed mice (four per home cage) following a 4-week acclimatisation period, during which the animals were fed a LFD. As we intended to measure the effect of the above-mentioned diets on energy intake and metabolic parameters using TSE Phenomaster cages (TSE systems), by housing the mice individually in these specialised cages, it was important to first determine the length of time the animals may need to be acclimatised to this new cage environment. This was investigated in study 1. In study 2, we assessed the effect of the LFD, the HFD and the HF-WPI diet on energy intake and metabolic parameters using the above-mentioned cages, following the pre-established acclimatisation period.

Study 1

Mice were fed a LFD for 7 weeks (n 8). In weeks 5 and 6, the animals were individually housed in TSE Phenomaster cages (TSE systems) for 3 d and energy intake and metabolic parameters were measured on days 2 and 3. At the end of the third day, the animals were placed back in their appropriate home cages. Food intake in the Phenomaster cages was measured using high-precision weighing sensor-associated feeding stations. O_2 consumption (ml/h per kg; VO_2) and CO_2 production (ml/h per kg, VCO_2) were measured by indirect open-circuit calorimetry. The sensors measured the levels of food consumed and VO_2 and VCO_2 in each cage every 9 min. A meal was defined as an intake greater than 0·01 g. Energy intake was calculated from 16·10 kJ/g for the LFD. The RER was calculated from VCO_2/VO_2. Locomotor activity was measured using a multi-dimensional infrared beam system, and it is defined as the total number of infrared beam breaks in the X-axis and Y-axis.

Study 2

The experimental protocol is outlined in Fig. 1. For 8 weeks, three weight-matched groups of mice were fed the LFD, the HFD or the HF-WPI diet (n 8). Body weights were measured weekly. In weeks 5 and 6, the energy intake and metabolic activity were measured in the individual mice using the TSE Phenomaster cages (TSE systems; Fig. 1). As mentioned earlier, the animals were housed in the Phenomaster cages for 3 d. Following a 2 d acclimatisation period (see the Results section for study 1), data were collected on the third day, after which the animals were placed back in their home cages. The parameters mentioned earlier were measured. Energy intake was calculated as described earlier for the LFD and using 19·80 kJ/g for the HFD and the HF-WPI diet. Heat production (kcal/h per kg) was calculated using the Weir equation[24] ($3{\cdot}941 \times VO_2 + 1{\cdot}106 \times VCO_2$) and converted to kJ/h per kg (1 kcal = 4·184 kJ). The food quotient (FQ), defined as the ideal diet-specific VCO_2:VO_2 ratio, was calculated for each diet as described previously[25]. In week 8, after a 6–8 h fast, body composition was determined by NMR using the Bruker minispec LF50H (Bruker Optics). Also, at that time point, plasma was collected from fasted anaesthetised mice to measure the glucagon-like peptide 1, insulin, glucose, leptin and TAG levels. The animals were then immediately culled by cervical dislocation, and tissues of interest were dissected and snap-frozen in liquid N_2 (liver and adipose tissue) or on dry ice (brain).

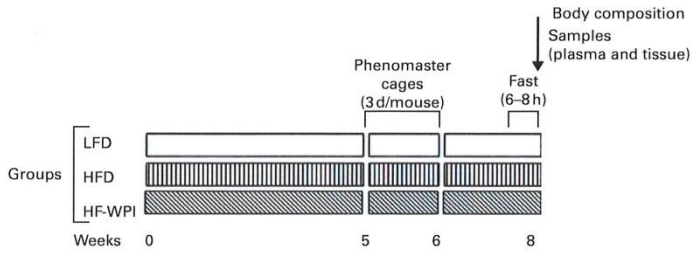

Fig. 1. Timeline of the dietary treatments and experimental measurements. C57BL/6J mice were fed for 8 weeks with diets containing 10 % energy as fat (low-fat diet, LFD) or 45 % energy as fat (high-fat diet, HFD) with either 20 % energy as casein (LFD and HFD) or whey protein isolate (high fat (HF)-WPI). To measure metabolic parameters at weeks 5 and 6, mice were individually housed in TSE Phenomaster cages (TSE systems) for 3 d with data being collected in the final 24 h of the housing period. In week 8, body composition was measured following a 6–8 h fast, and then plasma and tissue samples were isolated.

Plasma hormones, glucose and TAG

Blood collected into BD Vacutainer EDTA tubes from fasted anaesthetised (65 mg/kg ketamine and 13 mg/kg xylazine) mice was treated with 70 mg/l aprotinin and 0·1 mM-diprotin A (Sigma) to prevent the degradation of plasma peptides by dipeptidyl aminopeptidase IV, trypsin and other related proteolytic enzymes. Blood was centrifuged at 2000 rpm and 4°C for 15 min, and plasma was isolated and stored at −80°C. ELISA were used to measure plasma glucagon-like peptide-1 (Linco Research), leptin and insulin (Crystal Chem) levels. Plasma glucose and TAG levels were measured using colorimetric assay kits (glucose: Calibochem; TAG: Wako Chemicals). Homeostatic model assessment of insulin resistance values were determined using the following formula[26]:

Fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/l)/22·5.

Liver TAG

Lipids from frozen liver samples (50 mg) were extracted according to the Folch method[27]. Briefly, lipids in each sample were extracted using a 2:1 (v/v) solution of chloroform–methanol and aliquots of the organic phase were collected, dried and resuspended in duplicate in the infinity TAG lipid-stable reagent (Thermo Scientific) or the LabAssay TAG kit reagent (Wako Chemicals). TAG levels in the samples were determined according to the manufacturer's instructions, and for samples resuspended in the infinity TAG lipid-stable reagent, lipid quantification was performed using a TAG chemistry calibrator (Pointe Scientific).

RNA extraction and complementary DNA synthesis

Total RNA was isolated from the hypothalamic and epididymal adipose tissues with diethylpyrocarbonate-treated distilled water using the RNeasy lipid tissue mini kit (Qiagen) according to the manufacturer's instructions. To remove any potential genomic DNA contamination, on-column DNase treatment (Qiagen) was performed during RNA isolation. Complementary DNA was synthesised from 1 μg of total RNA using 2·5 ng/μl of random hexamer primers (Bioline), 0·5 mM-deoxyribonucleotide triphosphate (Promega), 32 nKat/μl of RNase inhibitor (Promega) and the First Strand Synthesis System containing the SuperScript II RT (Invitrogen), according to the manufacturer's instructions. A parallel reaction without the inclusion of the RT enzyme was also performed as a negative control.

Real-time PCR

The amplification of complementary DNA was performed in the Lightcycler 480 system (Roche) using 0·25 μM primers (Eurofins MWG Operon), 1 μl complementary DNA and the Lightcycler 480 SYBR Green I Master kit (Roche), according to the manufacturer's instructions. Real-time PCR conditions were as follows: 95°C for 10 min followed by fifty cycles at 95°C for 10 s; 60 or 55°C for 5 s; 72°C for 15 s. The primer sequences used are given in Table 1. Melting curve analysis allowed the validation of the authenticity of the real-time PCR products. Automated sequencing was performed to verify the sequences of these PCR products. Data obtained as C_p values were normalised to the expression of *18S* and *β-actin* according to $\Delta\Delta C_p = \Delta C_p$ target gene $- \Delta C_p$ housekeeping gene. For both the adipose tissue[28–31] and the hypothalamus[32–34], *18S* and *β-actin* have been shown to

Table 1. Sequences of primers used for real-time PCR

Genes	Forward primer (5′—3′)	Reverse primer (5′–3′)
PPARγ	5′-tcaggtttgggcggatgc-3′	5′-tcagcgggaaggactttatgtatg-3′
FASN	5′-tccacctttaagttgccctg-3′	5′-tctgctctcgtcatgtcacc-3′
LPL	5′-tgtctaactgccacttcaaccac-3′	5′-gggcacccaactctcatacattc-3′
GLUT4	5′-ggcctgcccgaaagagtc-3′	5′-aggagctggagcaaggac-3′
β-3AR	5′-cgccttcaacccggtcatctactg-3′	5′-ggtggactctgcctggcttcaac-3′
INSR	5′-gatttccccaacgtctcctctac-3′	5′-caatgcggtacccagtgaagtg-3′
CPT1b	5′-cgagaggggcggactgagactg-3′	5′-ggctaggcggtacatgtttggtg-3′
POMC	5′-gggcaagcgctcctactccatg-3′	5′-ctcgccttccagctccctcttg-3′
ObR	5′-gaccaccgaacacaaccgatgac-3′	5′-acacctagctggcgaaaaactgaag-3′
TNF-α	5′-tggcctccctctcatcag-3′	5′-acttggtggtttgctacgac-3′
CD68	5′-cacttcgggccatgtttctcttg-3′	5′-aggggctggtaggttgattgtcgtc-3′
FATP1	5′-ccggtgtggtggctgctcttctc-3′	5′-gctgccatctccccgccataaatg-3′
β-Actin	5′-agagggaaatcgtgcgtgac-3′	5′-caatagtgatgacctggccgt-3′
18S	5′-aggaccgcggttctattttgttggg-3′	5′-atgctttcgctct-ggtccgtcttg-3′

PPARγ, peroxisome proliferator activated receptor; *FASN*, fatty acid synthase; *LPL*, lipoprotein lipase; *GLUT4*, Glucose transporter 4; *β-3AR*, β-3 adrenergic receptor; *INSR*, insulin receptor; *CPT1b*, carnitine palmitoyltransferase 1b; *POMC*, pro-opiomelanocortin; *ObR*, leptin receptor; TNF-α, Tumour necrosis factor alpha; *CD68*, cluster of differentiation 68; *FATP1*, fatty acid transporter 1.

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be the appropriate housekeeping genes. The relative gene expression was calculated using $2^{-\Delta\Delta C_T}$, and it is shown in comparison with that of the LFD group.

Immunoblot analysis

Adipose tissue samples (approximately 1 ml of lysis buffer per 200 mg, *n* 3) were homogenised in a lysis buffer (50 mM-HEPES, 150 mM-NaCl, 1 mM-EDTA, 1 mM-ethylene glycol tetraacetic acid, 1% Nonident P40, 0·5 mM-dithiothreitol and 0·1 mM-Na_3VO_4), containing protease inhibitors (0·1 mM-phenylmethylsulphonyl fluoride, 2 μg aprotinin/ml, 2 μg leupeptin/ml, 0·02 mM-NaF and 0·025 mM-NaPPi), and centrifuged at 12 000 ***g*** for 10 min to remove insoluble debris. Protein concentrations were analysed using the bicinchoninic acid reagent (Pierce Biotechnology). Protein lysate (20 μg) was mixed with a 4 × sample buffer (333 mM-Tris–HCl, 3% SDS, 26·7% glycerol, 130 mM-dithiothreitol and 0·2% Bromophenol Blue) and heated for 10 min at 95°C before loading onto a NuPAGE® 4–12% Bis–Tris gel (Invitrogen). Protein bands were separated using SDS-PAGE in the 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer and transferred onto polyvinylidene difluoride membranes (BioRad). A Tris-buffered saline solution containing 0·1% Tween-20 was used as the wash buffer, which was supplemented with 2% bovine serum albumin (Sigma) and 3% non-fat dry milk (Marvel; Premier Foods Limited) for the blocking solution and with 1% bovine serum albumin and 1% non-fat dry milk for the antibody diluent. The blocked membranes were exposed to a 1:500 dilution of the primary antibody against the insulin receptor (INSR) β (sc-711; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibody (1:8000 dilution; Jackson Immuoresearch) was used to visualise the bound primary antibody. The membranes were stained with a 1:15 000 dilution of a β-actin–horseradish peroxidase antibody (A3854; Sigma) to correct for sample loading. Visualisation was performed using the enhanced chemiluminescence Western blotting Substrate Kit (Pierce Biotechnology).

Statistical analysis

All data are presented as means with their standard errors. Differences between the experimental groups were analysed by a one-way or two-way ANOVA with Tukey's *post hoc* pairwise comparisons. Body weight differences over 8 weeks were analysed by a two-way repeated-measures ANOVA with *post hoc* pairwise comparisons. Statistical analysis of immunoblot analysis data was performed using a multiple-comparisons Kruskal–Wallis test followed by Mann–Whitney *U* tests for individual comparisons. Significance was accepted at $P<0·05$, and the statistical analysis was performed using the GraphPad Prism version 3.03 (GraphPad Software, Inc.), Minitab version 15 (Minitab, Inc.) and Sigma Stat version 3.1 (SyStat Software, Inc.).

Results

Body weight and composition

Body weight trajectories over the 8-week treatment period showed that the mice on the HFD and the HF-WPI diet were significantly heavier than the LFD group from

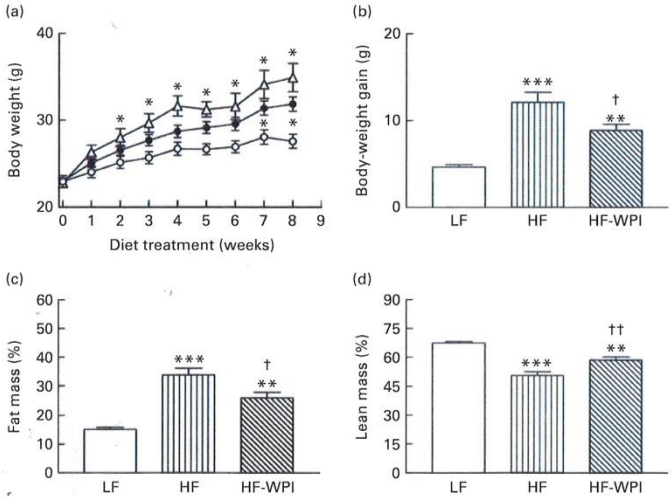

Fig. 2. Effect of feeding a low-fat (LF, –○–), a high-fat (HF, –△–) or a HF with whey protein isolate (HF-WPI, –●–) diet for 8 weeks upon (a) body weight, (b) body-weight gain, (c) total fat mass and (d) total lean mass in C57BL/6J mice. Values are means (*n* 8 per group), with standard errors represented by vertical bars. Mean value was significantly different from that of the group fed the LF diet: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. Mean value was significantly different from that of the group fed the HF diet: † $P<0·05$, †† $P<0·01$.

the second and seventh weeks, respectively (Fig. 2(a)). Importantly, the HF-WPI group gained significantly less body weight than the HFD group ($P<0·05$; Fig. 2(b)). In week 8, the total body fat (%) in the HF-WPI diet-fed mice was significantly lower than that in the HFD-fed mice ($P<0·05$; Fig. 2(c)), but the total body fat (%) was still greater than that in the LFD-fed mice ($P<0·001$). Furthermore, the total lean mass (%) was higher in the HF-WPI group than in the HFD-fed group ($P<0·05$; Fig. 2(d)), and as anticipated it was lower in the HFD- and HF-WPI diet-fed mice than in the LFD group ($P<0·001$).

Plasma hormone, glucose and TAG, and hepatic TAG levels

In week 8, plasma leptin concentrations were higher in the HFD and HF-WPI groups than in the LFD group ($P<0·05$), while plasma TAG levels were greater in the HF-WPI group than in both the LFD and HFD groups ($P<0·05$; Table 2). There was no significant difference in the plasma glucose, insulin, homeostatic model assessment of insulin resistance and glucagon-like peptide-1 levels among the three diet groups (Table 2). Hepatic TAG levels (mg/g liver) were determined using the infinity TAG lipid-stable reagent or the LabAssay TAG kit (Wako Chemicals) following the extraction of the lipids. The data generated using the former revealed that the HFD-fed mice had a higher hepatic TAG level than the LFD group ($P<0·05$; Table 2). While hepatic TAG levels in the HF-WPI diet-fed mice were lower than those in the HFD group, the values did not differ significantly from those of either the LFD or HFD group. In contrast, the data generated using the LabAssay TAG kit (Wako Chemicals) showed that the hepatic TAG values (mg/g liver) for the HF-WPI diet (58·68 (SEM 3·92))-fed mice were significantly lower than those for the HFD group (90·9 (SEM 8·65)) ($P<0·05$), while the values for the HF-WPI group were not significantly different from those for the LFD group (46·65 (SEM 3·09)).

Energy intake, energy expenditure and RER

Study 1. The LFD-fed mice were individually housed in the Phenomaster cages during weeks 5 and 6 of the 7-week dietary challenge. In the 3 d housing period in the Phenomaster cages, the energy intake, VO_2, RER and locomotor activity in the animals were similar on days 2 and 3 (Fig. S1, available online). We interpreted these data to suggest that by day 2 the mice had been acclimatised to the new Phenomaster cage environment and that data gathered on day 3 represent accurate measures of their energy intake and metabolic activity. Previous studies have also used a 48 h period for acclimatisation, before measuring the metabolic and feeding behaviours in mice using similar specialised cages[35,36].

Study 2. Energy intake and metabolic activity were measured as described earlier in weeks 5 and 6 of the 8-week dietary challenge. Energy intake was lower during the dark phase for the HFD group compared with both the HF-WPI diet- and LFD-fed mice ($P<0·05$; Fig. 3(a)). The average meal size over the same period was not significantly different between the LFD- and HFD-fed mice, while the HF-WPI diet-fed mice had a greater average meal size than both the LFD and HFD groups ($P<0·05$; Fig. 3(b)). The meal number during the dark phase was reduced for both the HFD and HF-WPI groups compared with the LFD-fed mice ($P<0·001$; Fig. 3(c)). No difference in energy intake-related parameters was found between all groups during the light phase (Fig. 3(a)–(c)). The FQ calculated for the LFD, HFD and HF-WPI diet are shown in Fig. 4(a). The RER of both the HFD- and HF-WPI diet-fed groups were lower than that of the LFD group in both the light and dark phases ($P<0·05$; Fig. 4(a)). The VO_2, heat production and locomotory activity during the dark phase of the HFD-fed mice were lower than those of the LFD group ($P<0·05$; Fig. 4(b)–(d)). In the HF-WPI diet-fed mice, these parameters did not differ significantly from those of either the LFD or HFD group during the dark phase. No differences in the above-mentioned parameters were observed between all groups during the light phase (Fig. 4(b)–(d)).

Adipose tissue gene and protein expression

The mRNA expression of *GLUT4* (Fig. 5(a)), *INSR* (Fig. 5(b)), β-3 adrenergic receptor (*β-3AR*; Fig. 5(c)) and fatty acid synthase (*FASN*; Fig. 5(d)) was significantly lower in the HFD group than in the LFD-fed mice ($P<0·05$). The mRNA

Table 2. Plasma levels of hormones and TAG and hepatic TAG accumulation
(Mean values with their standard errors, *n* 7–8)

	Low fat		High fat		High-fat WPI	
Groups...	Mean	SEM	Mean	SEM	Mean	SEM
Leptin (ng/ml)	3·07[a]	0·34	33·78[b]	6·27	18·79[b]	5·05
GLP-1 (pmol/l)	6·63	0·28	6·00	0·13	6·15	0·32
Insulin (ng/ml)	0·21	0·01	0·31	0·04	0·26	0·04
Glucose (nmol/μl)	10·65	1·40	11·60	1·00	12·18	0·49
HOMA-IR	2·67	0·39	4·17	0·62	3·46	0·32
Plasma TAG (mg/l)	949·3[a]	75·4	1127·0[a]	51·8	1464·7[b]	77·3
Liver TAG (mg/g liver)	48·61[a]	5·55	91·48[b]	20·06	59·35[a,b]	5·29

WPI, whey protein isolate; GLP-1, glucagon-like peptide 1; HOMA-IR, homeostatic model assessment of insulin resistance.
[a,b] Mean values with unlike superscript letters were significantly different ($P<0·05$).

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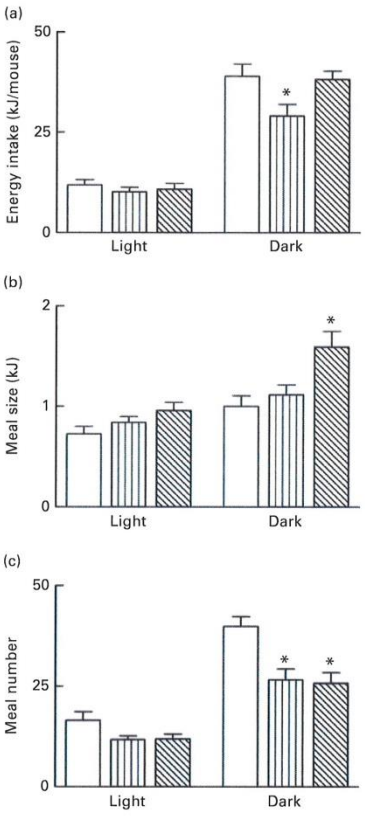

Fig. 3. Effect of feeding a low-fat (LF, □), a high-fat (HF, ▥) or a HF with whey protein isolate (HF-WPI, ▨) diet for 5 to 6 weeks upon (a) energy intake (b) meal size and (c) meal number in C57BL/6J mice. Experimental data, collected from individual mice at 9 min intervals over a 24 h period using TSE Phenomaster cages (TSE systems), are shown for the light and dark phases. Values are means (n 8 per group), with their standard errors represented by vertical bars. (a) * Mean value was significantly different from those of the LF and HF-WPI groups ($P<0·05$). (b) * Mean value was significantly different from those of the LF and HF groups ($P<0·05$). (c) * Mean value was significantly different from that of the LF group ($P<0·05$).

expression of *INSR* and *GLUT4* was increased in the HF-WPI diet-fed mice compared with the HFD-fed mice ($P<0·05$), such that the expression was not significantly different from that of the LFD group (Fig. 5(a) and (b)). Similarly, the mRNA expression of *β-3AR* in the HF-WPI group was increased compared with the HFD group, but did not reach statistical significance. The mRNA expression of *FASN* was not affected by the HF-WPI diet (Fig. 5d). No significant difference in the expression of the following genes was found between all the three diet groups: *PPARγ* (1·00 (SEM 0·17) in the LFD group *v.* 0·93 (SEM 0·24) in the HFD group *v.* 1·09 (SEM 0·18) in the HF-WPI group); lipoprotein lipase (*LPL*: 1·00 (SEM 0·16) in the LFD group *v.* 1·37 (SEM 0·21) in the HFD group *v.* 1·54 (SEM 0·20) in the HF-WPI group); carnitine palmitoyltransferase 1b (*CPT1b*: 1·00 (SEM 0·15) in the LFD group *v.* 1·24 (SEM 0·22) in the HFD group *v.* 1·24 (SEM 0·14) in the HF-WPI group); fatty acid transporter protein 1 (*FATP1*: 1·00 (SEM 0·13) in the LFD group *v.* 0·83 (SEM 0·13) in the HFD group *v.* 0·93 (SEM 0·10) in the HF-WPI group); *TNF-α* (1·00 (SEM 0·14) in the LFD group *v.* 2·35 (SEM 0·82) in the HFD group *v.* 1·43 (SEM 0·31) in the HF-WPI group); cluster

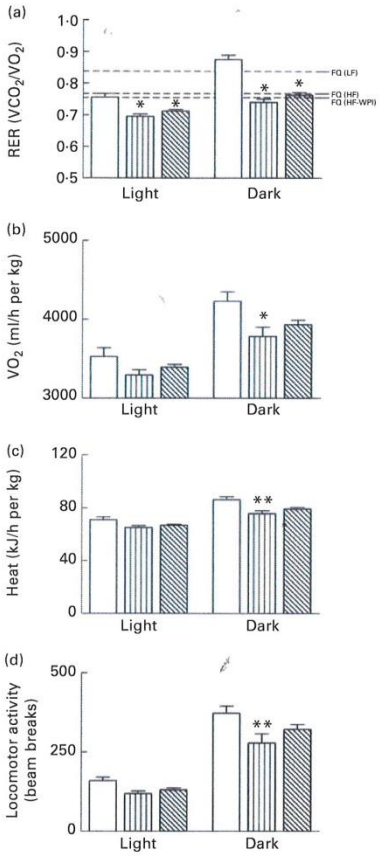

Fig. 4. Effect of feeding a low-fat (LF, □), a high-fat (HF, ▥) or a HF with whey protein isolate (HF-WPI, ▨) diet for 5 to 6 weeks upon (a) RER, (b) oxygen consumption (VO_2), (c) heat production and (d) locomotor activity in C57BL/6J mice. Experimental data, collected from individual mice at 9 min intervals over a 24 h period using TSE Phenomaster cages (TSE Systems), are shown for the light and dark phases. The food quotient (FQ; ----) used in (a) is defined as the ideal diet-specific VCO_2:VO_2 ratio, and it was calculated for each diet as described previously[25]. Values are means (n 8 per group), with standard errors represented by vertical bars. Mean value was significantly different from that of the group fed the LF diet: * $P<0·05$, ** $P<0·01$.

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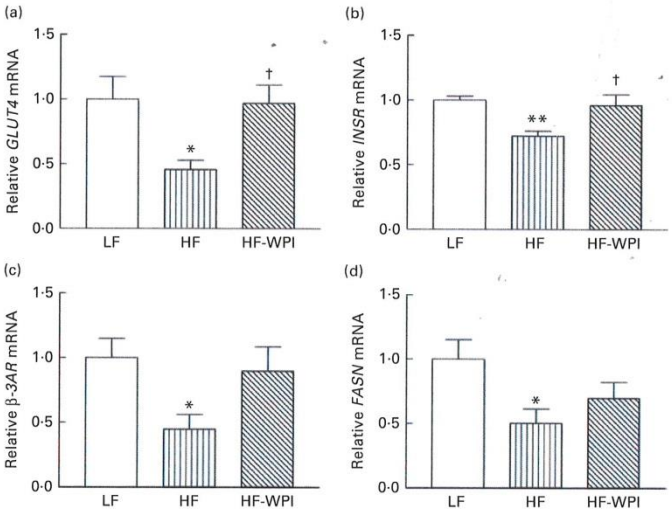

Fig. 5. Effect of feeding a low-fat (LF), a high-fat (HF) or a high-fat with whey protein isolate (HF-WPI) diet for 8 weeks upon the epididymal adipose tissue mRNA expression of (a) *GLUT4*, (b) insulin receptor (*INSR*) (c) β-3 adrenergic receptor (*β-3AR*) and (d) fatty acid synthase (*FASN*) in C57BL/6J mice. The mRNA expressions were normalised using *18S* and *β-actin* according to $\Delta\Delta C_p = \Delta C_p$ target gene $- \Delta C_p$ housekeeping gene. The gene expressions were calculated using $2^{-\Delta\Delta C_p}$, and they are shown in comparison with that of the LF group. Values are means (n 7–8 per group), with their standard errors represented by vertical bars. Mean value was significantly different compared with the LF group: * $P<0{\cdot}05$, ** $P<0{\cdot}01$. † Mean value was significantly different compared with the HF group ($P<0{\cdot}05$).

of differentiation 68 (*CD68*: 1·00 (SEM 0·17) in the LFD group *v.* 2·95 (SEM 1·05) in the HFD group *v.* 1·37 (SEM 0·28) in the HF-WPI group).

The association between the diet and the expression of *INSR* was further explored by investigating the protein expression of INSR-β in the epididymal adipose tissue (Fig. 6). The intake of the HFD decreased the protein expression of INSR-β compared with the intake of the LFD, similar to mRNA expression, although the decreased protein expression did not reach statistical significance (Fig. 6). The protein expression of INSR-β did not differ between the HFD and HF-WPI groups (Fig. 6).

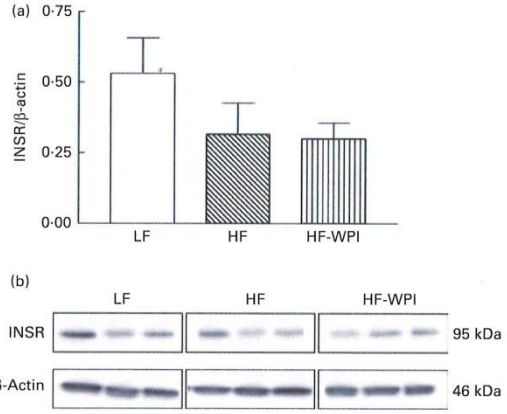

Fig. 6. Effect of feeding a low-fat (LF), a high-fat (HF) or a high-fat with whey protein isolate (HF-WPI) diet for 8 weeks upon the epididymal adipose tissue expression of insulin receptor-β (INSR-β), as measured by the immunoblot analysis. The protein expression of INSR-β relative to the expression of β-actin is shown in (a) and the images of the corresponding immunoblot are shown in (b). Values are means (n 3 per group), with their standard errors represented by vertical bars.

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Hypothalamic tissue gene expression

Given the key central effects on feeding and adipocyte metabolism[37,38], we next focused our attention on the hypothalamus and investigated whether the diets affected the expression of energy balance-related genes in this region. The data showed that the HFD group had increased mRNA expression of pro-opiomelanocortin (*POMC*), leptin receptor (*ObR*), *INSR*, *CPT1b*, *PPARγ* and *CD68* compared with the LFD-fed mice ($P<0{\cdot}001$; Fig. 7(a)–(f), respectively). Similarly, an increase in the mRNA expression of *TNF-α* was observed with HFD feeding ($P<0{\cdot}05$; Fig. 7(g)). The mRNA expression of *ObR*, *INSR*, *CPT1b* and *TNF-α* was significantly lower in the HF-WPI-fed mice than in the HFD group ($P<0{\cdot}05$; Fig. 7(b)–(d) and (g)), with no significant difference in expression being observed for *POMC* and *CD68* between the two groups. The mRNA expression of *PPARγ* in the HF-WPI diet-fed mice also showed a trend towards a decrease compared with the HFD values ($P=0{\cdot}06$). The observed mRNA expression of *ObR*, *INSR*, *CPT1b*, *PPARγ* and *CD68* was still elevated in the HF-WPI group compared with the LFD group ($P<0{\cdot}05$).

Discussion

Mammals are able to defend perturbations in their body weight and adiposity by a complex interaction between central and peripheral tissues. The intake of a HFD is known to shift the defended body weight to a higher set point through changes in the mechanisms regulating energy balance[39]. To better understand how WPI may influence this shift in energy balance during an 8-week HFD feeding trial, we compared energy balance-related parameters in LFD-, HFD- and HFD with WPI (HF-WPI)-fed mice at weeks 5 and 6 and at week 8. In agreement with recent findings[10,20–22], WPI reduced HFD-induced weight gain and adiposity. Interestingly, the HF-WPI diet-fed mice had increased energy intake but unaltered energy expenditure-related parameters compared with the HFD-fed mice. WPI also influenced hypothalamic and adipose tissue gene expression in the latter mice. To our knowledge, the present study is the first to show that WPI has an impact on energy intake by affecting the meal size and also causes changes to hypothalamic cellular activity in HFD-fed mice.

High-fat feeding in mice has been shown to have a differential impact upon energy intake in a temporal fashion, with no

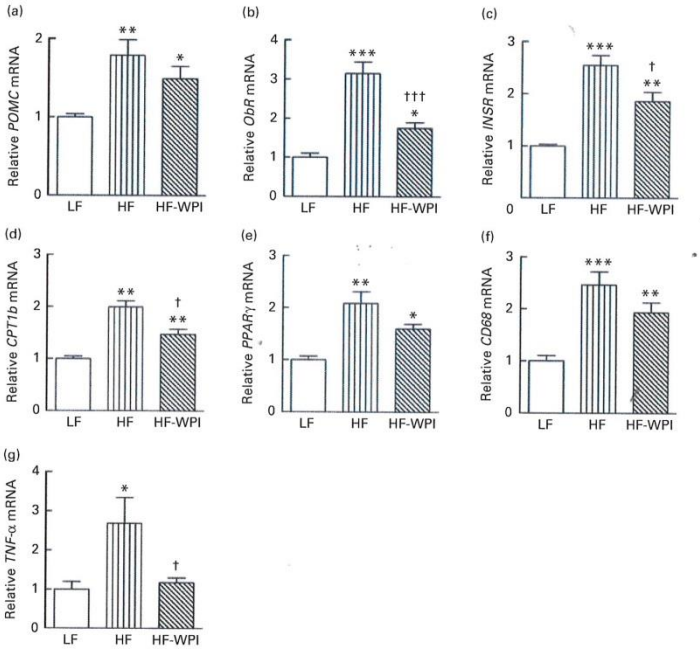

Fig. 7. Effect of feeding a low-fat (LF), a high-fat (HF) or a high-fat with whey protein isolate (HF-WPI) diet for 8 weeks upon the hypothalamic tissue mRNA expression of (a) pro-opiomelanocortin (*POMC*), (b) leptin receptor (*ObR*), (c) insulin receptor (*INSR*), (d) carnitine palmitoyltransferase 1b (*CPT1b*), (e) *PPARγ*, (f) cluster of differentiation 68 (*CD68*) and (g) *TNF-α* in C57BL/6J mice. The mRNA expressions were normalised using *18S* and *β-actin* according to $\Delta\Delta C_p = \Delta C_p$ target gene $- \Delta C_p$ housekeeping gene. The relative gene expressions were calculated using $2^{-\Delta\Delta C_p}$, and they are shown in comparison with that of the LF group. Values are means (n 6–8 per group), with their standard errors represented by vertical bars. Mean value was significantly different compared with the LF group: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$. Mean value was significantly different compared with the HF group: † $P<0{\cdot}05$, ††† $P<0{\cdot}001$.

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difference being observed in weeks 1–4, a significant reduction in energy intake during weeks 5–12 and a progressive increase in energy intake in week 13 onwards compared with the LFD-fed controls[5]. Consistent with these findings, during weeks 5 and 6, we found a reduced energy intake in the HFD-fed mice compared with the mice fed the LFD. We further observed that this reduction is due to the dark-phase consummatory behaviour, possibly resulting from the reduction in meal number. The data suggest that mice fed the HFD are attempting to regulate their energy intake at weeks 5 and 6 during the diet-induced shift in body weight (set point). The intake of WPI, in place of casein, increased HFD-induced dark-phase energy intake to a level comparable with that observed in the LFD group, probably by increasing the meal size. The differential effects of WPI and casein on HFD-induced energy intake may arise due to the differential kinetics of digestion and/or metabolism of the two proteins and the associated neuroendocrine changes, given that whey proteins are known to be digested fast, thus releasing the associated bioactive components into the circulatory system much quicker than that observed with casein digestion[11,18]. Previous studies have shown that WPI reduces energy intake compared with other dietary proteins in the lean state[12,13], with one study suggesting that whey proteins increase inter-meal interval compared with soya proteins[13]. In the obese state, the effects of WPI on energy intake appear to be lost, yet dietary proteins still attenuate HFD-induced obesity[10,20–23]. The discrepancies between these data and those of the present study, in particular, with regard to energy intake, may be related to the differences in dietary macronutrient and micronutrient composition (45 % energy as fat *v.* 60 % energy as fat, casein *v.* soya as the control and altered Ca content in the diet) and/or the study duration (8 weeks *v.* up to 20 weeks) that were used to assess the energy balance impact of whey proteins[19]. It is also possible that some of these discrepancies may have arisen due to the fact that we subjected the group-housed mice to a single housing environment of the TSE Phenomaster cages (TSE systems) to measure their energy intake. Indeed, individual housing can induce stress in C57BL/6J mice, resulting in a decreased food intake by 3 h following exposure to the new environment[40]. However, the effect of the novelty stress is lost after 6 h in the new environment as the food intake in these mice is similar to that of the controls. To minimise any such effect of single housing on physiological parameters in the present study, we allowed the mice a 2 d acclimatisation period in the new TSE Phenomaster cages (TSE systems) before measuring their energy balance-related parameters. Indeed, our preliminary investigation (study 1) revealed that on days 2 and 3 of the housing period, the mice consumed a similar energy content and exhibited a similar metabolic activity (Fig. S1, available online).

We, as others[25,41], have found an association between FQ, RER and energy balance. In a state of 'perfect' energy and nutrient balance (energy homeostasis), the FQ equals the RER. In the present study, the RER was much lower than the FQ for all dietary groups during the light phase. This is expected given that during this phase the mice consumed less energy and had reduced metabolic (VO_2 and heat) and locomotor activities compared with those observed during the dark phase. The potential mismatch between energy intake and energy expenditure that causes fat oxidation, as indicated by the RER being lower than the FQ, is likely to represent a mechanism to obtain the required energy to sustain metabolic activity in the mice during the light phase[41]. During the dark phase, the RER of the HFD group was close to the FQ, but the RER of the LFD group was much higher than the FQ. This indicates that for the HFD group, energy intake is closer to energy utilisation, while for the LFD group, a much greater energy intake is required to meet the energy demands. This suggestion is supported by the data showing that both energy intake and overall metabolic (VO_2 and heat) and locomotor activities in the mice on the HFD were much lower than those observed in the LFD-fed mice. The data support the suggestion that mice fed the HFD for 5 to 6 weeks are attempting to regulate their energy supply with their energy demands to maintain their body weight, in a way similar to what the LFD-fed mice do, but at a set point much higher than that of the latter group[39]. Interestingly, during the dark phase, the mice on WPI had a RER almost equal to their theoretical FQ, yet the expected association between energy intake and parameters linked to energy expenditure did not exist, as these mice had a much higher energy intake than the HFD-fed mice, but had metabolic and locomotor activities similar to those of the HFD-fed mice. The present data suggest that WPI does not have a significant impact upon the metabolic activity in the HFD-fed mice. This is not in agreement with the data from another study[10], in which it has been shown that HFD-fed mice drinking WPI-supplemented water had increased VO_2 levels and mitochondrial respiration rates compared with the HFD controls. However, unlike in the present study, the two dietary groups in the Shertzer *et al.*[10] study were consuming disproportionate amounts of protein due to WPI being present in the drinking water in addition to the protein received in the diet. Therefore, the increased metabolic activity observed in the WPI group in the above-mentioned study may simply have resulted from the increased protein intake-induced metabolism rather than due to an effect of the protein source. In the absence of an elevated metabolic activity, energy consumed by the HF-WPI-fed mice in the present study could be utilised for another biological activity, such as muscle metabolism, resulting in an increased total lean mass (%) in the HF-WPI group compared with the HFD group by week 8.

Feeding the HFD for 8 weeks increased the overall fat mass compared with LFD feeding, which concurs with previous studies[3,5]. Feeding WPI to the HFD-fed mice decreased the overall fat content in the body, with decreased TAG accumulation in the liver, although the statistical significance of this decrease varied depending upon the type of assay kit used to measure the lipid content in the liver or upon the efficiency of the Folch lipid extraction method[42]. The results are nonetheless consistent with previous findings reported in the literature[10,22] that WPI reduces liver TAG levels. The effects of the dietary challenges on tissue lipid metabolism were

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also reflected at the level of gene expression. We showed that HFD feeding decreased the expression of *INSR*, *GLUT4* and *FASN* in the epididymal adipose tissue compared with LFD feeding. Given the involvement of these genes in adipocyte lipogenesis[43,44], the data suggest that a HFD decreases endogenous TAG production in the epididymal adipose tissue. Interestingly, we, as others[45–47], observed a reduction in the expression of *β-3AR* in the epididymal adipose tissue with HFD feeding. As β-3AR plays an important role in energy homeostasis due to its impact on lipolysis and thermogenesis[45–47], it is likely that this reduction in gene expression coupled with the reduced activity of the protein product that is known to occur in HFD-fed mice[45–47] contributes to the increased TAG accumulation and hence adiposity in these mice. Similarly, the HFD-induced increase in the expression of lipid-responsive *PPARγ*[48,49] and *CPT1b*[50,51] in the hypothalamus is consistent with the recent findings that the HFD increases fatty acid accumulation in the brain[52,53]. Thus, the present data suggest that HFD-fed mice have greater lipid storage in multiple tissues compared with the LFD-fed mice, potentially leading to the shift in their defended body weight to a higher level. The reduction in the overall body and hepatic fat content described here with the HF-WPI diet feeding has been reported by other studies, which have shown the effects of WPI on adipocyte cross-sectional area and liver TAG levels in HFD-fed mice[22]. In fact, compared with a diet with casein as the protein source, a whey protein diet fed to rats reduced the expression and activity of key lipogenic enzymes in the liver[54]. We further suggest that lipid accumulated in the hypothalamus may also be decreased with WPI intake because of the significantly lower expression of *CPT1b* in the hypothalamus coupled with a trend towards a decreased expression of *PPARγ* ($P=0{\cdot}06$). These cellular changes occurred in a background of elevated plasma TAG levels, suggesting reduced plasma lipid uptake and/or storage in the tissues in the HF-WPI-fed mice compared with the HFD controls. The present investigation revealed that WPI does not affect the expression of *LPL* or *FATP1* that are involved in fatty acid extraction[55] and transport[56] to the epididymal adipose tissue, respectively, although we cannot rule out the possibility that WPI may have affected the protein expression or activity of the above-mentioned genes, causing the HF-WPI-fed mice to have elevated plasma TAG levels but reduced adiposity compared with the HFD-fed mice. To explore the possibility that WPI may have influenced lipid storage, we investigated genes involved in lipid metabolism within the adipose tissue. The lack of a WPI effect on the adipose tissue expression of *FASN* and *CPT1b* may suggest that adipose tissue-specific lipogenesis and β-oxidation pathways[50] are unaffected by WPI intake. Interestingly, we observed a trend towards an increase in the expression of *β-3AR* in the epididymal adipose tissue of the HF-WPI-fed mice. A longer duration of WPI intake (21 weeks) was found to significantly increase the adipose tissue expression of *β-3AR* compared with that of the control HFD group[57]. These data suggest the possibility that WPI increases lipolysis in adipocytes via the activation of β-3AR, possibly arising as a consequence of the effects of dietary protein on the central nervous system, given that central nervous system outflow to the adipose tissue activates β-3AR[37,38,58,59].

HFD-induced development of obesity is associated with increased inflammation[60–62] and is accompanied by functional peripheral and central resistance to both leptin[5,63–66] and insulin[3,53,67–69]. Evidence from the present study also suggests that the components of the leptin and insulin signalling pathways acting in the hypothalamus and the adipose tissue may be affected by the HFD. Feeding of the HFD for eight weeks increased the expression of *CD68* and *TNF-α* in the hypothalamus. In parallel, we observed an increased expression of *INSR* in the hypothalamus and a reduced expression of *INSR* and *GLUT4* in the adipose tissue with HFD feeding. These findings are consistent with the suggestion that insulin resistance is partly instigated by an inflammatory response to a HFD[69–71]. The increased expression of the leptin receptor and its downstream target *POMC* in the hypothalamus of the HFD-fed mice compared with the LFD-fed controls corroborates with previous data[63,72,73]. The changes in the expression of the leptin receptor may be a homeostatic mechanism attempting to defend against HFD-induced body-weight gain, in a background of elevated plasma leptin, hypothalamic inflammation and increased PPARγ expression, which contributes to central leptin resistance[48,74]. The increased hypothalamic tissue expression of genes linked to the leptin and insulin signalling pathways in response to a HFD may thus represent an elevated threshold needed to initiate physiological responses of these hormones[39,53,65]. In the epididymal adipose tissue and in the hypothalamus, WPI reversed the HFD-associated effects on the mRNA expression of *INSR* (both tissues), *GLUT4* (adipose) and leptin receptor (hypothalamus), although the expression of the INSR-β isoform in the adipose tissue did not differ from that of the HFD group. Differences in *INSR* mRNA and INSR-β protein expression could have arisen due to the effects of the diet on the transcription, translation and/or processing of the mature precursor protein or may have also arisen due to the differences in the sensitivity of the techniques (real-time PCR *v* immunoblotting) used to measure gene expression. Despite these differences, WPI-induced changes in the expression of genes linked to the leptin and insulin signalling pathways were observed to occur in concert with reduced tissue lipid accumulation and potentially lower inflammation, specifically in the hypothalamus, since the expression of *TNF-α* in the hypothalamus was significantly lower in the HF-WPI diet-fed mice than in the HFD-fed mice. The gene expression profile in the WPI-fed mice may thus represent a lower threshold (or higher sensitivity) to leptin and insulin action, which may allow the central nervous system outflow to the adipose tissue to activate and normalise the expression of *β-3AR* as that observed in week 8 of the HF-WPI dietary challenge. The investigation of gene expression in specific nuclei within the hypothalamus may allow future investigators to further define the role of this region in mediating the effects of WPI on the adipose tissue.

In summary, we showed that WPI causes an overall reduction in fat mass in HFD-fed mice, normalises their energy intake, possibly by affecting the meal size, and influences their hypothalamic and adipose tissue expression of genes linked to leptin and insulin signalling pathways and lipid metabolism. Further work is needed to determine how WPI appears to prevent plasma TAG storage in the tissues and whether this contributes to potentially increased leptin and insulin sensitivity in the hypothalamus and the adipose tissue. Overall, the present data collected at two different time points (at weeks 5/6 and week 8) are consistent with the suggestion that WPI partially or completely reverses the HFD-induced physiological and cellular changes, which may therefore represent a state of reduced susceptibility to weight gain on a HFD.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513003396

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Appendix D

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Protein Quality and the Protein to Carbohydrate Ratio within a High Fat Diet Influences Energy Balance and the Gut Microbiota In C57BL/6J Mice

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Abstract

Macronutrient quality and composition are important determinants of energy balance and the gut microbiota. Here, we investigated how changes to protein quality (casein versus whey protein isolate; WPI) and the protein to carbohydrate (P/C) ratio within a high fat diet (HFD) impacts on these parameters. Mice were fed a low fat diet (10% kJ) or a high fat diet (HFD; 45% kJ) for 21 weeks with either casein (20% kJ, HFD) or WPI at 20%, 30% or 40% kJ. In comparison to casein, WPI at a similar energy content normalised energy intake, increased lean mass and caused a trend towards a reduction in fat mass ($P = 0.08$), but the protein challenge did not alter oxygen consumption or locomotor activity. WPI reduced HFD-induced plasma leptin and liver triacylglycerol, and partially attenuated the reduction in adipose FASN mRNA in HFD-fed mice. High throughput sequence-based analysis of faecal microbial populations revealed microbiota in the HFD-20% WPI group clustering closely with HFD controls, although WPI specifically increased *Lactobacillaceae/Lactobacillus* and decreased *Clostridiaceae/Clostridium* in HFD-fed mice. There was no effect of increasing the P/C ratio on energy intake, but the highest ratio reduced HFD-induced weight gain, fat mass and plasma triacylglycerol, non-esterified fatty acids, glucose and leptin levels, while it increased lean mass and oxygen consumption. Similar effects were observed on adipose mRNA expression, where the highest ratio reduced HFD-associated expression of UCP-2, TNFα and CD68 and increased the diet-associated expression of β3-AR, LPL, IR, IRS-1 and GLUT4. The P/C ratio also impacted on gut microbiota, with populations in the 30/40% WPI groups clustering together and away from the 20% WPI group. Taken together, our data show that increasing the P/C ratio has a dramatic effect on energy balance and the composition of gut microbiota, which is distinct from that caused by changes to protein quality.

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Introduction

It is widely recognised that levels of obesity and related clinical conditions such as diabetes, stroke, hyperlipidemia and cardiovascular disease are increasing worldwide [1]. Importantly, the development of obesity increases the set point at which the body weight, more specifically body fat, is defended, thus making its reversal difficult to achieve [2,3]. As such, there is an increased research interest to develop effective treatments for this disease.

Dairy proteins belonging to the whey fraction (a by-product of cheese manufacture) have been increasingly tested for their potential anti-obesity effect, specifically for their ability to reduce high fat diet (HFD)-associated body weight and fat mass gain [4–6]. Shi *et al.*, [7] showed that replacing 5%, 50% or 100% of the dietary casein protein-derived energy content with a lactoperoxidase and lactoferrin-enriched whey protein isolate (WPI) caused a proportional suppression of body weight gain in HFD fed mice. We have previously demonstrated that a WPI-related reduction in body weight and fat mass gain in HFD fed mice was accompanied by a normalisation of energy intake and complete or partial reversal of energy balance-related gene expression in the adipose tissue and the hypothalamus [8]. While these data suggest that whey proteins have specific-effects on energy balance, such effects appear be modified by the macronutrient composition in the diet [9]. In the latter study, it was shown that increasing the lipid to carbohydrate ratio within a whey protein-rich diet significantly reduced energy intake and bodyweight gain in rats. Collectively, these data suggest that protein quality and macronutrient composition are important determinants of energy balance.

Interestingly, diet is also an important factor in determining the composition of the gut microbiota [10,11] and specific gut microbiota signatures are associated with obesity phenotypes in animals and humans [12–14]. Notably, studies have shown specific whey proteins to possess anti-microbial activity [15–17], and that

the digestive process itself facilitates the formation of potent antimicrobial whey-derived peptides, such as pepsin catalysed lactoferrin to lactoferricin [18]. A study by Sprong *et al.*, [19] demonstrated that in comparison to casein, whey protein intake increased levels of lactobacilli and bifidobacteria in a rat model of colitis. However, in a more recent study, whey protein intake was found to have no influence on gut microbiota composition in mice fed a HFD for 7 or 13 weeks [20]. Several key unanswered questions are; could whey proteins specifically influence the gut microbiota composition associated with prolonged high fat feeding, and would any changes relate to energy balance? Could changes to protein to carbohydrate ratio within a HFD vary the gut microbiota profile and energy balance in a different way to changes to protein quality?

To assess WPI specific effects on above parameters, we subjected male C57BL/6J mice to 21 weeks of either a low fat diet (LFD) with 20% kJ casein or a HFD with 20% kJ casein or WPI. In addition, using two additional HFD dietary groups on 30 or 40% kJ WPI, we evaluated the impact of increasing the protein to carbohydrate (P/C) ratio within the HFD on parameters of interest. Our data show that WPI has a specific effect on energy balance and gut microbiota, while increasing the P/C ratio within the HFD leads to dramatic alterations in energy balance, body composition, metabolic health and the composition of the gut microbiota.

Materials and Methods

Ethics Statement

All research involving mice was licensed under the Cruelty to Animal Act 1876 and received ethical approval from the University College Cork Animal Ethics Review Committee (#2011/005).

Animals

Male 3–4 week old C57BL/6J mice (Harlan, Oxon, UK) were group housed either 5 per cage (Study 1) or 4 per cage (Study 2) in individually ventilated cages and acclimatised for four weeks in a light (06:00–18:00), temperature (21±1°C) and humidity (45–65%) controlled environment with free access to water and a low fat diet (LFD; 10% kJ fat and 20% kJ casein; #D12450, Research diets; New Brunswick, NJ, USA).

Experimental protocol

Two studies were performed to assess how the WPI-derived energy content within a HFD (study 1) or LFD (study 2) impacts on energy balance-related parameters in mice over a 21 week (study 1) or 7 week period (study 2).

Study 1: Following the acclimatisation period, weight-matched dietary groups were maintained on the LFD or switched to either a HFD (45% kJ fat and 20% kJ casein; #D12451) or a HFD with WPI (Alacen™ 895 NZMP, New Zealand) at an energy content of 20% kJ (HFD-20% WPI), 30% kJ (HFD-30% WPI) or 40% kJ (HFD-40% WPI) (Table S1) (n = 10) for a total of 21 weeks. Body weights were measured weekly. Energy intake in group housed mice was measured by weighing the food hopper each week until week 16. During weeks 17–20, energy intake and metabolic activity in individual mice was measured using TSE Phenomaster cages (TSE systems, Bad Homburg, Germany). Following this analysis and prior to re-housing the mice in home cages, faecal pellets were collected from individual mice for examination of microbial composition via pyrosequencing and subsequent bioinformatic analysis. At the end of the experimental period, mice were fasted for 6 hours and the body composition was measured using the Bruker minispec LF50H (Bruker optics, Ettlingen, Germany). Mice were then anesthetised using ketamine (65 mg/kg bodyweight) and xylazine (13 mg/kg bodyweight). Blood was collected from anesthetised mice into vacutinater EDTA tubes (BD, USA) and treated with Aprotinin (500,000 KIU/L final concentration; Sigma, Ireland) and Diprotin A (0.1 mM final concentration; Sigma, Ireland) to protect plasma peptides from proteolytic degradation. Plasma was isolated from blood by centrifugation at 2000 rpm at 4°C for 15 mins. Mice were sacrificed by cervical dislocation, and tissues of interest were dissected and snap frozen in liquid nitrogen (liver, adipose and stomach) or on dry ice (brain). Plasma and tissue samples were stored at −80°C until analysis.

Study 2: Weight matched mice were provided for 7 weeks with either the LFD or a LFD with WPI replacing the casein protein (LFD-WPI; 10% kJ fat and 20% kJ WPI) (n = 8). Body weights were measured weekly. Energy intake and metabolic activity in individual mice was measured during weeks 5 and 6 using the TSE Phenomaster system. After the analysis, mice were re-housed, as before, in the home cages and the experiment was terminated at the end of week 7.

Analysis of metabolic parameters

The TSE Phenomaster cages comprised an open-circuit indirect calorimetry system with gas sensing units to measure oxygen consumption (ml/h/kg) (VO_2) and CO_2 production (ml/h/kg) (VCO_2). The cages also contained high precision sensor associated-feeding baskets to accurately measure food intake (g), with a meal defined as intake over 0.01 g. A multi-dimensional infrared beam system allowed the measurement of locomotor activity, which was defined as the total number of infrared beam breaks in the X and Y axis. Mice were singly housed in TSE Phenomaster cages for a total of 3 days, with data collected during the final 24 hours, following a 2 day acclimatisation to the new cage environment. The acclimatisation period was established based on the data from our previous study [8]. Heat production (kcal/h/kg) in individual mice was calculated using the Weir equation ($3.941\times VO_2 + 1.106\times VCO_2$)[21]), and this was converted to kJ/h/kg using 1kcal = 4.184 kJ. The respiratory exchange ratio (RER) was calculated by VCO_2/VO_2. Energy intake was calculated from food intake measurements using the energy content of the diets supplied by the manufacturer.

Microbial DNA extraction, amplification and high throughput DNA sequencing

Total metagenomic DNA was extracted from individual faecal samples using QIamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), after an additional bead-beating step. Bacterial composition was determined by sequencing of 16s rRNA amplicons (V4-V5 region; 408nt long) generated by a separate PCR reaction for each sample (in triplicate) using universal 16S primers, where, the forward primer (5′-AYTGGGYD-TAAAGNG), with attached molecular identifier tags between 454 adapter sequence and target-specific primer sequence, and reverse primer V5 (5′-CCGTCAATTYYTTTRAGTTT) [22], were used along with Biomix Red (Bioline, London UK). The template DNA was amplified under the following PCR conditions for a total of 35 cycles: 94°C for 2 minutes and 1 minute respectively (initialization and denaturation), 56°C for 60 seconds (annealing) and 72°C for 60 seconds (elongation), proceeded by a final elongation stage of 2 minutes. Negative control reactions with PCR grade water in place of template DNA were used to confirm a lack of contamination. Amplicons were pooled and cleaned using the AMPure XP purification system (Beckman and Coulter,

Takeley, UK) and DNA concentration was determined using the NANODROP 3300 Fluorospectrometer (Thermo Scientific, USA) coupled with the Quant-it™ Picogreen® dsDNA Assay Kit (Invitrogen, Paisley, UK). Equal volumes of each sample were then pooled together and underwent a final cleaning and quantification stage. Amplicons were sequenced in-house on a Roche GS FLX Titanium platform.

Bioinformatics

Raw sequencing reads were 'de-noised' using traditional techniques implemented in the Ribosomal Database Project Pyrosequencing (RDP) Pipeline with ambiguous bases, non exact primer matches and reads shorter than 150 bp being excluded. Trimmed FASTA files were then BLASTed against a previously published 16S-specific database using default parameters. The resulting files were then parsed using the MEGAN software package, which assigns reads to the National Centre for Biotechnology Information (NCBI) taxonomies via the lowest common ancestor algorithm. Results were filtered prior to tree construction and summarization by the use of bit scores from within MEGAN where a cut-off bit score of 86 was employed [23,24]. The QIIME software suite was employed to achieve clustering of sequence reads into operational taxonomic units (OTUs) [25]. Chimeric OTUs were removed using the ChimeraSlayer program [26] and phylogenetic trees constructed using the FastTreeMP tool [27]. Beta diversity values were calculated based on Bray Curtis, weighted and unweighted UniFrac distances, and the KING viewer was used to visualise resulting PCoA plots [28,29]. Sequence reads were deposited in the European Nucleotide Archive (EHA) under the accession number PRJEB4636.

Plasma analysis

Colorimetric assays were used to measure plasma levels of glucose (Calibochem, Darmstadt, Germany), triacylglycerol (TAG; Wako Chemicals, Richmond, VI, USA) and non-esterified fatty acids (NEFA; Abcam, Cambridge, UK). Commercially available ELISA kits were used to analyse plasma levels of insulin, leptin (Crystal Chem, Downers Grove, IL, USA), glucagon-like peptide 1 (GLP-1; Millipore, St. Charles, MO, USA) and corticosterone (Enzo Life sciences, Farmingdale, NY, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was determined using the formula: fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/L)/22.5 [30]. To measure plasma amino acid levels, samples were first deproteinised by mixing with equal volumes of 24% (w/v) tri-chloroacetic acid. The samples were then allowed to stand for 10 minutes before been centrifuged at 14400×g (Microcentaur, MSE, UK) for 10 minutes. Supernatants were mixed with 0.2 M sodium citrate buffer, pH 2.2, and the plasma concentration of amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, UK) fitted with a Jeol Na^+ high performance cation exchange column.

Liver TAG analysis

Total lipids from liver samples (approx. 50 mg) were extracted as described previously [8] using the Folch extraction method [31]. Briefly, total lipids were extracted using 2:1 (v/v) chloroform: methanol solution, into which a 0.88% NaCl solution was added before centrifugation at 2000 rpm and 4°C for 30 mins. Aliquots of the organic phase were collected, dried and re-suspended in the LabAssay TAG reagent (Wako Chemicals, Richmond, VI, USA) to measure TAG levels using LabAssay TAG kit according to the manufacturer's protocol.

Real-Time PCR analysis

Total RNA was isolated from tissues using RNeasy mini (liver and stomach) or RNeasy lipid mini (adipose and hypothalamus) kits (Qiagen, Hilden, Germany) according to manufacturers' instructions. RNA was treated with DNase (Qiagen, Hilden, Germany) during RNA isolation to eliminate any possible genomic DNA contamination. Reverse transcription of 1 μg of RNA was performed using 2.5 ng/μl random hexamer primers (Bioline, London, UK), 0.5 mM dNTP (Promega, Madison, VI, USA), 2 U/μl RNase inhibitor (Promega, Madison, VI, USA), and the Superscript II first stand system (Invitrogen, Carlsbad, CA, USA) according to manufactures' instructions. Gene expression was measured by the Roche Lightcycler 480 system (Rotkreuz, Switzerland) via amplification of 1 μl complementary DNA using the Lightcycler SYBR Green I Mastermix kit (Roche, Penzberg, Germany) and 2.5 μM gene specific primers (Eurofins MWG operon, Ebersberg, Germany) in a 10 μl total reaction volume. Primer sequences used are listed in Table S2. PCR conditions were; 10 mins at 95°C, followed by 50 cycles of 95°C for 10 s, 58–65°C for 5 s and 72°C for 15 s. Authenticity of PCR products was determined by melting curve analysis and by automated sequencing. Crossing point (Cp) of fluorescence signals were used to calculate target gene expression by $2^{-\Delta\Delta Cp}$, following normalisation against housekeeping gene according to $\Delta\Delta Cp = \Delta Cp$ target gene $- \Delta Cp$ housekeeping gene. Housekeeping genes used were β-actin (liver, stomach and hypothalamus), YWHAZ (liver and hypothalamus), 18-S (adipose) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (adipose and stomach). Relative gene expression is shown compared to the LFD group.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between experimental dietary groups were analysed by one-way or two-way ANOVA followed by pairwise comparisons using *tukey* or *bonferroni* post hoc tests, respectively. Body weight trajectories were analysed by two-way repeated measures ANOVA with *bonferroni* post hoc tests. Non-parametric data was compared by Kruskal-Wallis ANOVA followed by *Dunn's* pairwise comparisons. Significance levels were set at $P \leq 0.05$, and statistical analysis was performed using Graphpad prism (ver. 5.04; San Diego, CA, USA) and Minitab (ver.15; State College, PA, USA).

Results

WPI inclusion or increasing the P/C ratio within a HFD alters body composition and plasma amino acids

Fig. 1A–B demonstrates that body weight gain of HFD-20%WPI fed mice was similar to HFD controls. However, intake of WPI was seen to have a specific effect on body composition, with HFD-20% WPI fed mice having an increased lean mass (%) ($P<0.05$), and a trend towards a reduction in fat mass (%) ($P=0.08$) compared to the HFD control group. Increasing the WPI derived energy content in the HFD to 40% and proportionally reducing the carbohydrate energy content led to a significant reduction in body weight gain compared to all other HFD-WPI groups ($P<0.001$), with observed values similar to that seen for the LFD group. This was accompanied by significantly reduced body fat mass and increased lean mass levels in the HFD-40% WPI fed mice compared to HFD control and other WPI diets groups ($P<0.001$), while the body composition of HFD-30% WPI group did not differ from that of the HFD-20% WPI group (Fig. 1C).

Comparison of the plasma amino acid profiles including those that could influence lean and fat mass, revealed an impact of WPI

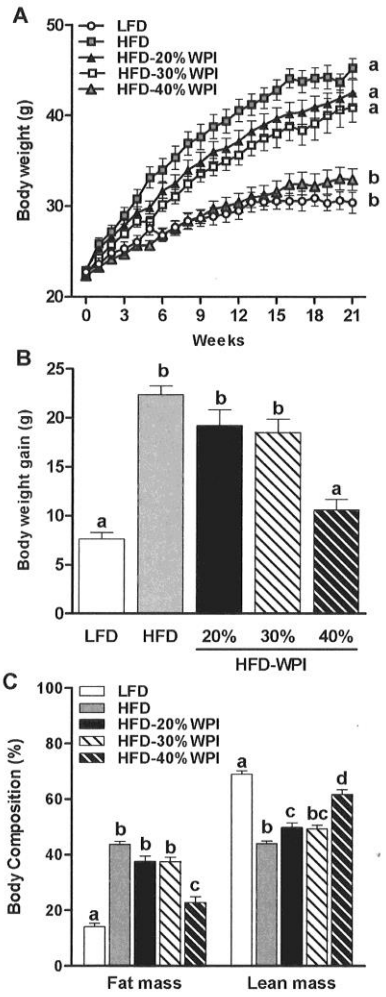

Figure 1. Impact of whey protein isolate and protein to carbohydrate ratio on body weight and composition. (A) shows the body weight trajectories of mice during 21 weeks of dietary treatment with a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Body weight gain (B) and body composition (C) of mice after 21 weeks on experimental diets are also shown. Data represent mean values ± S.E.M. (n = 10 per group). Groups that do not share a common letter are significantly different at $P<0.05$.
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and the P/C ratio (Table 1). WPI specific effects were observed on glutamic acid, aspartic acid and glycine, which either decreased (glutamic acid and aspartic acid) or increased (glycine) compared to HFD fed mice ($P<0.05$) (Table 1). Changes in macronutrient ratio in HFD-40%WPI, decreased plasma histidine, phenylalanine, serine and threonine levels compared to the lowest P/C ratio (20% WPI) ($P<0.01$) (Table 1).

WPI-enriched HFD normalised energy intake, while increasing the P/C ratio accentuated metabolism

The cumulative energy intake (MJ) for the dietary groups (2 cages/group, all with n = 5 mice), measured over the first 16 weeks did not differ between LFD, HFD and HFD-20% WPI groups (24.73±2.70 vs. 27.69±1.54 vs. 29.40±0.31, respectively). In contrast, data gathered by individually housing the mice in TSE Phenomaster cages in weeks 17–20 demonstrated that the energy intake of the HFD-WPI groups was greater than that of the HFD control group during both the light and dark phases ($P<0.05$), while being similar to that of the LFD group (Fig. 2A). Increasing the P/C ratio had no significant effect on cumulative energy intake (MJ) in HFD fed mice up to week 16 (20%WPI, 29.40±0.31 vs. 30%WPI, 30.01±0.62 vs. 40%WPI, 26.71±0.15). Energy intake measurements from TSE Phenomaster cages corroborated this data (Fig 2A). There was also no significant effect on meal number or meal size of altering the P/C ratio (i.e. between WPI groups; Fig 2B–C).

The HFD-20% WPI diet had no impact on VO_2, heat production, locomotor activity or respiratory exchange ratio (RER) when compared to HFD fed mice (Fig. 3A–D). Increasing the P/C ratio was found to impact on energy expenditure with HFD-40% WPI fed mice having significantly increased levels of dark phase VO_2 compared to HFD-20 and 30% WPI fed mice ($P<0.001$) (Fig 3A). A similar change in heat production was observed between the groups, albeit data was only significant between HFD-40% and HFD-30% WPI groups ($P<0.05$) (Fig 3B). There was no effect of WPI or P/C ratio on locomotor activity (Fig 3C). RER values of all HFD groups were lower than the LFD group in both the light and dark phases, consistent with increased fat metabolism ($P<0.001$) (Fig 3D).

Investigation of the above parameters in mice fed a LFD with WPI or casein for 7 weeks (study 2) revealed that WPI does not influence body weight, energy intake, VO_2, locomotor activity or RER in a low fat background (Figure S1A–D).

Increasing the protein to carbohydrate ratio attenuated adverse metabolic impact of HFD

Specific effects of WPI and the P/C ratio were observed on lipid metabolism-related gene expression and on tissue lipid deposition. Firstly, the decrease in epididymal adipose tissue fatty acid synthase (FASN) mRNA expression with HFD feeding, was somewhat attenuated by WPI challenge ($P<0.05$), with no added benefit of increasing the P/C ratio on expression of this gene (Fig 4A). Notably, the epididymal mRNA expression of a number of other genes were altered by the P/C ratio, specifically, fatty acid transporter 1 (FATP1), beta-3 adrenergic receptor (β3-AR), peroxisome proliferator-activated receptor gamma (PPARγ), uncoupling protein 2 (UCP-2) and lipoprotein lipase (LPL) ($P\leq 0.05$) (Fig 4). In the liver, WPI specifically reduced TAG levels (Table 2) and the mRNA expression of fatty acid binding protein 1 (FABP1) compared to HFD fed mice (Table 3). The highest P/C ratio (40% WPI) significantly decreased mRNA levels of cluster of differentiation 36 (CD36) and PPARγ ($P<0.05$) (Table 3), dramatically reduced liver TAG levels compared to 20/30% WPI fed mice (Table 2), and normalised the elevated plasma levels of TAG and NEFA observed with HFD feeding ($P\leq 0.05$) (Table 2). Finally, the mRNA level of lipid metabolism-related

Table 1. Plasma amino acid levels (μmol/L) in mice fed a 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1].

	HFD	20% WPI	30% WPI	40% WPI	P value
Alanine	170.91±8.82^{a}	163.83±11.59^{ab}	137.44±9.72^{ab}	127.15±8.46^{b}	<.05
Arginine	41.74±4.82	38.41±7.16	56.39±7.60	39.08±4.34	NS
Aspartic acid	7.47±1.25^{a}	3.63±0.51^{b}	3.49±0.51^{b}	4.85±1.02^{ab}	<.05
Cyteine	11.95±1.34	8.87±1.37	8.93±1.33	7.86±1.72	NS
Glutamic acid	90.76±3.42^{a}	74.74±1.89^{b}	75.25±1.86^{b}	71.25±2.69^{b}	<.001
Glycine	106.22±4.28^{a}	131.43±3.57^{b}	116.45±4.52^{ab}	131.92±2.69^{b}	<.001
Histidine	75.43±2.12^{a}	74.93±2.98^{a}	68.21±3.35^{ab}	64.21±2.51^{b}	<.05
Isoleucine	213.81±8.87	207.87±9.09	195.27±5.74	186.78±10.17	NS
Leucine	91.86±9.66	81.36±6.65	66.93±6.69	69.71±10.21	NS
Lysine	113.81±5.88	108.18±8.30	102.57±8.39	88.29±4.07	NS
Methionine	23.94±1.77	23.65±0.75	21.05±0.97	20.40±1.08	NS
Phenylalanine	46.92±1.70^{a}	41.13±1.13^{ab}	38.23±1.52^{bc}	33.30±2.35^{c}	<.001
Proline	118.26±10.14	127.63±9.63	106.04±11.02	104.39±11.93	NS
Serine	68.18±5.69^{ab}	74.98±2.29^{a}	62.42±2.57^{ab}	55.73±2.12^{b}	<.01
Threonine	92.71±2.93^{a}	87.63±2.49^{a}	78.29±2.95^{ab}	69.28±3.96^{b}	<.01
Tyrosine	40.29±2.47^{a}	36.37±1.12^{ab}	33.76±1.54^{ab}	29.63±2.77^{b}	<.01
Valine	154.51±8.65^{a}	130.59±5.99^{ab}	118.40±6.69^{b}	113.16±9.49^{b}	<.01

[1]Data are means ± SEM (n = 7–10). In each row values without a common letter significantly differ, $P<0.05$;NS, non-significant.
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carnitine palmitoyltransferase 1a-c (CPT1a-c), fatty acid transport protein 5 (FATP5) and PPARα, in tissues of interest, was not influenced by the dietary challenges (Tables 3–4).

Increasing the P/C ratio reduced plasma glucose levels, particularly in the 40% WPI group ($P<0.05$) (Table 2). In parallel HOMA-IR values were also reduced ($P<0.05$), but the change in plasma insulin concentration did not reach statistical significance (Table 2). At a cellular level, the highest P/C ratio normalised the HFD-induced reduction in adipose expression of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1), and partially prevented the HFD-induced reduction in glucose transporter 4 (GLUT4) (Fig. 4B) ($P<0.001$). In the hypothalamus, WPI specifically increased IR mRNA expression ($P<0.05$), as did P/C ratio, with the highest P/C ratio having the greatest impact (Table 4). Epididymal adipose tissue mRNA expression of inflammatory markers, namely tumour necrosis factor (TNF)-α and cluster of differentiation (CD) 68 only responded to the highest P/C ratio, which significantly reduced the expression of both in a HF background (Fig. 4B) ($P<0.001$). In the hypothalamus, whilst TNFα mRNA was elevated by HFD feeding, neither WPI nor the P/C ratio influenced its levels, although there was a trend towards a decrease for the highest P/C ratio (40% WPI) (Table 4). None of the dietary challenges influenced hepatic glucose transporter 2 (GLUT2), IRS-1 and TNF-α mRNA expression (Table 3) or hypothalamic IRS-1 mRNA expression (Table 4).

The increased plasma leptin concentration in response to the HFD was significantly blunted by WPI intake with dramatic reductions seen at the highest P/C ratio ($P<0.001$) (Table 2). Yet, the hypothalamic expression of genes known to be responsive to plasma leptin levels were unaffected, specifically mRNA levels of leptin receptor (ObR), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and growth hormone secretagogue receptor (GHS-R) (Table 4). In addition, gastric mRNA expression for the orexigenic hormone ghrelin was not found to significantly differ between all dietary treatment groups (1.00±0.37, LFD vs. 1.11±0.40, HFD vs. 0.73±0.35, 20%-WPI vs. 0.29±0.28, 30%-WPI vs. 1.07±0.45, 40%-WPI). Plasma corticosterone levels were also elevated with HF feeding, but were not influenced by protein source (WPI or casein) or P/C ratio (Table 2). Similarly, there was no effect of WPI on the HFD-associated suppression of adipose 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) (Fig 4B), or the HFD-induced increase in glucocorticoid receptor (GCCR) in the hypothalamus (Table 4).

WPI inclusion or increasing the P/C ratio within a HFD altered the composition of gut microbiota

A total of 251,395 V4–V5 16s rRNA sequence reads were generated which corresponded to an average of 50,279 reads per diet group or 5,130 reads per animal. α-diversity values were calculated for biodiversity (Shannon index), species richness (Chao1) and the number of species relative to the abundance in the sample (Simpson diversity index). When α-diversity values were compared by diet group, the only difference observed was a significantly higher microbial richness (Chao1) within the HFD microbiota compared to the HFD-30% WPI ($P=0.028$). Principal coordinate analysis (based on unweighted UniFrac distances) (Fig. 5) of the sequence data highlighted a clustering of the LFD, HFD and HFD-20% WPI group microbial populations, while HFD-30% and 40% WPI groups clustered in close proximity to each other and distinctly from the LFD, HFD and HFD-20% WPI group clusters. Indeed, the LFD diet with casein as a protein source clustered most closely with the HFD containing the casein protein.

Phylogenetic analysis revealed several significant microbial population shifts between the HFD control and WPI groups (Table 5). At the family level, all WPI diet groups had significantly increased proportions of *Lactobacillaceae* and significantly decreased proportions of *Clostridiaceae* compared to the HFD control group. *Bifidobacteriaceae* populations were increased in both the HFD-20%

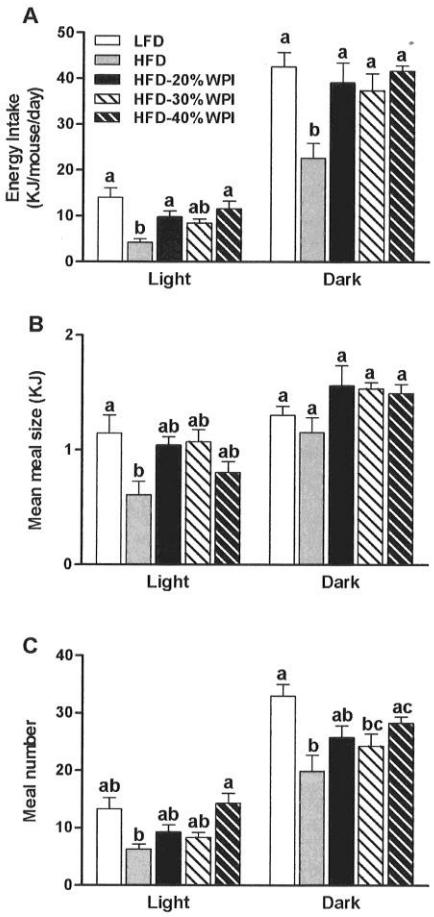

Figure 2. Impact of whey protein isolate and protein to carbohydrate ratio on energy intake. Energy intake (A) and feeding behaviour (meal size & meal number) (B–C) was measured using TSE Phenomaster cages at 17–20 weeks for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data collected from individual mice at 9 minute intervals over a 24 hour period are shown as mean values ± SEM (n = 8–10 per group) for light and dark phases. In light and dark phase, groups that do not share a common letter are significantly different at $P<0.05$.
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WPI and HFD-30% WPI diet groups compared to the HFD control, while in contrast they were significantly lower in the HFD-40% WPI group compared to the HFD control. The aforementioned patterns were also observed with respect to the corresponding genera (*Lactobacillus*, *Clostridium* and *Bifidobacterium* respectively) (Table 5). Also at genus level, proportions of *Rikenella*

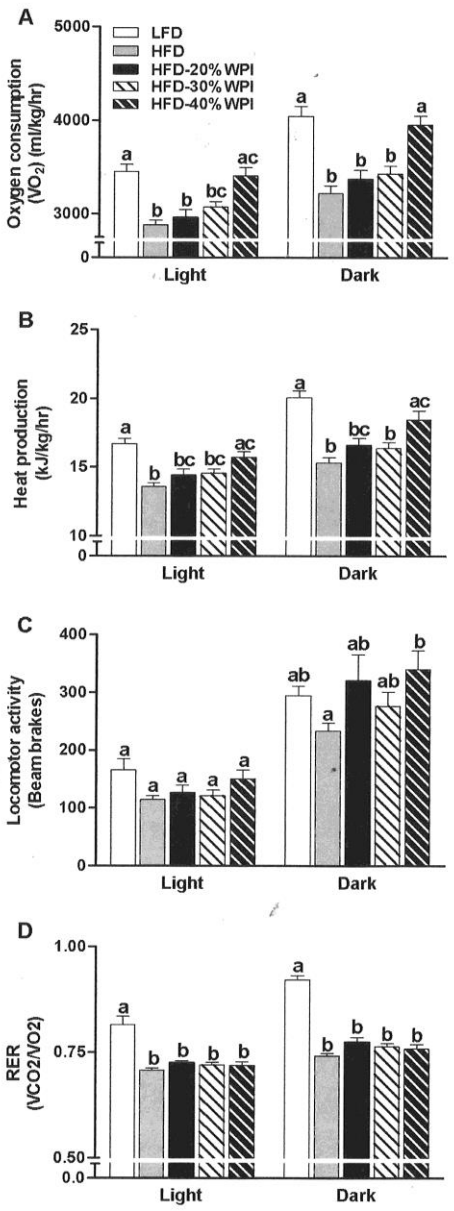

Figure 3. Impact of whey protein isolate and protein to carbohydrate ratio on metabolic activity. Metabolic activity was

measured using TSE Phenomaster cages at 17–20 weeks for mice on either a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD) or a HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Experimental data for (A) oxygen consumption (VO_2), (B) heat production, (C) locomotor activity and (D) respiratory exchange ratio (RER), collected from individual mice at 9 minute intervals over a 24 hour period, are shown as mean values ± SEM (n = 8–10 per group) for light and dark phases. In light and dark phase, groups that do not share a common letter are significantly different at $P<0.05$.
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were significantly higher in the HFD-40% WPI group compared to HFD-20/30% WPI groups, while proportions of *Peptostreptococcus* were significantly higher in the HFD-40% WPI group than in any other diet group (Table 5). Specific comparison of the microbiota of the HFD-20% WPI and HFD control was deemed particularly important given that the changes occurring here reflected changes resulting specifically from the presence of whey protein, rather than casein, in the diet, and not simply a change in the P/C ratio in the diet. In addition to the changes in the *Lactobacillus*, *Clostridium* and *Bifidobacterium* populations (and associated families) referred to above, it was also noted that proportions of *Desulfovibrio* and *Mucisprillum* (genus) were increased in the HFD-20% WPI relative to HFD control animals.

Discussion

The key findings of this study are that WPI has a specific effect on HFD-induced energy intake, metabolic health and gut microbiota composition. Additionally, with the exception of energy intake, increasing the P/C ratio, by increasing WPI dietary content, was seen to dramatically alter the above parameters.

Energy intake

Cumulative energy intake measured up to week 16 did not significantly differ between LFD and HFD fed mice. While this is consistent with data reported elsewhere [20], HF feeding has also been shown to increase or decrease energy intake in rodents [32,33]. Differences between data reported may relate to variances in diet composition including fat source/composition, or it may be

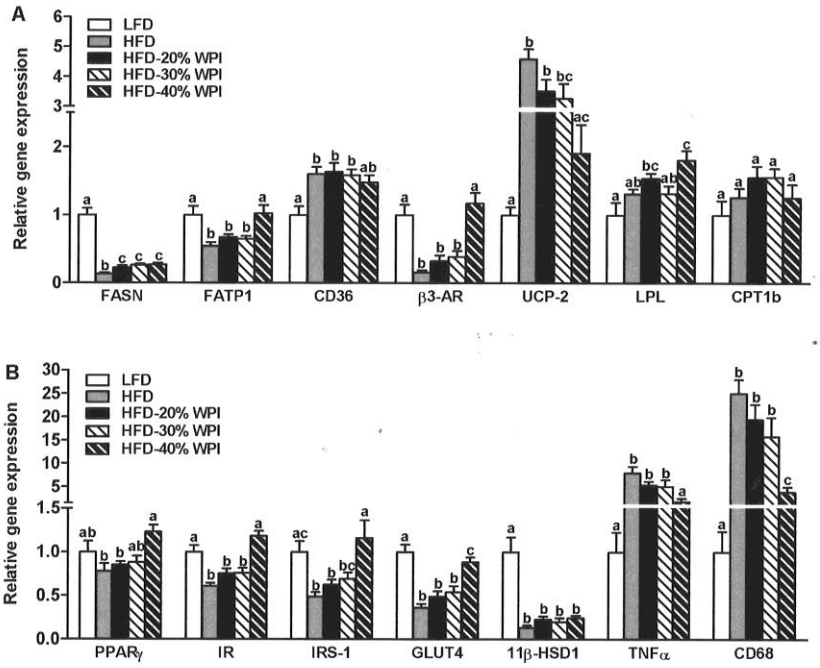

Figure 4. Impact of whey protein isolate and protein to carbohydrate ratio on adipose cellular activity. Epididymal adipose tissue gene expression was investigated in mice after 21 weeks on a 10% kJ low fat diet (LFD), 45% kJ high fat diet (HFD), or HFD with 20, 30 or 40% kJ whey protein isolate (WPI). Relative mRNA expression is shown for (A) fatty acid synthase (FASN), fatty acid transporter 1 (FATP1), cluster of differentiation 36 (CD36), beta-3 adrenergic receptor (β3-AR), uncoupling protein 2 (UCP-2), lipoprotein lipase (LPL) and carnitine palmitolytransferase 1b (CPT1b), and (B) for peroxisome proliferator activated receptor gamma (PPARγ), insulin receptor (IR), insulin receptor substrate 1 (IRS-1), glucose transporter 4 (GLUT4), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), tumour necrosis factor alpha (TNF-α) and cluster of differentiation 68 (CD68). Data represent mean values ± SEM (n = 9–10 per group). Gene expression is shown relative to the LFD control group set at 1.00. Groups that do not share a common letter are significantly different at $P<0.05$.
doi:10.1371/journal.pone.0088904.g004

Table 2. Tissue lipid parameters and plasma levels of hormones and metabolites in mice fed a 10%kJ low fat diet (LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1].

	LFD	HFD	20% WPI	30% WPI	40%WPI	P value
Liver TAG (mg/g tissue)	71.47±9.17^{a}	139.03±6.92^{b}	104.94±5.88^{c}	107.21±8.83^{c}	70.82±7.64^{a}	<.001
Leptin (ng/ml)	4.41±0.75^{a}	84.69±3.17^{b}	53.78±5.67^{c}	51.12±4.69^{c}	15.01±4.07^{a}	<.001
TAG (mg/dl)	39.85±2.91^{a}	56.90±6.31^{b}	45.15±4.32^{ab}	52.36±3.91^{ab}	40.19±3.96^{a}	<.05
NEFA (mmol/L)	0.37±0.09^{a}	0.67±0.07^{b}	0.64±0.05^{b}	0.63±0.06^{b}	0.36±0.05^{a}	<.01
Corticosterone (ng/ml)	150.0±21.7^{a}	319.0±48.6^{b}	286.8±31.9^{ab}	334.1±36.7^{b}	277.1±35.5^{ab}	<.01
GLP-1 (pM)	25.03±2.47	33.15±2.83	28.07±2.3	27.88±1.98	24.03±2.25	NS
Glucose (mmol/L)	9.14±1.24^{a}	15.68±0.83^{b}	13.92±0.95^{b}	14.08±1.05^{b}	9.25±0.77^{a}	<.001
Insulin (ng/ml)	0.29±0.05	0.55±0.08	0.40±0.10	0.37±0.04	0.31±0.06	NS
HOMA-IR	2.48±0.67^{a}	9.79±1.07^{b}	6.81±2.58^{ab}	6.20±0.95^{ab}	3.04±0.64^{a}	<.01

[1]Data are means ± SEM (n = 5–10). In each row results without a common letter significantly differ, $P \leq 0.05$ NS, non-significant. TAG, triacylglycerol. NEFA, non-esterified fatty acids. GLP-1, glucagon-like peptide 1. HOMA-IR, homeostasis model assessment of insulin resistance.

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due to differences in the palatability of the LFD used as the control [33,34].

In this study, there was a discrepancy in energy intake in HFD fed mice depending on the housing environment (single or group housed). In contrast to the group house environment up to week 16, HFD fed mice when individually housed in metabolic cages during weeks 17–20 showed a hypophagic response compared to LFD fed mice. It is possible that these differences may be related to the accuracy of the method used to measure food intake in group versus single housed mice, although if this was an error due to methodology, then it would likely to have influenced all dietary groups equally and not just the HFD group. Alternatively, the different behavioural responses could be result of social isolation, which has been shown to decrease energy intake and elevate plasma corticosterone levels [35,36]. However, socially isolated mice adapt to the new environment and consume similar amounts of food as pre-adapted singularly housed mice 6 hr post-novelty stress, but interestingly 24hr later, their food intake reduces significantly once again in the new environment, suggesting that stress of social isolation could extend up to 24 h [36]. We showed that group housed mice on a LFD when placed in isolation adapt to the new environment and continue to consume similar amounts of food by day 2 and 3 in the new location [8] and consequently used the day 3 time point to measure energy intake in this study. Rodents on a HFD have been shown to display increased anxiety [37], and have an over-active hypothalamic-pituitary-adrenal axis [38,39] resulting in elevated plasma corticosterone levels, as demonstrated here. This could explain why HFD fed mice are more susceptible to stress stimuli with more pronounced reductions in energy intake compared LFD fed counterparts subjected to the same stress levels [40–42]. Consequently, in a HFD background, it's possible that social isolation-induced stress responses could have had a greater impact on energy intake with effects lasting up to the 3 day housing period as used in this study. Given the finding that whey proteins such as lactoferrin and α-lactalbumin and native whey protein itself reduce stress [43–47], it is possible that replacing the casein protein with an equivalent WPI content caused normalisation of energy intake in HFD-fed mice by affecting a specific feeding behaviour related mechanism(s), with increasing the WPI protein-derived bioactives having no further effect. This WPI effect on energy intake appeared be specific to the HFD-induced neuroendocrine state because mice on the LFD with WPI showed similar energy intake to casein diet-fed controls. Since neither WPI nor increasing the P/C ratio influenced plasma corticosterone levels, adipose expression of 11β-HSD1 or hypothalamic expression of GCCR in HFD fed mice, it is possible that WPI may have affected other central mechanisms mediating stress responses not investigated in this study [48–50] either independently or in combination with key mechanisms regulating energy balance. Given that leptin decreases meal size and number [51–53], and WPI reduced the HFD-induced increase in plasma leptin levels, it's possible that WPI-derived bioactives could have specifically influenced circadian rhythm of leptin production and/or action within the neuroendocrine state of HFD-fed mice in a socially isolated environment. Additionally, the reduction in plasma amino acids associated with WPI intake (see below), could also have acted as a possible central trigger to increase energy intake in WPI groups compared to HFD control in the single house environment.

HFD feeding has been shown to cause a gain of weight in rats up to the duration of a test period lasting 76 weeks, with animal's body weight gain responding to changes to the dietary fat content introduced at various time points [54]. Lin *et al.*, [32] demonstrated that mice on a HFD for 19 weeks are responsive to intracerebroventricular administration of leptin. These data suggest that energy balance related mechanisms are able to respond to energetic challenges even after prolonged high fat intake. High protein intake within a HFD suppresses energy intake [55,56] albeit not consistently [4,57], and in our study, the P/C ratio did not alter energy intake in either housing environment. This could be a result of the quantity or composition of the macronutrient used in the test diets. Indeed, data from human trials showed that increasing protein dietary content (10/15% to 30%) only decreased energy intake when the carbohydrate content was kept constant [58,59]. This further highlights the importance of designing appropriate experimental diets with the correct macronutrient composition for uncovering the energy balance related impact of the dietary component under investigation.

Metabolic health

Replacement of the casein protein with an equivalent energy content of WPI (i.e. 20%) did not specifically alter metabolic activity, heat production or locomotor activity in HFD or LFD fed mice. In contrast, Acheson *et al.*, [60] showed that whey has a greater thermic effect than casein or soy in humans. These

Table 3. Relative hepatic gene expression in mice fed a 10%kJ low fat diet (LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1].

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
CD36	1.00±0.20^{a}	2.85±0.34^{b}	2.98±0.45^{b}	2.17±0.25^{ab}	1.50±0.25^{a}	<.05
PPARγ	1.00±0.12^{a}	3.00±0.57^{b}	2.30±0.49^{b}	1.88±0.46^{b}	0.85±0.13^{a}	<.001
FABP1	1.00±0.09^{ab}	1.27±0.10^{b}	0.86±0.06^{ac}	1.27±0.17^{bc}	0.71±0.09^{a}	<.001
IRS-1	1.00±0.15^{a}	0.58±0.04^{b}	0.69±0.05^{ab}	0.68±0.06^{ab}	0.79±0.06^{ab}	<.01
CPT1a	1.00±0.08	0.85±0.08	0.97±0.06	0.90±0.13	0.86±0.10	NS
GLUT2	1.00±0.09	0.76±0.04	1.02±0.08	0.97±0.09	0.89±0.05	NS
FATP5	1.00±0.21	0.89±0.07	0.88±0.07	1.16±0.07	1.24±0.12	NS
FASN	1.00±0.36	0.46±0.14	0.54±0.19	0.40±0.06	0.32±0.04	NS
PPARα	1.00±0.04	1.13±0.05	1.21±0.05	1.17±0.10	1.28±0.11	NS
TNFα	1.00±0.26	0.84±0.15	1.12±0.18	0.80±0.09	0.71±0.10	NS

[1]Data are means ± SEM (n = 7–10). In each row results without a common letter significantly differ, $P<0.05$; NS, non-significant. Gene expression shown relative to the LFD control group set at 1.00.CD36, cluster of differentiation 36; PPARγ, peroxisome proliferator activated receptor gamma; FABP1, Fatty acid binding protein 1; IRS-1, Insulin receptor substrate 1; CPT1a, carnitine palmitoyltransferase 1a; GLUT2, Glucose transporter 2; FATP5, Fatty acid transporter 5; FASN, Fatty acid synthase; TNF-α, Tumour necrosis factor alpha.

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differences in data may be related to the fact that the latter study only investigated an acute post-prandial response to a defined test meal, or it may relate to how different species (humans versus mice) digest and metabolise dietary proteins. Shetzer *et al*, found that mice consuming a HFD and WPI-supplemented drinking water have enhanced oxygen consumption compared to mice drinking unsupplemented water [4]. In this instance, the increased metabolic activity may have arisen due to the increased protein intake (proteins from diet and from WPI supplemented water). In fact, this corroborates with the data presented here, which show that increasing the P/C ratio resulted in increased energy expenditure (VO_2 and heat production) and dark phase locomotors activity, resulting presumably from the increased catabolism of ingested dietary protein, coupled with thermic effects of WPI compared to casein [60] and/or due to increased deposition of lean mass with WPI content [61–63]. Interestingly, Zhang *et al.* [64] showed that HFD fed mice on leucine-supplemented drinking water have reduced fasting plasma levels of aspartic acid, glutamic acid, and phenylalanine, as well as increased VO_2 and reduced adiposity compared to HFD controls. Given the influence of leucine on WPI-induced muscle hypertrophy [65,66] and its unique ability to regulate the translation of protein synthesis [67], it is possible that the elevated leucine content found normally in the WPI diets may have enhanced muscle protein synthesis by directing other amino acids towards protein synthesis and/or catabolism [68,69], with the required energy been derived possibly from fat catabolism [70]. Consistent with the latter suggestion, we found an increased lean mass and a trend towards a reduction in fat mass with decreased plasma levels of several amino acids, but not leucine, when the casein protein in a HFD was replaced with WPI or when the P/C ratio in the HFD was increased.

WPI intake appeared to cause a trend towards a reduction in fat mass, and in the liver this manifested as a WPI specific reduction

Table 4. Relative hypothalamic gene expression in mice fed a 10%kJ low fat diet (LFD), 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1].

	LFD	HFD	20% WPI	30% WPI	40% WPI	P value
IR	1.00±0.03^{ab}	0.97±0.07^{a}	1.22±0.04^{bc}	1.37±0.04^{cd}	1.55±0.08^{d}	<.05
GCCR	1.00±0.09^{a}	1.40±0.10^{b}	1.34±0.07^{ab}	1.30±0.08^{ab}	1.42±0.09^{b}	<.05
TNFα	1.00±0.05^{a}	1.76±0.09^{b}	1.57±0.13^{b}	1.47±0.14^{ab}	1.40±0.25^{ab}	<.01
POMC	1.00±0.08	0.99±0.06	1.01±0.06	0.98±0.06	1.00±0.08	NS
NPY	1.00±0.04	1.02±0.09	0.90±0.06	0.88±0.05	0.92±0.08	NS
ObR	1.00±0.11	0.92±0.05	0.99±0.05	1.07±0.07	0.95±0.05	NS
GHS-R	1.00±0.07	0.98±0.06	0.98±0.08	1.02±0.11	1.04±0.09	NS
PPARγ	1.00±0.36	1.07±0.04	0.95±0.05	0.99±0.05	0.98±0.07	NS
CPT1c	1.00±0.09	0.92±0.04	0.91±0.01	0.97±0.06	0.86±0.04	NS
IRS-1	1.00±0.04	0.99±0.04	0.97±0.02	1.06±0.08	1.00±0.06	NS

[1]Data are means ± SEM (n = 5–10). In each row values without a common letter significantly differ, $P<0.05$; NS, non-significant. Gene expression shown relative to the LFD control group set at 1.00. IR, Insulin receptor; GCCR, Glucorticoid receptor; TNF-α, Tumour necrosis factor alpha; POMC, Pro-opiomelancortin; NPY, Neuropeptide Y; ObR, Leptin receptor; GHS-R, Growth hormone secretatgogue receptor; PPARγ, peroxisome proliferator activated receptor gamma; CPT1c, carnitine palmitoyltransferase 1c; IRS-1, Insulin receptor substrate 1.

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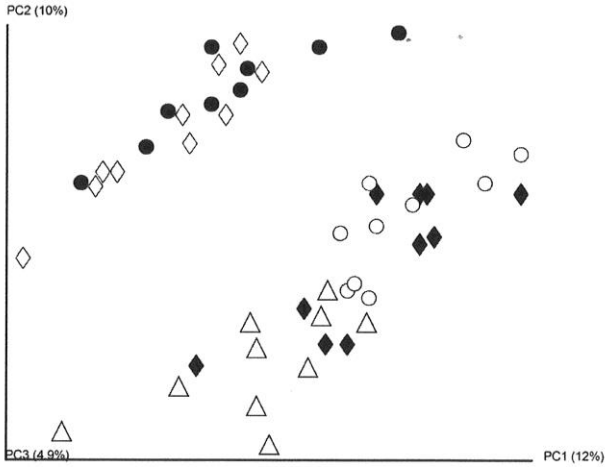

Figure 5. Impact of whey protein isolate and protein to carbohydrate ratio on the gut microbiota composition. Principal Coordinate analysis (PCoA) of unweighted Unifrac distances of the 16srRNA sequences, demonstrating where sequences cluster according to diet group. Data were generated from analysis of faecal samples collected from mice on 10% kJ low fat diet (LFD, Δ) or 45% kJ high fat diet (HFD, ♦) or a HFD with 20% kJ whey protein isolate (HFD-20% WPI, ○), 30% kJ WPI (HFD-30% WPI, ◇) or 40% kJ WPI (HFD-40% WPI, ●)(n = 10).
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in TAG levels, which was accompanied by the suppression of FABP1 mRNA expression, similar to previous findings [4,6,71,72]. In the epididymal adipose tissue, WPI prevented the HFD-induced FASN gene expression, albeit a recent study reported that WPI does not affect the weight of the epididymal tissue in HFD fed mice, but instead causes a reduction in subcutaneous fat pad weight [20]. These data suggest that WPI affects cellular activity in the liver and in specific adipose tissue depots. While it has been suggested that whey protein may facilitate enhanced postprandial chylomicron clearance via an alteration in LPL expression/activity [73,74], here we did not find a WPI specific effect on LPL expression or plasma TAG levels, but we did observe that intake of the highest P/C ratio (40%-WPI) led to an increase adipose tissue LPL mRNA expression which was accompanied by significant reduction in plasma TAG levels, and a complete reversal of genes involved in lipid accumulation (PPARγ), fatty acid transport (FATP1), and lipolysis (β3-AR). Given that HF feeding/obesity down-regulates β3-AR mRNA expression [75], our data suggests an increased adipocyte lipolysis, and reduction in adipose TAG storage in HFD-40%WPI fed mice. Yet the endogenous CPT1b-associated β-oxidation pathway and the UCP-2-associated pathway in epididymal adipose tissue seem to be unaffected (CTP1b) or suppressed (UCP-2) by raising the P/C ratio. This data raises the possibility that the free fatty acids generated from the potentially increased availability of β3-AR in the adipose may have been re-directed for utilisation by other physiological processes active in HFD-40% group, possibly leading to the increased metabolic activity (VO_2) observed in the animals. It is also noteworthy that WPI has been shown to increase faecal fat excretion compared to casein [5], which may have also contributed to the decreased plasma TAG and NEFA seen here with intake of the highest P/C ratio diet (HFD-40%WPI).

Given the link between HFD-induced obesity, low-grade inflammation and insulin resistance [76,77], one could argue that the dramatic reduction in fat mass observed with the highest P/C ratio may underlie the effects on inflammatory markers in the adipose tissue (TNFα and CD68) and the hypothalamus (TNFα), along with simultaneous changes in expression of genes involved in insulin signalling (IR, IRS-1 and GLUT4 in the adipose, IRS-1 in liver and IR in the hypothalamus), and the reduction in plasma glucose in these mice. Improvements to insulin sensitivity with WPI have been reported previously [9,78], but our data suggested that only an increased P/C ratio in the HFD facilitated improvements to insulin signalling pathway associated gene expression, particularly in the adipose, in parallel with reduced HOMA-IR values.

Composition of gut microbiota

While many of the effects described above may be due to direct WPI or P/C ratio-host interactions, the effect of WPI and P/C ratio on the composition of the gut microbiota may also play an important role in adiposity and weight gain in these animals. Here, high throughput sequencing based analyses of faecal microbial populations revealed the clustering of the microbiota from animals in receipt of 30 and 40% WPI diets away from those in receipt of 20% kJ WPI or HFD-casein diets. Tranberg *et al* [20] recently suggested that the efficient absorption of dairy whey proteins in the small intestine may explain the absence of changes in the faecal microbiota. This may explain the clustering of the microbiota from animals fed 20% WPI or HFD-casein diets in our study. However, it is apparent that the high concentrations of WPI present in the 30 and 40% WPI diets employed in our study had a more profound effect, possibly due to additional whey proteins finding their way to the large intestine and/or the overall change in the P/C ratio in the diet. Consumption of the 30 and 40% WPI diets did not result in a shift in the microbiota toward that of the LFD animals and thus the effects on weight gain are not simply due to an overall

Table 5. Gut microbiota composition as % of reads in mice fed a 45%kJ high fat diet (HFD) or HFD with 20%, 30% or 40% kJ whey protein isolate (WPI) for 21 weeks[1].

	HFD	20% WPI	30% WPI	40% WPI
Phylum				
Proteobacteria	0.36^{a}	0.63^{b}	0.34^{ab}	0.32^{a}
Actinobacteria	0.63^{a}	1.82^{b}	3.79^{b}	0.36^{c}
Deferribacteres	0.57^{a}	1.61^{b}	1.56^{ab}	2.03^{b}
Family				
Desulfovibrionaceae	0.12^{a}	0.31^{b}	0.21^{ab}	0.23^{ab}
Rikenellaceae	6.71^{ab}	7.54^{b}	3.9^{a}	6.4^{ab}
Bacteroidaceae	0.44^{a}	0.42^{a}	0.16^{b}	0.21^{b}
Lactobacillaceae	0.21^{a}	3.03^{b}	4.6^{b}	2.14^{b}
Bifidobacteriaceae	0.43^{a}	1.71^{b}	3.66^{b}	0.22^{c}
Deferribacteraceae	0.57^{a}	1.59^{b}	1.32^{ab}	2.03^{ab}
Peptostreptococcaceae	0.62^{a}	1.79^{a}	1.54^{a}	8.01^{b}
Succinivibrionaceae	0.13^{a}	0.15^{a}	0^{b}	0^{b}
Clostridiaceae	1.31^{a}	0^{b}	0^{b}	0^{b}
Veillonellaceae	0.02^{a}	0.12^{b}	0^{a}	0^{a}
Genus				
Anaerobiospirillum	0.13^{a}	0.15^{a}	0^{b}	0^{b}
Desulfovibrio	0.07^{a}	0.22^{b}	0.17^{ab}	0.15^{ab}
Alistipes	4.33^{ab}	4.41^{a}	2.24^{b}	3.76^{ab}
Rikenella	1.04^{ab}	0.49^{b}	0.68^{b}	1.08^{b}
Bacteroides	0.44^{a}	0.4^{a}	0.16^{b}	0.21^{ab}
Oscillibacter	0.24^{a}	0.67^{ab}	0.42^{ab}	0.52^{b}
Lactobacillus	0.2^{a}	3.03^{b}	4.6^{b}	2.39^{b}
Bifidobacterium	0.43^{a}	1.71^{b}	3.66^{b}	0.22^{c}
Mucispirillum	0.57^{a}	1.61^{b}	1.56^{ab}	1.92^{ab}
Coprococcus	0.11^{ab}	0.23^{b}	0.06^{a}	0.06^{ab}
Turicibacter	0.56^{a}	0.35^{a}	0.15^{ab}	0^{b}
Clostridium	1.3^{a}	0^{b}	0^{b}	0^{b}
Peptostreptococus	0.1^{a}	0.14^{a}	0.12^{a}	0.78^{b}

[1]Data are means ± SEM (n = 10). Statistically significant differences generated using the Kruskal-Wallis algorithm. In each row values without a common letter significantly differ, $P \leq 0.05$.

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conversion to a LFD-like microbiota. Specific taxonomic changes were also noted in response to the different diets. In all cases dietary WPI resulted in significant increases in *Lactobacillaceae/Lactobacillus* and decreases in *Clostridiaceae/Clostridium*. Increased proportions of *Lactobacillus* have previously also been observed in a study of individuals following a regime of calorie restriction and exercise [79]. However, in contrast, increased proportions of *Lactobacillus* have also been noted in HFD fed rats [80] and diet-induced obese mice [81]. While specific species of *Lactobacillus* have been associated with both lean and obese gut microbiota profiles and also to play a role in obesity and immune response regulation [82–84], due to the length of the 16S sequences generated and the high degree of sequence homology, we cannot assess changes in proportions of *Lactobacillus* at the species level. An increase in the proportions of *Bifidobacteriaceae/Bifidobacterium* was also observed in both the HFD-20% WPI and HFD-30% WPI compared to the HFD group. This result, combined with the aforementioned increases in *Lactobacillaceae/Lactobacillus*, mirror those reported by Sprong *et al* who suggest that whey proteins act as grow factors for certain species of bacteria by an amino acid composition mediated mechanism [19]. This pattern did not extend to the HFD-40% WPI group suggesting that, at these high protein levels, other factors are at play. Our observations are also consistent with previous findings that high proportions of the class *Clostridiales* are associated with the gut microbiota of animals fed a HFD [85], while fasting reduces the levels of *Clostridium* [86]. Notably, *Clostridiaceae* can produce short chain fatty acids as a product of their metabolism [87], which can play an important role in the regulation of immune cells and has been associated with inflammation and obesity [88]. These differences, as well as others in the *Proteobacteria Actinobacteria Deferribacteres* (phylum), *Desulfovibrionaceae Deferribacteraceae Veillonellaceae* (family), *Desulfovibrio* and *Mucispirillum* taxa in the HFD-20% whey protein relative to HFD controls (20% casein) are particularly notable as these reflect changes resulting from the specific presence of whey proteins in the diet, in place of casein, rather than changes in the P/C ratio. Changes in relative proportions may be attributed to (a) the ability of bacteria to utilise whey proteins as a growth medium, (b) the anti-microbial activity of whey protein/peptide components, (c) decreased competition as a result of the whey proteins/peptides antimicrobial activity or (d) whey protein mediated changes in the host. Ultimately, the question of cause versus effect remains unanswered, and so while the changes to the microbiota observed may contribute to the mechanisms involved in controlling weight gain, further studies with, for example germ free animals, will be required to determine this definitively.

WPI effects on energy balance from a whole animal context

Focusing on the experimental data gathered between weeks 17–21, during which we measured metabolic parameters, faecal microbial population, body composition and tissue and plasma level of energy balance related parameters, it is clear that WPI intake increased energy intake associated with the HFD, without altering energy expenditure, as measured by VO_2 and locomotor activity. However, the body composition and body weight in the HFD-WPI group does not appear to reflect a positive energy balance, as animals showed a trend towards a reduction in fat mass and increased lean mass. It is noteworthy in this regards that WPI has been shown to increase faecal fat excretion [5] and we also observed some subtle changes in the gut microbiota at a phylogenic level that are associated with non-obese states, raising the possibility of a reduced intestinal TAG absorption in the HFD-WPI groups with increased energy intake, leading to similar body weight trajectories as HFD controls. Increasing the P/C ratio by changing WPI from 30 to 40% did not alter energy intake but significantly accentuated energy expenditure with a concurrent dramatic change in physiology.

In summary, our results show that WPI specifically normalises energy intake, increases lean mass and causes a trend towards a reduction in fat mass associated with prolonged high fat feeding. Raising the P/C ratio had no effect on energy intake but augmented metabolic activity and beneficially altered gene expression profiles for lipid metabolism, inflammation and insulin signalling, particularly in the adipose tissue. High throughput analysis of gut microbiota revealed distinct changes in microbial populations with increased P/C ratio causing clustering of 30/40% WPI groups together and distinct from those of HFD and 20% WPI groups, but with specific phylogenetic differences existing between the latter groups. These data indicate that changes to P/C ratio have a dramatic effect on energy balance and the composition of gut microbiota distinct from that seen with

changes to protein source. Future studies should focus on determining whether the effects demonstrated for highest P/C ratio are specific to the WPI content, a consequence of macronutrient change, or both.

Supporting Information

Figure S1 Effect of a 10%kJ low fat diet with 20%kJ casein (LFD) or 20%kJ whey protein isolate (LFD-WPI) on (A) body weight, (B) energy intake (C) oxygen consumption (VO_2) and (D) locomotor activity, which were measured in individual mice at 9 minute intervals over a 24 hour period using TSE Phenomaster cages.
(DOC)

Table S1 The composition of the low fat diet (LFD), high fat diet (HFD) and HFD with 20% kJ, 30% kJ or 40% kJ whey protein isolate (WPI)[1].
(DOC)

Table S2 Sequences of mouse specific primers used in real-time PCR analysis[1].
(DOC)

Author Contributions

Conceived and designed the experiments: LM KNN JFC PDC PS RPR POC GF HMR. Performed the experiments: LM PS POC. Analyzed the data: LM KNN JFC PDC PS RPR POC GF HMR. Contributed reagents/materials/analysis tools: KNN PDC PS POC HMR. Wrote the paper: LM KNN JFC PDC PS RPR POC GF HMR. Ethical Approval: KNN JFC RPR GF HMR.

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