A HIGH PROPORTION OF DIETARY CASEIN ATTENUATES THE OBESOGENIC EFFECT OF HIGH-FAT DIETS IN C57BL/6J MICE

ASTRID ELISE HASSELBERG

MASTER THESIS IN HUMAN NUTRITION

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LIST OF ABBREVATIONS

AA	Amino acid
ANOVA	Analysis of variance
ATP	Adenosin-5'-trifosfat
AUC	Area under curve
BCAA	Branched chain amino acids
BAT	Brown adipose tissue
BMI	Body mass index
BW	Body weight
cAMP	Cyclic-adenosine monophosphate
CCK	Cholecystokinin
DAUC	Decremental area under curve
DIO	Diet-induced obesity
ELISA	Enzyme-linked immunsorbent assay
ETDA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
eWAT	Epididymal white adipose tissue
OGTT	Oral glucose tolerance test
hBAT	Human brown adipose tissue
HF/HP	High fat and high protein
HF/HS	High fat and high sucrose
HP	High protein
iBAT	Interscapular brown adipose tissue
IAUC	Incremental area under curve
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue

LF	Low fat
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
PB	Phosphate buffer
PET	Positron emission tomography
SEM	Standard error of the mean
SNS	Sympathetic nervous system
TAG	Triacylglyceride
UCP1	Uncoupling protein-1
VHF	Very high fat
WAT	White adipose tissue
WHO	World Health organization

ABSTRACT

The obesity epidemic is on the rise, and currently represents the largest global health threat. Westernization and advances in food production have led to a contemporary diet comprised of 49 % energy from carbohydrates, 35 % from fats and 16 % from proteins. Former studies have demonstrated the weight-reducing benefits of increasing the amount of dietary protein at the expense of sucrose in high-fat diets, but the impact of specific proteins has not been elucidated. An unpublished study from our group discovered that different protein sources exert dissimilar obesogenic effects in mice when included in a high-protein diet. While proteins from pork promoted obesity development and proteins from cod had an intermediate effect, casein was the only protein to protect against diet-induces obesity. Moreover, the study registered a higher energy intake in the mice fed pork compared to the mice fed cod.

We undertook our current study to investigate the obesogenic effect of different proteins and the satiating effect of cod proteins in a high-fat high-protein diet. Additionally, we aimed to expound on the various mechanisms by which different proteins affect obesity development. Thus, obesity-prone C57BL/6J mice were fed high-fat high-protein diets with casein, cod or pork as the protein source, with one pork group being pair fed with the group fed cod. Furthermore, we found it interesting to explore the weight-reducing effects of the same protein sources on diet induced obese mice. Hence, obese C57BL/6J mice were fed low-fat diets with casein, cod or pork as the protein source. In agreement with earlier studies, our results demonstrated that casein was the only protein to attenuate the obesogenic effect of a high-fat diet. On the contrary, mice fed pork gained a significant amount of fat mass and had an impaired glucose tolerance. Furthermore, our results demonstrated that cod-protein had an intermediate effect. In contrast to previous observations, the cod proteins did not promote satiety in a higher degree than proteins from pork. In our second animal experiment, we observed a significantly higher weight loss and an improved glucose tolerance in obese mice fed a low fat diet with casein. Collectively, our results underscore that the dietary protein source is an important factor to abrogate the development of obesity, and implies that it might be beneficial to increase the amount of dietary casein at the expense of pork proteins.

1. INTRODUCTION

1.1 OVERWEIGHT AND OBESITY

Overweight and obesity is defined as an excessive or abnormal accumulation of fat that may lead to multiple co-morbidities (WHO).

1.1.1 Quantification of overweight and obesity

Body mass index (BMI) is a widely used method for diagnosing and classifying overweight and obesity, which provides a number that indicate general body fatness (WHO 2012). To calculate BMI, the body weight in kilograms is divided by the square of height in meters (kg/m^2). WHO provides a classification of BMI, where a BMI of 25 kg/m^2 or higher is considered overweight, and a BMI of 30 kg/m^2 or above is classified as obese. Although BMI is a good indicator of a person's general health status, it does not consider factors such as bone density, muscle-and fat mass ratio or body shape. Waist-to-hip ratio can be measured to obtain information regarding fat deposition, and it accounts for differences in body shape (Karelis, St-Pierre et al. 2004). Having a pear shaped body, which is most common with women, is considered to lower the risk of developing obesity-related diseases due to subcutaneous fat deposition. Individuals with an apple shaped body are at a higher risk of developing cardiovascular diseases and diabetes type 2, as they have increased visceral fat deposition (Haslam and James 2005). A more precise measurement of fat depots may be obtained by dual-energy X-ray absorptiometry (DEXA) or magnetic resonance imaging (MRI) (James 2004). Nevertheless, these methods are expensive to perform and are not of general clinical relevance.

1.1.2 Prevalence of overweight and obesity

The prevalence of overweight and obesity has doubled since the 1980s, with an estimated 1.4 billion adults being overweight and 500 million adults being obese in 2008 (WHO 2012). Islands in the South Pacific have the highest rate of adult obesity worldwide (>50%), followed by large countries such as Mexico (32.8%) and the USA (31.8%) (FAO 2013). In Norway the average body weight has increased by 6 kg since 1985 and today more than half of the adult population is overweight and 15-20 % is obese (Folkehelseinstituttet 2012). Childhood overweight is also on the rise, particularly in developing countries (WHO 2012). More than 30 million overweight children under the age of five live in developing countries while 10 million live in developed countries. Hence, to define overweight and obesity as a high-income country problem is no longer viable, as the prevalence is rising more rapidly in middle-and low-income countries. Underweight has been a global challenge for decades, however, 65% of the world's population currently live in countries where obesity and overweight causes more deaths than underweight.

1.1.3 Causative factors of overweight and obesity

The primary cause of the increasing prevalence of overweight and obesity is an energy imbalance between calories consumed and calories expended. One of the contributing factors to reduced energy expenditure is the modern sedentary lifestyle. Additionally, there has been dramatic changes in food production and consumption in the past centuries (FAO 2013). Food production has evolved from self-sufficient farming, to industrial production of more energy-dense foods containing higher amounts of sugar, omega-6 fatty acids and salt. In addition to energy imbalance, genetic factors can affect the prevalence of obesity on many levels such as appetite control, energy metabolism and hereditary traits (Pelleymounter, Cullen et al. 1995). A relation between social class and risk of developing obesity has also been established, along with migration and other behavioural factors (Ball, Mishra et al. 2003; Rankinen, Zuberi et al. 2006). The causes of overweight and obesity are intricate, and as varied as the people it affects.

1.1.4 The pathophysiology of overweight and obesity

Excess body weight is considered to be the fifth leading risk factor of global deaths and is attributable to a series of associated disorders (Guh, Zhang et al. 2009). Overweight and obesity are associated with development of insulin resistance and type 2 diabetes along with cardiovascular diseases as atherosclerosis and stroke. Additionally, obesity promotes secretion of inflammatory cell signalling proteins which increase the risk of developing various types of cancer, including colon and oesophageal cancer (Redinger 2007). Obesity may also lead to female reproductive problems and infertility, due to increased secretion of fertility-associated hormones (Brothers, Wu et al. 2010). Furthermore, sleep apnea and osteoarthritis due to excess weight are common in obese individuals. This cluster of co-morbidities is currently the largest global health threat.

1.2 THE ADIPOSE ORGAN

The adipose organ consists of intermingled white and brown adipocytes. While white adipocytes are mainly used as a lipid storage that functions as fuel between meals, the brown adipocytes burn fat to produce heat.

1.2.1 White adipose tissue (WAT)

White adipose tissue (WAT) consists of unilocular white adipocytes containing a single large lipid droplet surrounded by a thin rim of cytoplasm (Cinti 2009). The lipid droplets are semi-liquid and primarily comprised of triacylglycerols (TAGs). In addition to function as an energy storage, it has been discovered that WAT is an active endocrine organ that secrete protein signals and factors (Kershaw and Flier 2004). These signals and factors include leptin, adiponectin, tumor necrosis factor α (TNF-α), proteins of the renin-angiotensin system, interleukin 6, plasminogen activator inhibitor-1 and resistin. Obesity is known to alter the endocrine function of adipose tissue and lead to an increased secretion of pro-inflammatory cytokines from macrophages (Weisberg, McCann et al. 2003). The elevated cytokine secretion induces chronic inflammation, which is considered as a causative factor for developing insulin resistance. (Xu, Barnes et al. 2003).

1.2.2 Brown adipose tissue (BAT)

Brown adipose tissue (BAT) is composed of multilocular brown adipocytes that store triglycerides in small vacuoles and numerous large mitochondria packed with laminar cristae (Cinti 2009). Unlike WAT, BAT uses lipids to produce heat through non-shivering thermogenesis. The process is activated by mitochondrial uncoupling protein-1 (UCP1), which is found solely in brown adipocytes (Cannon, Hedin et al. 1982; Frontini, Rousset et al. 2007). UCP1 allows the uncoupling of protons from oxidative phosphorylation and adenosin-5'-trifosfat (ATP) synthesis, resulting in thermogenesis. The metabolic activity of BAT is regulated via release of norepinephrine from the sympathetic nervous system (SNS) which binds to G-protein coupled β-adrenergic receptors in BAT (Townsend and Tseng 2012). Activation of the receptor triggers production of cyclic-adenosine monophosphate (cAMP) and protein kinase A (PKA) activation. Activated PKA leads to phosphorylation and activation of hormone sensitive lipase (HSL), which accelerates free fatty acid (FFA) release from stored triglycerides. FFAs enters the mitochondria and are used for either β-oxidation or activation of UCP1-induced thermogenesis.

Occurrence of brown adipose tissue

Brown adipose tissue is a mammalian prerogative, which provides the ability to regulate body temperature through non-shivering thermogenesis. Although BAT was serendipitously discovered in the 16th century, its physiological properties were not supported until the 1960s. Metabolically active BAT has been known to exist in the interscapular region of small animals and infants, but the discovery of UCP1 positive brown adipose tissue (hBAT) in human adults is a fairly recent discovery. In 2009, metabolically active hBAT in adults was detected by using positron emission tomography (PET) and glucose tracers (Cypess, Lehman et al. 2009; van Marken Lichtenbelt, Vanhommerig et al. 2009; Virtanen, Lidell et al. 2009). This recent discovery rekindled the interest in human adipose biology and its potential therapeutic benefits.

Transcriptional regulation of brown and "beige" adipocytes

Brown adipocytes are believed to derive from precursor cells that express the Myf5+ gene, which also gives rise to white adipocytes and skeletal muscle (Seale, Bjork et al. 2008; Sanchez-Gurmaches, Hung et al. 2012). Furthermore, brown precursor cells express a gene signature similar to muscles and a related mitochondrial protein expression. In addition to activation of brown adipocytes, it has been established that stimuli by cold acclimatization or β-adrenergic agonists induce the appearance of a different UCP1-expressing adipocyte in WAT; the "beige" or "brite" adipocyte (Cousin, Cinti et al. 1992; Guerra, Koza et al. 1998; Wu, Bostrom et al. 2012). While brown adipocytes are found in specific anatomical depots and express high levels of UCP1 in an unstimulated state, the "beige" adipocytes appear intermingled in WAT and only express UCP1 when stimulated (Petrovic, Walden et al. 2010; Wu, Cohen et al. 2013). "Beige" adipocytes derive from transdifferentiation of mature white adipocytes, *de novo* differentiation of precursor cells or a combination of these phenomena. Cinti and colleagues have shown that cold-induced browning in WAT is mainly due to transdifferentiation of mature white adipocytes (Cinti 2009), while recent in vivo mapping studies have unveiled Myf5 negative brown adipocytes in WAT after adrenergic stimulation (Timmons, Wennmalm et al. 2007; Seale, Bjork et al. 2008). The findings of Timmons and colleagues suggest that "beige" adipocytes may have a dissimilar origin then the classical brown adipocyte, but a unanimous conclusion has not yet been presented. A description of both transcriptional theories on brown and "beige" adipocyte regulation is presented in figure 1.1.

In summary, browning of white adipose tissue may represent a new therapeutic target in the prevention of obesity and its co-morbidities. Several animal studies have shown an increased amount of "beige" adipocytes in obesity-resistant mice, but human studies are still at its inception (Xue, Rim et al. 2007; Vitali, Murano et al. 2012).
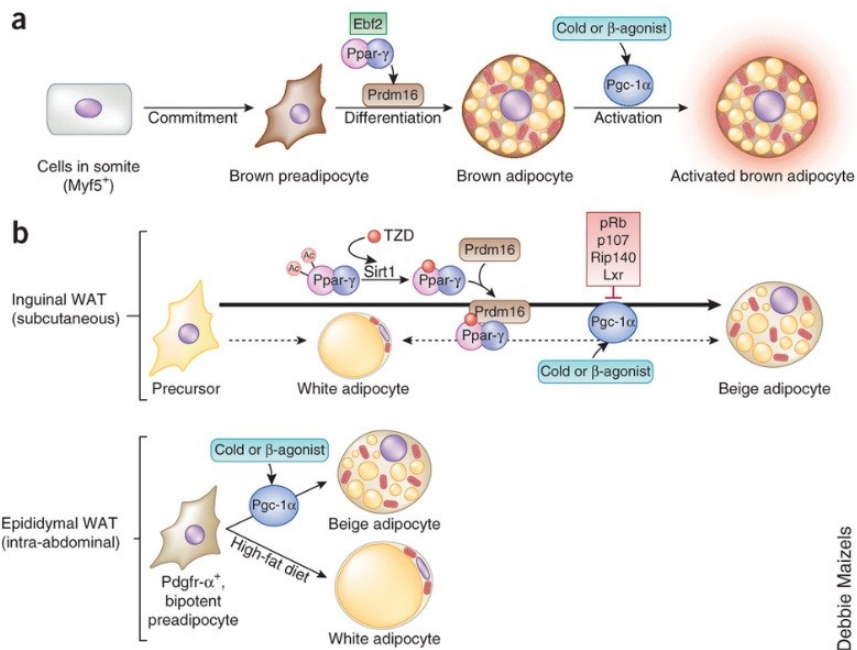
Figure 1.1: *Transcriptional regulation of brown and beige adipocyte development (a) Brown adipocytes are derived from a Myf5-expressing progenitor population. Ebf2 cooperates with Ppar-γ to promote the expression of Prdm16, which drives a brown-fat cell fate. Thermogenesis in mature brown adipocytes is activated by norepinephrine (NE), a β3 agonist, released from sympathetic neurons. NE signals through β-adrenoreceptors to increase the expression and activity of Pgc-1α, a transcriptional coactivator that coordinates gene programming in response to activation. (b) In inguinal fat, β-adrenergic stimulation triggers predominantly de novo differentiation of precursor cells (large arrow). We leave open the possibility that under some conditions, mature white fat cells can transdifferentiate into beige cells (small dashed arrow). In epididymal WAT, caloric excess causes bipotent progenitors to differentiate into white adipocytes, whereas β-adrenergic activators stimulate beige adipocyte development. TZD agonists of Ppar-γ promote beiging both by increasing the stability of Prdm16 and through the Sirt1-dependent deacetylation of Ppar-γ, which recruits Prdm16 to Ppar-γ target genes. β-adrenergic signaling drives the expression and activity of Pgc-1α in beige adipocytes. Pgc-1α is targeted by numerous repressors to block beige adipocyte development. Ac, acetylation.* Figure and figure text are adapted from (Harms and Seale 2013).

1.3 WEIGHT REDUCTION AND OBESITY PREVENTION

1.3.1 Macronutrients

The main cause for obesity development is a long-term energy imbalance, although the true understanding of its causative factors and treatment options remains uncertain. The contemporary Western diet is energy dense and contains a high level of carbohydrates, especially starches and processed carbohydrates. By introducing more dietary carbohydrates, the intake of protein has been dramatically reduced when compared to typical Stone-age and Hunter-gatherer diets (Cordain, Eaton et al. 2005; Eaton 2006). In addition to a more sedentary lifestyle, the shift in dietary macronutrient composition may contribute to the increasing prevalence of obesity and type 2 diabetes. While the Norwegian Health Authorities recommend a diet high in carbohydrates and low in fat to prevent obesity, many alternative approaches have emerged (Helsedirektoratet 2014). The definition of the perfect dietary macronutrient-balance is widely debated, with each combination presenting their pros and cons. New dietary trends emerge continuously, and lately high-protein diets have gained attention as a popular method to lose weight.

1.3.2 Low-fat diets

Low fat (LF) diets are based on the restriction of dietary fat, specifically saturated -and trans fatty acids. Limiting the dietary fat has been a popular way of reducing calorie intake, given that fat is the most energy-dense macronutrient (9 kcal/g). Traditionally a LF diet consists of 10-20 E% from proteins, 45-60 E% from carbohydrates and 25-40 E% from fat (Helsedirektoratet 2014). In obese and overweight individuals, calorie restricted LF diets have induced substantial weight loss, reduced cholesterol levels and improved glucose tolerance (Sacks, Bray et al. 2009) . However, LF diets have been observed to promote gluttony in a higher degree than HP diets. The protein-leverage hypothesis proposes that protein intake is prioritized over the intake of the other macronutrients, resulting in a higher energy-intake in diets low in protein (Sorensen, Mayntz et al. 2008). Furthermore, the weight loss ratio between fat-and lean mass produced by low-fat diets has been debated, and many advocate for a higher percentage of dietary protein (Leidy, Carnell et al. 2007).

1.3.3 High-protein diets

High protein (HP) diets were previously most common amongst bodybuilders and athletes, but have lately gained increased interest as a method for weight management. There is no specific definition of a HP diet, but an intake of >1.6 g/kg or >25 E% protein can be regarded as high (Eisenstein, Roberts et al. 2002). Studies and meta-analyses have demonstrated that both calorie-restricted and *ad libitum* HP diets promote weight loss in the form of fat, improve glucose homeostasis and maintains lean mass in human subjects (Skov, Toubro et al. 1999; Layman, Boileau et al. 2003; Krieger, Sitren et al. 2006). Similar evidence has been presented in studies with rats, where a long-term HP diet reduced white adipose tissue mass and lowered basal concentrations of triglycerides, glucose and insulin (Lacroix, Gaudichon et al. 2004). The potential negative effects of a high protein intake on renal function and urinary calcium loss is widely discussed, but little evidence have supported a connection between these factors (Eisenstein, Roberts et al. 2002). The protein source is also of importance in a HP diet, due to variations in macro- and micronutrient composition (Gilbert, Bendsen et al. 2011). While dairy products are rich in calcium, oily fish is rich in ω-3 fatty acids and meats from terrestrial animals are rich in saturated fat. The mutual characteristics of animal proteins are that they contain all 9 essential amino acids necessary for protein turnover, and are thereby classified as complete proteins.

The effect of protein on satiety

The weight regulating effects of HP diets may be attributed to increased satiety and thereby a reduced energy intake (Blouet, Mariotti et al. 2006; Westerterp-Plantenga, Lemmens et al. 2012). Various physiological mechanisms have been proposed to explain the satiating effect of proteins, including gastric hormones and circulating amino acids. Animal studies with rats have demonstrated that proteins are the most potent food stimulant for the release of the peptide hormone cholecystokinin (CCK) compared to fat and carbohydrates (Liddle, Green et al. 1986; Douglas, Woutersen et al. 1988). CCK is known for its appetite suppressing abilities through slowing down gastric emptying, which may lead to a reduced energy intake (Little, Horowitz et al. 2005).

In addition to gastric hormones, circulating amino acids in plasma might be recognized as a satiety signal. High circulating levels of the essential amino acid tryptophan has been linked to a higher satiety sensation in lean men, likely due to tryptophan's role in the synthesis of the appetite modulating neurotransmitter serotonin (Uhe, Collier et al. 1992). The branch chained amino acid (BCAA) leucine has been known to stimulate mammalian target of rapamycin (mTOR) signalling via the hormone leptin and thereby increase the sense of satiety in the ventromedial nuclei in the hypothalamus (Cota, Proulx et al. 2006). Moreover, a rat study illustrated another positive connection between leucine and high levels of the adipose tissue-derived hormone leptin (Lynch, Gern et al. 2006). A 40 % reduction in leptin secretion was observed in rats receiving a leucine-deficient meal, whereas no differences were seen when other amino acids were eliminated. All combined, the satiating effects modulated by HP diets may explain a share of its effects on weight management and energy intake.

The effect of protein on energy expenditure

An alternative process by which the HP diets may exercise their weight-stabilizing effects is through diet-induced thermogenesis (DIT). The thermic effect of food is based on the energy required for digestion, absorption and disposal of ingested nutrients (Halton and Hu 2004). Ingestion of protein-rich foods has been known to promote a higher diet-induced thermogenesis (20-30%) compared to carbohydrates (5-10 %) and fat (0-3 %) (Tappy 1996). In contrast to most nutrients the body has no storage capacity for amino acids, which results in immediate metabolic processing through gluconeogenesis, ureagenesis and protein turnover (Giordano and Castellino 1997; Mikkelsen, Toubro et al. 2000). Thus, HP diets may affect energy expenditure by preserving muscle mass and maintaining protein turnover despite fluctuations in body weight. Furthermore, energy expenditure may be increased by an up-regulation of UCP1 in white adipose tissue. The amino acid tyrosine is a precursor to norepinephrine, which may stimulate UCP1 expression and increase energy expenditure via β-adrenoreceptor stimulation (Cannon and Nedergaard 2004). Conclusively, it is plausible that a HP diet increases energy expenditure more than diets rich in fat or carbohydrates.

The effect of protein on insulin metabolism

The metabolic hormones insulin and glucagon may also influence the weight-regulating effect of HP diets. Insulin is known to exert an anabolic effect in healthy individuals by promoting fat and glucose storage, impeding fat oxidation and repressing gluconeogenesis (Woerle, Meyer et al. 2003; Cherrington 2005). The obesogenic effect of insulin is confirmed by reports showing that mice lacking insulin receptors in adipose tissue or have no expression of the pancreatic *Ins2* gene is protected against diet-induced obesity (Bluher, Michael et al. 2002; Mehran, Templeman et al. 2012). HP diets have been known to improve insulin sensitivity compared to diets rich in carbohydrate, and increase plasma levels of glucagon (Madsen, Pedersen et al. 2008). While carbohydrates are the main stimulants for insulin secretion, various protein sources appear to have different insulinotropic properties. Ingestion of dairy proteins have been known to induce a greater insulin response than beef and fish proteins, possibly due to an increment in plasma amino acids mediated by the BCAAs (Gannon, Nuttall et al. 1988; Calbet and MacLean 2002). Furthermore, leucine has been identified as the sole amino acid to interact directly with the insulin pathway and maintain glucose homeostasis (Devkota and Layman 2010). On the contrary, other studies have shown a connection between high levels of BCAAs in fasting plasma and development of insulin resistance in mice fed a high-fat diet (Newgard 2012). Cod proteins contain high amounts of the organic acid taurine, a potent antioxidant. Administration of taurine has been linked to an improved insulin sensitivity in rats with insulin resistance and type 2 diabetes by inhibiting pancreatic β-cell oxidation and increasing the secretion of cholesterol into bile acid (Nakaya, Minami et al. 2000; Nandhini, Thirunavukkarasu et al. 2005). The effect of different proteins on insulin metabolism is currently under debate, and no definitive answer has been presented.

1.4 INTRODUCTION TO THE STUDY

New dietary trends emerge continuously, and recently high-protein diets have gained more attention as a method for weight-regulation. A review by Westerterp-Plantega confirmed the beneficial effects of high-protein diets on weight loss and energy expenditure, as well as its modulating effects on satiety sensation (Westerterp-Plantenga 2008).

Several studies from our group and elsewhere have demonstrated that a high amount of dietary protein attenuates obesity development and reduces energy intake in mice (Madsen, Pedersen et al. 2008; Ma, Liaset et al. 2011; Lillefosse, Tastesen et al. 2013). Additionally, high-protein diets have been associated with increased cAMP signaling, which allows metabolic processes associated with fasting to occur in a fed state (Madsen, Pedersen et al. 2008). Furthermore, an up-regulation of *Ucp1* in iWAT has been observed in mice given a high amount of dietary protein, which can possibly be related to the observed weight loss in humans maintaining a high-protein diet.

The most frequently used protein in feeding trials with rodents is casein, while commonly used proteins in a human diet also include eggs, fish and various meats from terrestrial animals. Hence, the use of a sole protein source in nutritional studies has produced little evidence regarding the obesogenic effect of various protein sources. In the light of these facts, a previous study in our group was conducted to investigate whether a high proportion of other protein sources were able to protect against diet-induced obesity in the same degree as casein. Consequently, obesity prone C57BL/6J mice were fed high-fat high-protein diets with soy, casein, cod or proteins from terrestrial animals (pork, chicken and beef). In concurrence with previous studies, casein was the only protein source that attenuated obesity development. On the contrary, the mice fed high proportions of beef, cod, pork or chicken became heavier than mice fed a high-fat high-sucrose reference diet. Last year, a follow-up study was conducted to investigate the adipogenic effect of protein:sucrose ratio in combination with casein, cod and pork proteins. Hence, paired groups of C57BL/6J mice were fed high-fat high-sucrose and high-fat high-protein diets. An increased amount of dietary sucrose elevated the body weight significantly in mice fed casein or pork, compared to the diet with a high amount of protein. Mice receiving cod protein did however not have significant variations between the groups. As in the previous study, casein was the only protein source that attenuated diet-induced obesity in a high-fat high-protein diet.

1.5 AIM OF THE STUDY

In view of the finding that of all protein sources tested, only a high proportion of dietary casein has been able to protect against diet-induced obesity, this study had one primary aim, and two secondary aims:

- Primarily, this study aimed to investigate if the dietary protein source is of importance in relation to obesity development, satiety sensation, energy expenditure and glucose tolerance.
- Secondly, we aimed to investigate if proteins from cod are more satiating then proteins from pork.
- Lastly, we aimed to investigate the effect of different protein sources on weight loss and improvement of glucose tolerance.

2. MATERIALS AND METHODS

2.1 THE ANIMAL EXPERIMENTS

Male C57BL/6JBomTac mice were chosen for these experiments due to their ability to develop obesity, hyperinsulinemia and hyperglycemia when fed a high-fat diet (Black, Croom et al. 1998). Additionally, the C57BL/6J strain retains a low adipose tissue mass when fed a LF diet (Petro, Cotter et al. 2004).

Figure 2.1: *Private photo of a C57BL/6J mouse.*

Ethical statement

All animal protocols used during the experiment were approved by the Norwegian Animal Health Authorities.

ANIMAL EXPERIMENT 1:

Fifty-four male C57BL/6J mice were obtained from Taconic Europe (Ejby, Denmark) at 8 weeks of age. The mice were acclimatized for one week on a LF diet before the feeding experiment started. After one week of acclimatization, the mice were weighed and scanned. The mice were then divided into 6 groups (n=9), making sure that each group had a similar body weight, lean mass and fat mass. Tests were performed during the feeding experiment, including collection of feces, an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT). The mice were fed the experimental diets for 12 weeks prior to termination.

ANIMAL EXPERIMENT 2:

Seventy male C57BL/6J mice were obtained from Taconic Europe (Ejby, Denmark) at 7 weeks of age. The mice were fed a very high-fat diet (VHF) for 13 weeks before the feeding experiment was initiated. The mice were then divided into 4 groups (n=13) that had a similar body weight, lean mass and fat mass. Feces were collected before and after the diet experiment as well as OGTTs and ITTs. The mice were fed the experimental diets for 6 weeks prior to termination. Animal experiment 2 was performed in collaboration with Nina Norberg.

Housing and feeding

The mice were housed in individual cages (Techniplast 1291) in a controlled environment throughout the experiments. Each cage was equipped with a house, nesting material, a chewing stick and wooden bedding (Scanbur Bedding Aspen, Norway). The animal room had a 12 hour light/dark cycle, and was thermoneutral (29.35 ± 2.3°C) with an average humidity of 37.9 %. The mice were fed three times a week, and their water was changed twice a week throughout the experiments.

Measurements

The mice were weighed every Monday morning on a Mettler Toledo weight. They were scanned with a Bruker Minispec LF50mq7.5 apparatus before each feeding experiment, halfway through the experiment, and again before termination. The scanner contains a magnetic field that differentiates lean mass, fat mass and free water. Feed and feed remnants were weighed each Monday, Wednesday and Friday. The wooden bedding was sifted biweekly, to collect and weigh spilled feed.

Diets

ANIMAL EXPERIMENT 1:

The six groups were given five different diets, presented in figure 2.2. Two groups were given either a low fat (LF) or a high-fat high-sucrose (HF/HS) reference diet, and four groups were given experimental high-fat high-protein (HF/HP) diets with different protein sources. Two of the groups receiving HF/HP diets were pair fed throughout the experiment.
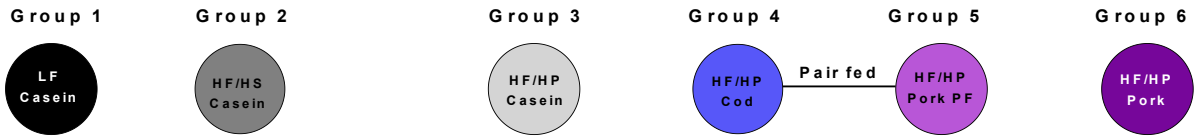
Figure 2.2: Grouping, diets and protein sources in animal experiment 1.

Casein sodium salt from bovine milk was bought from SIGMA (lot number BCBF8389V). Pork sirloin was obtained from H. Brakstad Eftf. AS meat processing and cod fillet was a gift from Lerøy seafood. The cod fillet and pork sirloin were freeze dried and pulverized at NIFES, mixed with the other diet ingredients in a Crypto Peerless EF20 blender and kept in a freezer throughout the experiment. The distribution of macronutrients in the diets are presented in figure 2.3 as energy percent (E %). For a detailed overview of the diet compositions, see appendix I table A.1.
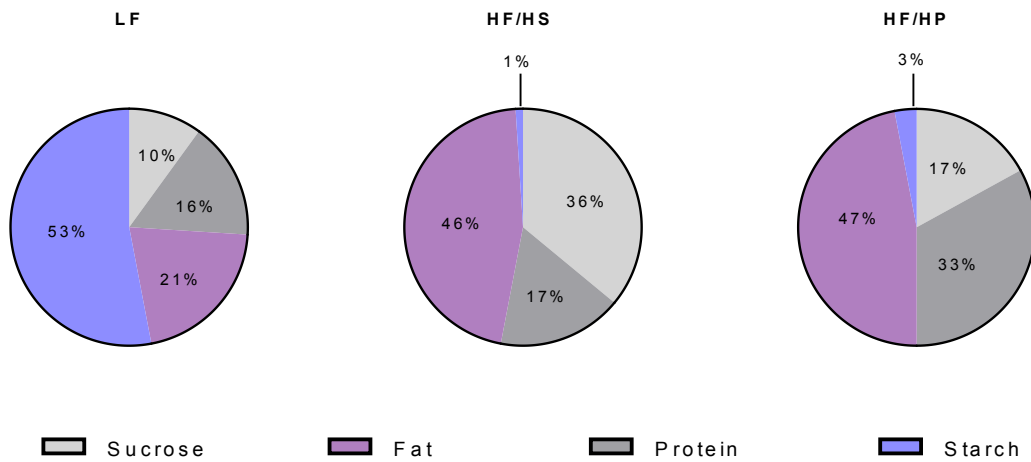
Figure 2.3: Distribution of fat, sucrose, protein and starch in the LF reference diet, HF/HS reference diet and the HF/HP experimental diets (E %).

ANIMAL EXPERIMENT 2:

After 13 weeks on a VHF diet, the four groups of mice were assigned to different diets. In the first week of experimental feeding, all groups were fed *ad libitum*. From week 2 group 1 received a VHF diet as a reference, and groups 2-4 were given 30 % calorie restricted LF diets with casein, cod or pork as the protein source. The protein sources and preparation of the LF diets were as described in experiment 1, while the VHF diet was obtained from Ssniff (Soest, Germany). The distribution of macronutrients in the LF diets were identical to the LF diet presented above in figure 2.3. For a detailed overview of the diet compositions, see appendix I table A.2. The distribution of macronutrients in the VHF diet is presented in figure 2.4 as energy percent (E %)
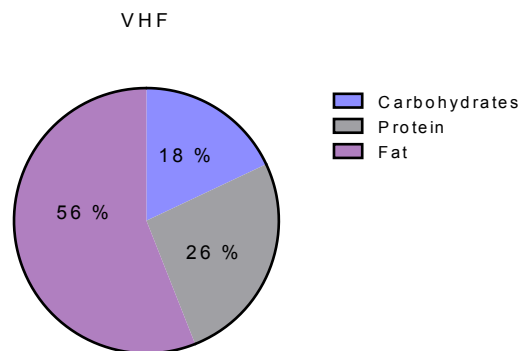
Figure 2.4: *Distribution of fat, protein and carbohydrates in the VHF diet (E %).*

Collection of feces

A collection of feces was performed to calculate apparent fat digestibility. The mice were transferred to clean cages with a sheet of paper or wooden bedding in the bottom, and fed as previously described. After one week the feces were collected and technicians analyzed the fecal fat content. Later, the apparent fat digestibility (AFD) was calculated using the following formula:

AFD= ((amount of fat eaten-amount of feces excreted)/amount of fat eaten) x 100%

Termination

Prior to termination, the mice were weighed and put in smaller cages. All the mice were in a randomly fed state.

The mice were anaesthetized with Isofluran (Isoba-vet, Schering Plough, Denmark) in a Univentor 400 Anesthesia Unit apparatus (Univentor Limited, Sweden). The sternum was cut open, and the mice were euthanized by cardiac puncture. Blood was collected with a syringe connected to a tube containing an EDTA anticoagulant. The blood samples were then centrifuged at 2500 x g for 5 minutes at 4 °C to separate the plasma from red blood cells and stored at - 80 °C foregoing analysis.

Tissue and organ harvesting

Three adipose tissue depots were excised during the termination: Visceral epididymal white adipose tissue (eWAT), inguinal subcutaneous white adipose tissue (iWAT) and intrascapular brown adipose tissue (iBAT). Additionally, the liver and pancreas were excised. The tissue and organ samples were weighed, and divided into bags/cassettes. Tissue samples for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analyses were freeze clamped and stored at -80 °C, and samples for histology were fixed in 4 % formaldehyde.

2.2 ORAL GLUCOSE TOLERANCE TEST (OGTT)

An OGTT test was performed to evaluate glucose tolerance. Before the test, the mice were moved to clean cages and fasted for six hours. They were then given an oral dose of (1 g/kg lean mass) glucose, distributed with a gavage needle. A small incision was made at their lateral tail vein to collect blood, and the blood glucose levels were measured in a fasted state and after 15, 30, 60 and 120 minutes with an automatic glucometer (Contour ®NEXT, Bayer). Blood samples (20 µl) were collected for further insulin analysis at T0, 15 and 30 min. The blood samples were centrifuged at 2500 x g for 5 minutes at 4 °C and the plasma was subsequently stored at -80 °C. The mice's tails were sterilized with 70 % EtOH at the end of the test to prevent infection. Fasting blood glucose- and serum insulin levels from the OGTT were later used to calculate HOMA-IR with the following formula:

(*Fasting blood glucose (mmol/L) x fasting plasma insulin (mU/L)/22.5*)

2.3 INSULIN TOLERANCE TEST (ITT)

An ITT was performed to evaluate insulin response. The mice were moved to clean cages with no food available during the test and an intraperintonal injection of insulin (0.1 U/kg lean mass) was given to each mouse. An incision was made at their lateral tail vein and blood glucose levels were measured at T0, 15 min, 30 min, 45 min and 60 min. The mice's tails were sterilized at the end of the test.

2.4 ELISA INSULIN KIT

The Mouse Insulin ELISA kit (DRG Instruments, GmbH, Germany) was used for quantitative determination of insulin in mouse plasma. Reagents are listed in appendix II, table A.3.

The ELISA kit and samples were thawed, and 10 µl of the calibrators and serum samples were transferred into a 96 well microplate. Enzyme Conjugate 11x and Enzyme Conjugate Buffer was mixed, and 100 µl was added to each well. The plate was then incubated on a shaker for two hours, allowing the insulin in the samples to react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the wells. Furthermore, the plate was washed to remove unbound enzyme labeled antibodies. Two hundred µl substrate TMB was then added into each well, allowing the antibody-bound enzymes to convert the uncolored TMB into a colored product. The reaction was stopped by adding Stop Solution to each well. Conclusively, optical density was measured at 450 and 620 nm with a spectrophotometric plate reader (iEMS Reader MF, Labsystems, Helsinki). A more detailed description can be obtained from the DRG® Insulin (Mouse) ultrasensitive ELISA protocol (DRG 2013)

2.5 HISTOLOGY

2.5.1 Fixation with paraformaldehyde and phosphate buffer (PB)

Samples of adipose tissue (iWAT, eWAT and eBAT) were excised and placed in plastic cassettes. The tissue samples were then fixated in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight. The tissues were transferred to PB with a few drops of paraformaldehyde the next day, and stored for 4 days at 4 °C. Paraformaldehyde was added to the PB to prevent enzymatic and bacterial tissue degradation.

2.5.2 Dehydration with ethanol and xylene

The PB was replaced with gradually increasing concentrations of ethanol, as presented below in table 2.1. When the tissues were completely dehydrated in 100% ethanol, the ethanol was replaced with xylene. Replacing ethanol with xylene is imperative, as xylene is soluble in both paraffin and alcohol.

Table 2.1: *Reagents and time span of each step in the dehydration process.*

Reagent	Time
75 % EtOH	45 min
95 % EtOH	2 x 45min
100 % EtOH	3 x 45 min
Xylene	2 x 45 min
Paraffin	overnight
Paraffin	2 x 15 min

2.5.3 Paraffin infiltration and embedding

The casettes containing the tissues were stored in liquid paraffin (Histowax, Histolab products AB, Sweden) over night. The following day tissues were embedded in paraffin using an EC 350 Paraffin embedding center (Microtom International GmbH, Germany). Liquid paraffin was poured into a metal mold, and the tissue sample was placed in the center. Subsequently, the bed of the cassette was placed on top of the mold and filled with paraffin. The paraffin was then solidified in a freezer, and the solid block was finally removed from the mould.

2.5.4 Sectioning and staining

A microtome (Leica RM2156, Germany) was used to cut 3 µm sections of the embedded tissues. The sections were placed in dissected water and transferred to glass slides to dry overnight. In order to analyze the slides with a microscope, the sections were stained with a combination of dyes. The reagents and time span of each step is presented in table 2.2.

Hematoxylin was used to stain the cell nucleus and eosin was used to stain the cytoplasm. After staining, the sections were mounted with a xylene based mounting medium (Microscopy, Entellan, Germany) and left to dry overnight. Chemicals and reagents used in the histological methods are listed in appendix III table A.4.

Table 2.2: Reagents and time span of each step in the rehydration-staining-dehydration process.

Reagent	Time
Xylene	2 x 10 min
100 % EtOH	2 x 10 min
95 % EtOH	2 x 5 min
75 % EtOH	5 min
50 % EtOH	5 min
ddH2O	Wash
Hematoxylin	2 min
H2O	Wash
Eosin	30 sec
95 % EtOH	Wash
ddH2O	1 min
50 % EtOH	2 min
75 % EtOH	2 min
95 % EtOH	2 x 2 min
100 % EtOH	2 x 5 min
Xylene	2 x 5 min

2.5.5 Microscopy

The cell morphology of iWAT and iBAT from the different groups was examined by using an Olympus BX 51 binocular microscope. A representative field of the section was photographed with an Olympus DP50 3.0 camera.

2.6 MEAL TOLERANCE TEST (MTT)

A meal tolerance test (MTT) was performed on a third set of young normal-weight mice. The mice were transferred to individual cages and fasted for approximately 16 hours. In the following morning, the lateral tail vein was punctured and blood glucose concentrations were measured using a glycometer (Ascensia, COUNTOUR, USA). Furthermore, 20 µl fasting blood samples were collected from each mouse prior to the test. The mice were then given access to 0.15 grams of either the LF, HF/HP casein, HF/HP cod or HF/HP pork diets for approximately 15 minutes.

Blood glucose concentrations were measured immediately after the food was removed and then again 15, 30, 60 and 120 minutes after food ingestion. Furthermore, 20 µl of blood was collected from each mouse at T15, T30 and T60. At completion of the MTT, the mice were placed back into their original cages. The collected blood was later used to measure plasma insulin levels by enzyme-linked immunosorbent assay (ELISA).

2.7 AMINO ACID ANALYSES

Laboratory technicians at NIFES performed analyses of amino acids. Amino acids in the diets were detected using a method of HCl hydrolysis, derivatization by AccQ.TagTM Ultra Derivatization Kit (Waters, Kjeller, Norway) and finally analysis by UPLC and UV-detection (Waters Aquity, Kjeller, Norway). Free amino acids in plasma were measured using ninhydrin detection on the Biochrom 30+ instrument (Cambridge, UK).

2.8 STATISTICAL ANALYSES

2.8.1 Microsoft excel 2013

Microsoft excel was used to process raw data, and calculate the standard error of the mean (SEM).

2.8.2 Graph Pad Prism 6

Graph Pad Prism 6 was used to test the normality of the data by using a D'Agostino-Pearson normality test and outliers were identified with Grubb's test. A one-way analysis of variance (ANOVA) with multiple comparisons was used to analyse differences between the experimental groups. The reference groups were excluded from the statistical analyses. P values <0.05 were considered as statistically significant.

3. RESULTS

3.1 FEEDING EXPERIMENT 1

Previous studies from our group have demonstrated that high-protein diets with casein as the protein source reduce feed efficiency and body weight gain (Madsen, Pedersen et al. 2008; Ma, Liaset et al. 2011). In order to evaluate the effect of different proteins on diet-induced obesity, C57BL/6J mice were fed the experimental high-protein diets presented in table A.1 for 12 weeks.

3.1.1 Body mass gain and obesity development

The body mass development and total body weight gain after 12 weeks of experimental feeding is presented as means ± SEM in figure 3.1.

Body mass development
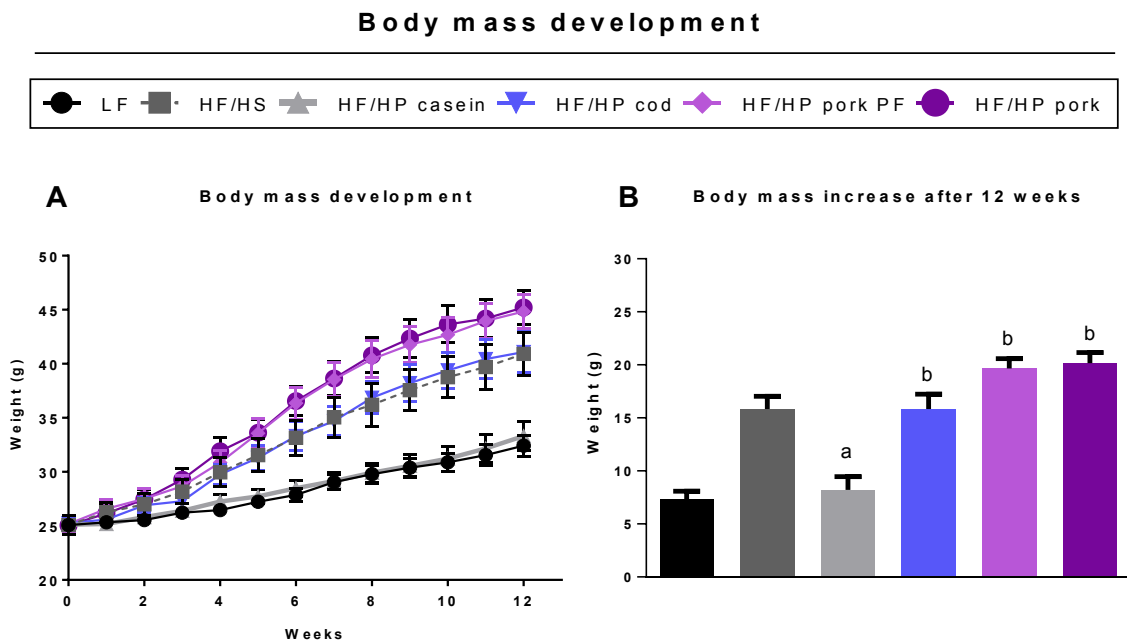
Figure 3.1: Body mass development (A) and weight gain (B) in groups (n=9) of C57BL/6J mice fed reference diets or HF/HP diets with casein, cod or pork as the protein source. **A:** Body mass development during the 12 weeks of experimental feeding. To compare the weekly body mass development, the data were analyzed with ANOVA repeated measures of the mean of each experimental group. **B:** Body mass increase after 12 weeks of experimental feeding. To compare the total body weight gain in the experimental groups, the data were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

As presented in figure 3.1 A, the different protein sources stimulated weight gain at different rates. After only 3 weeks of experimental feeding, the mice fed HF/HP casein had a significantly lower weight gain than the HF/HP pork group. Furthermore, ANOVA analysis at 5 weeks showed a significantly lower weight gain in the HF/HP casein group compared to both HF/HP groups fed pork. After 6 weeks of feeding, the casein-fed mice had a significantly lower weight gain than all the other experimental groups. In summary, the group fed proteins from pork had the highest weight gain, while cod protein had an intermediate effect. Casein was the only protein source that exerted a weight-stabilizing effect, which concurs with previous unpublished results from our group.

The total weight gain was of statistical significance in both pork fed groups and the group fed cod when compared to casein (figure 3.1 B). However, the differences in weight gain in the pair fed groups was not of statistical significance. In summary, the various protein sources had different effects on body weight development.

MRI scans of fat and lean mass

To determine if the weight gain was due to an increased amount of lean- or fat mass, the mice were MRI scanned prior to the feeding experiment and after 6 and 9 weeks of feeding. The results from the MRI scan in week 9 are presented in figure 3.2 as means ± SEM.

When casein was used as a protein source, the lean mass (fat-free body mass) was significantly higher than in both groups fed pork (Fig 3.2 A). However, there were no significant differences between the group fed cod protein and the other groups. As illustrated in figure 3.2 B, the group receiving casein as the protein source had significantly less fat mass than the groups fed pork or cod protein. A significant difference was also seen between the cod and both pork fed groups, with the pork groups having the highest fat mass. The data illustrates that protein source affect both lean and fat mass development, though the weight gain must be attributed to the increased fat mass.
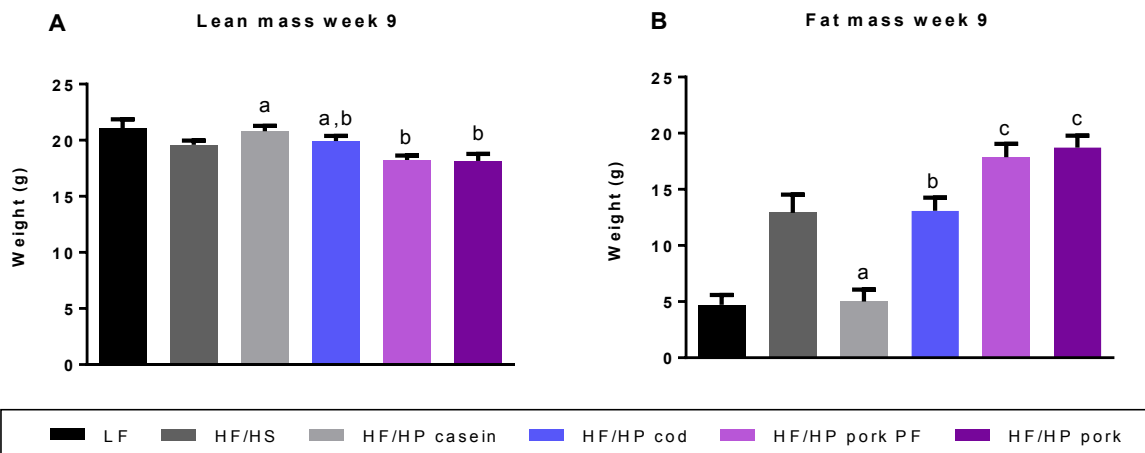
Figure 3.2: Lean mass (A) and fat mass (B) after 9 weeks on the experimental diets. **A and B:** Differences in lean- and fat mass were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

Adipose tissue depots

Three fat mass depots were examined by excising and weighing the adipose tissue depots during termination. Visceral (eWAT) and subcutaneous (iWAT) white adipose tissue was collected, in addition to brown adipose tissue (iBAT).

As presented in figure 3.3 A, the group fed casein had a significantly lower eWAT mass then both pork-fed groups and the group fed cod. The group fed cod protein had a lower eWAT mass than the groups fed pork, though it was not of statistical significance. The iWAT masses of casein-fed mice were also significantly lower than in the group fed cod, as well as both groups fed pork (Fig 3.3 B). Furthermore, a significant difference was seen between the group fed cod and the two pork-fed groups. When casein was used as a protein source, the iBAT weight was significantly lower than in the pork group (Fig 3.3 c). The iBAT masses of the groups fed cod or pork PF were not of statistical significance when compared to the other groups. In conclusion, the dietary proteins affected fat-deposition differently.
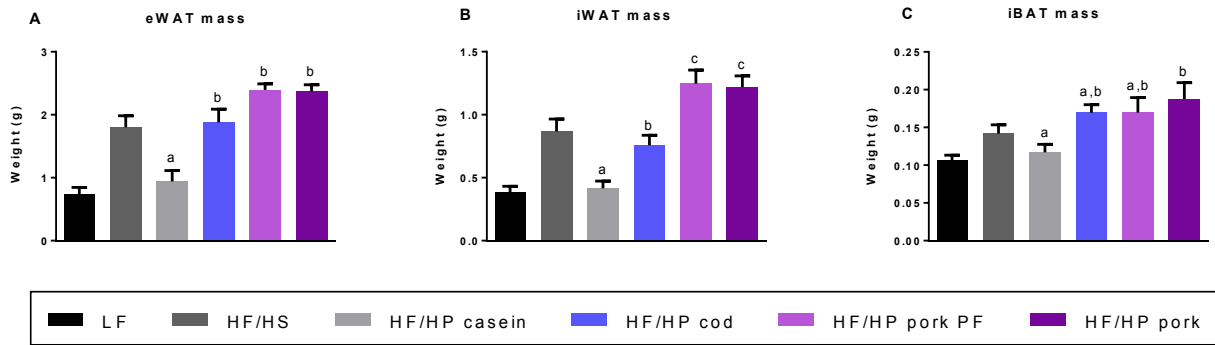
Figure 3.3: Masses of eWAT (A), iWAT (B) and iBAT (C) after 12 weeks on the experimental diets. **A, B and C**: Group variations in eWAT, iWAT and iBAT mass were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

Adipocyte size

The adipocyte size in one white (iWAT) and brown (iBAT) fat depot was examined by staining and photographing a representative part of a section.

The photographs illustrate that the casein-fed mice had the smallest white adipocytes compared to the hypertrophic cells seen in cod, and especially in pork. While the iBAT in the casein group mainly consists of multilocular adipocytes, the iBAT from the pork group solely consists of unilocular "white" adipocytes. Thus, the different protein sources also affected adipose tissue development at a cellular level.

Figure 3.4: Adipocyte morphometry in subcutaneous adipose tissue (iWAT) and intrascapular brown adipose tissue (iBAT) in mice fed casein, cod or pork as the protein source (magnified 40x).

Liver mass

To assess the effect of different proteins on other organ masses, the livers were excised during termination.

As presented in figure 3.5 the mice fed casein had a significantly lower liver mass compared to the cod-fed group. However, no significant differences were found in the two groups fed pork. Although the experimental diets affected liver mass differently, the variations do not constitute a major difference in total body weight gain.

The pancreas was also excised and weighed during termination, but the differences between groups were not statistically significant. The figure of pancreas masses is presented in appendix IV table A.5.
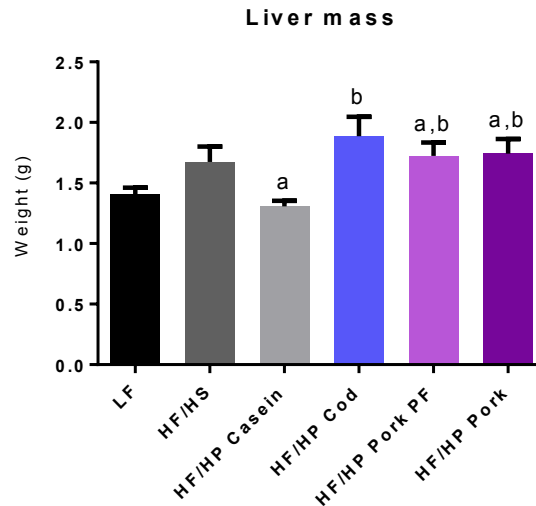
Figure 3.5: Liver masses in the different groups. Differences in liver mass were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

3.1.2 Glucose tolerance and insulin sensitivity

Oral glucose tolerance test (OGTT)

To evaluate the impact of dietary protein source on glucose tolerance, fasting mice were subjected to an oral glucose test (OGTT) after 10 weeks of experimental feeding. The results from the OGTT are presented in figure 3.6 as means ± SEM.

According to the OGTT data, glucose tolerance appears to be related to body weight in most groups (Fig 3.6). Before glucose administration, there were no significant differences between the experimental groups (Fig 3.6 C). All the groups peaked 15 minutes after glucose ingestion, but neither the measurements at 15 minutes (Fig 3.6 D) nor the calculated delta insulin (Fig 3.6 E) showed significant variations. A significant variation was observed after 60 minutes, where the casein group had a significantly lower blood glucose concentration than both pork groups. After 120 minutes all the groups had an equal or lower blood glucose concentration compared to their starting values. In summary, the pork groups had the most elevated glucose levels during the OGTT, while the casein group had the lowest. The mice in the cod group had intermediate blood glucose concentrations.
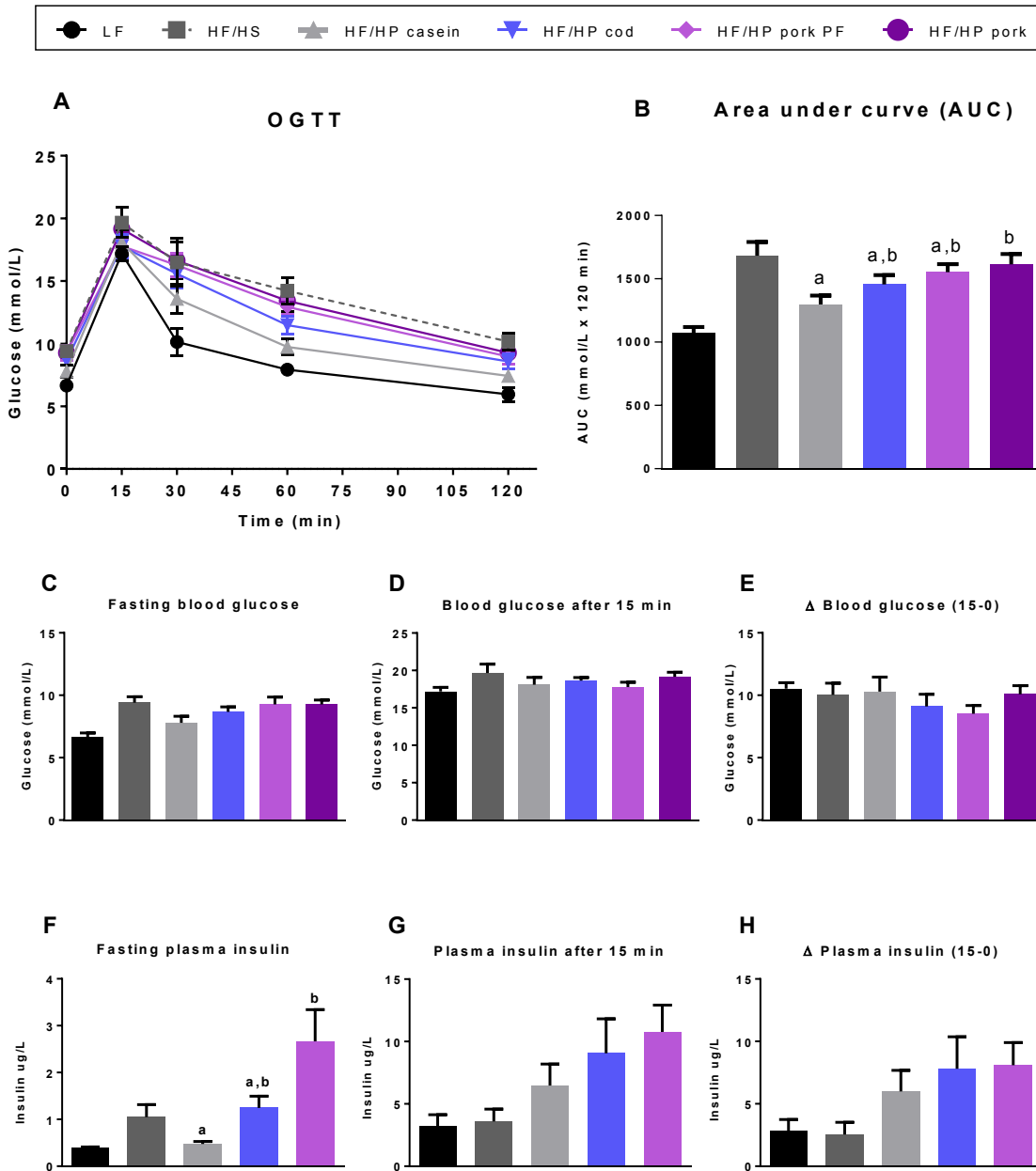
Figure 3.6: Oral glucose tolerance test (OGTT) on fasting mice (6 h) performed after 10 weeks on the experimental diets. **A**: Blood glucose levels during the OGTT. Blood glucose levels from T30-T120 were analyzed with ANOVA repeated measures with multiple comparisons of the mean of each experimental group. **B:** Calculated area under curve. **C:** Fasting blood glucose. **D:** Blood glucose after 15 minutes **E:** Delta blood glucose. **F:** Fasting plasma insulin. **G:** Glucose stimulated insulin secretion after 15 minutes. **H:** Delta insulin. **B-H:** Differences in blood glucose -and insulin levels were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

A one-way ANOVA analysis of the glucose AUC showed that the group fed casein had a significantly better glucose clearance compared to the pork group (Fig 3.6 B). The pork PF group and the group fed cod also had an elevated AUC compared to casein, but neither were significant.

The insulin secretion during the OGTT was measured in fasting mice, after 15 minutes and the delta insulin was calculated (Fig 3.6 F-H). Fasting insulin levels were significantly elevated in the pork PF group compared to the casein-fed group, while the cod group had an intermediate insulin level (Fig 3.6 F). Furthermore, figure 3.6 G illustrates that the glucose stimulated insulin secretion (GSIS) after 15 minutes was elevated in the cod group and pork PF group, but none of the variations between the groups were significant. The delta insulin levels revealed that the group fed pork had secreted most insulin from 0-15 minutes, but no significant results were obtained (Fig 3.6 H).

Insulin tolerance test (ITT)

An insulin tolerance test was performed after 11 weeks of experimental feeding to explore the connection between different proteins and the development of insulin-resistance. The results from the ITT are presented in figure 3.7 as means ± SEM.
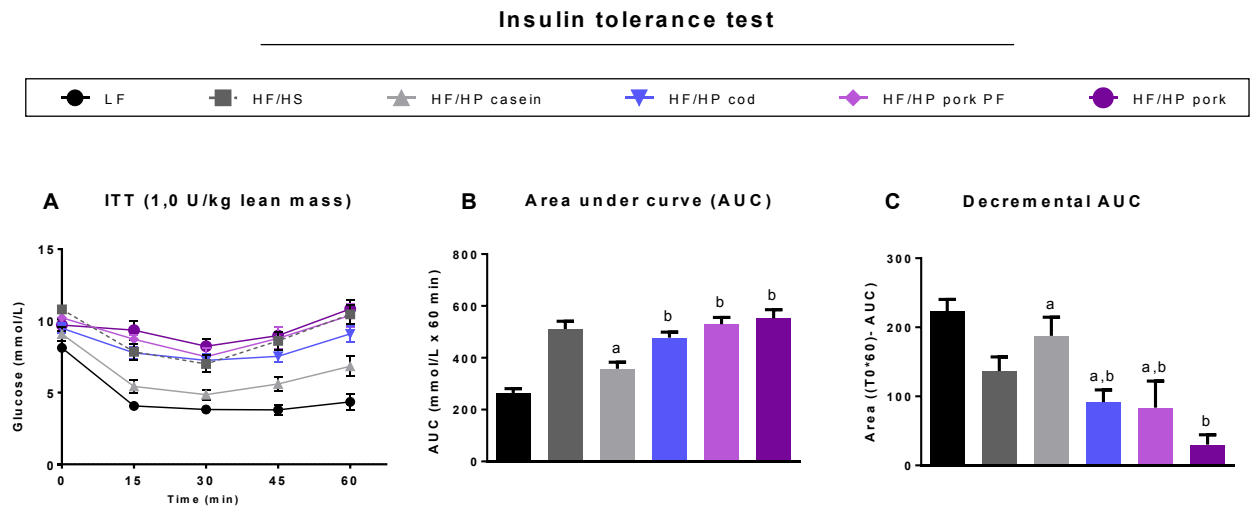
Figure 3.7: Insulin tolerance test (ITT) performed after 11 weeks of feeding. **A**: Blood glucose levels during the ITT. Blood glucose levels from T0-T60 were analyzed with ANOVA repeated measures with multiple comparisons of the mean of each experimental group **B:** Calculated area under curve (mmol/L x 60 min). **C:** Calculated decremental AUC ((T0*60) - AUC). **B and C:** The AUC and decremental AUC were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

The results from the insulin tolerance test (ITT) showed an association between a high body weight and a lowered insulin sensitivity (Fig 3.7). There were no significant differences in blood glucose levels at the start of the test, but the group fed casein had significantly lower blood glucose levels then the other experimental groups at 15 and 30 minutes after insulin injection. Further ANOVA analyses at 45 and 60 minutes showed that the casein-fed group had significantly lower blood glucose levels then both groups fed pork.

The calculated AUC revealed that the casein group had a significantly better glucose uptake in response to insulin compared to the other groups (Fig 3.7 B). There were no significant variations between the other groups, although the groups fed pork had a slightly higher AUC then the cod-fed group.

By calculating decremental AUC (DAUC) and thereby eliminating the differences in fasting glucose levels, the group variations became more prominent (Fig 3.7 B). The mice fed casein had a significantly higher drop in blood glucose in response to the insulin than the pork group, while the cod- and pork PF groups had no significant variations.

HOMA-IR

To further evaluate insulin sensitivity, a homeostasis model assessment – insulin resistance (HOMA-IR) index was calculated. A high HOMA-IR score is correlated with insulin resistance or type 2 diabetes. The calculated HOMA-IR scores are presented in figure 3.8 as mean ± SEM.
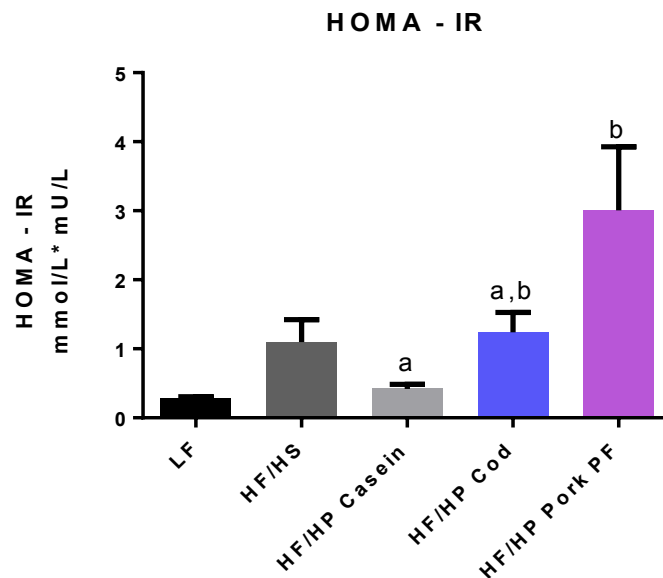
Figure 3.8: Calculated HOMA-IR scores. The HOMA-IR scores were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

As presented in figure 3.8 the mice fed casein had a significantly lower HOMA-IR score compared to the pork PF group. The cod-fed group had a lower HOMA-IR score compared with the pork PF group, but it did not reach statistical significance. These findings indicate that the protein types exert different insulinotropic effects.

Meal tolerance test

A meal tolerance test (MTT) was performed on young mice with a normal weight to further investigate what effects the experimental diets exert on insulin secretion. The blood glucose levels and calculated AUCs are presented in figure 3.9 as means ± SEM.
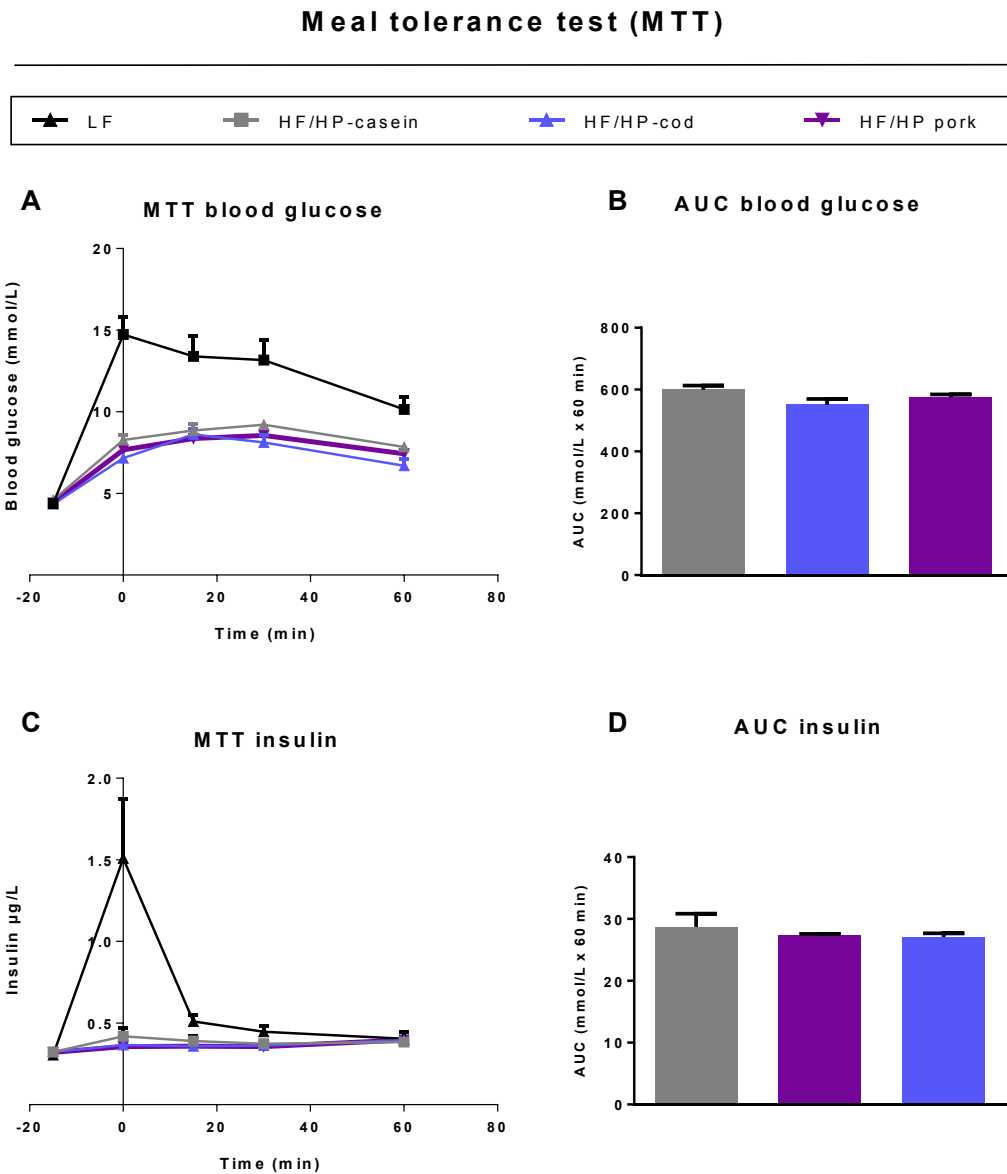
Figure 3.9: Meal tolerance test (MTT). A: Blood glucose curve from the MTT. B: Blood glucose AUC. C: Insulin curve from the MTT. D: Insulin AUC. The results are presented as mean ± SEM.

As presented in figure 3.9, the MTT did not provide any significant results when the experimental groups were compared.

3.1.3 Energy intake and feed efficiency

To investigate if the differences in body weight was due to a varied feed intake, the total calorie intake was calculated. Additionally, feed efficiency was calculated to determine the obesogenic effect of the different proteins.

Energy intake and feed efficiency
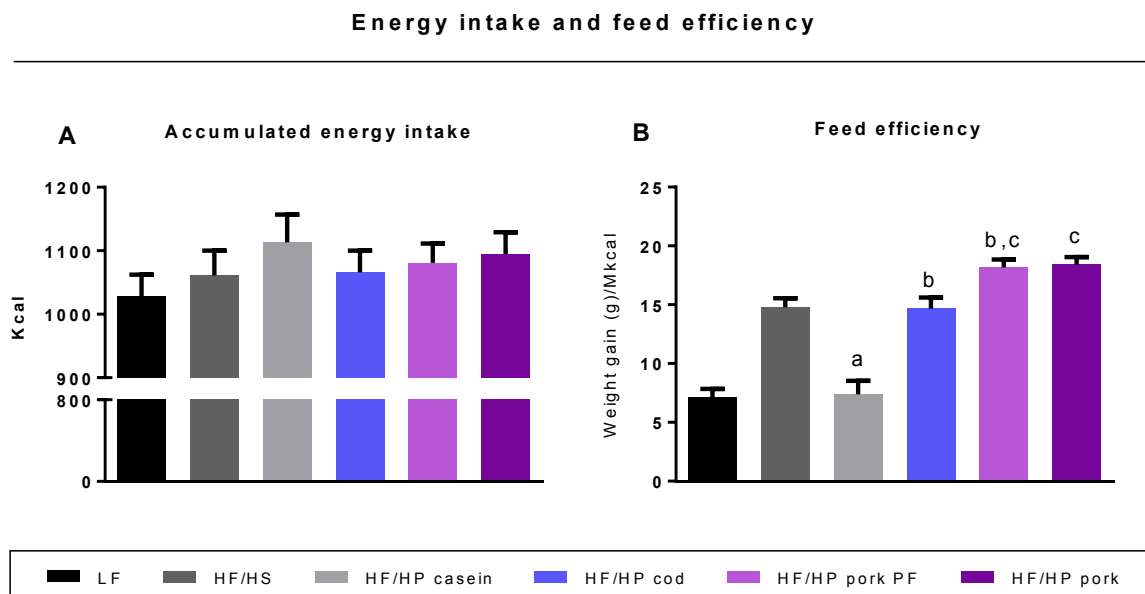
Figure 3.10: Energy intake after 12 weeks of feeding (A) and feed efficiency (B). **B:** Differences in feed efficiency were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

Differences in feed intake were not significant, though the group fed casein had a slightly higher calorie intake than the other groups (Fig 3.10 A). The two pair-fed groups, cod and pork PF, had a variation of 15 kcal in energy intake after 12 weeks of feeding. In contrast to the energy intake, the group fed casein had significantly lower feed efficiency compared to the other groups (Fig 3.10 B). Furthermore, the cod group had a significantly lower feed efficiency then the pork group. However, there were no significant differences between the pair-fed groups.

Apparent fat digestibility

To evaluate if the observed weight gain was caused by less fat excretion, feces were collected and their fat content was analyzed.
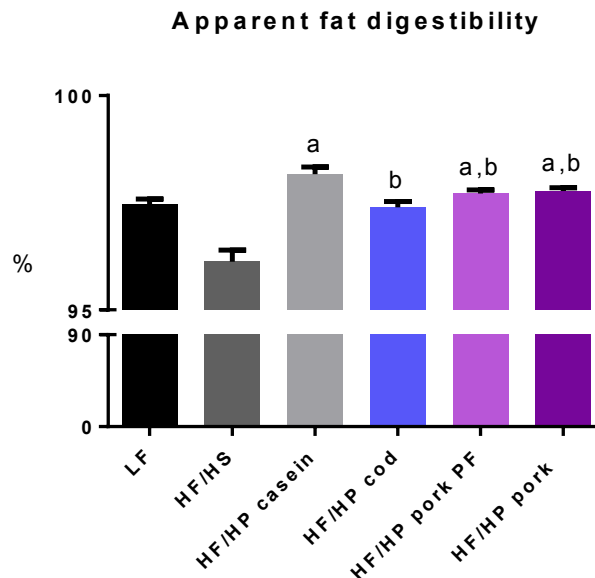
Figure 3.11: Apparent fat digestibility. Differences in apparent fat digestibility were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

Interestingly, we observed that the casein fed group had digested significantly more fat then the group fed cod (Fig 3.11). The pork-fed groups had an intermediate fat digestibility, but was not statistically different compared to the other groups.

3.1.4 Plasma analyses

Free amino acids in plasma

To uncover group variations in the free amino acid pool, non-fasting mouse plasma samples were sent for analysis. Both amount and types of free amino acids may be of importance in the development of obesity, due to their different effects on satiety and energy expenditure.

Free amino acids in non-fasting mouse plasma (µmol AA/100 ml plasma)
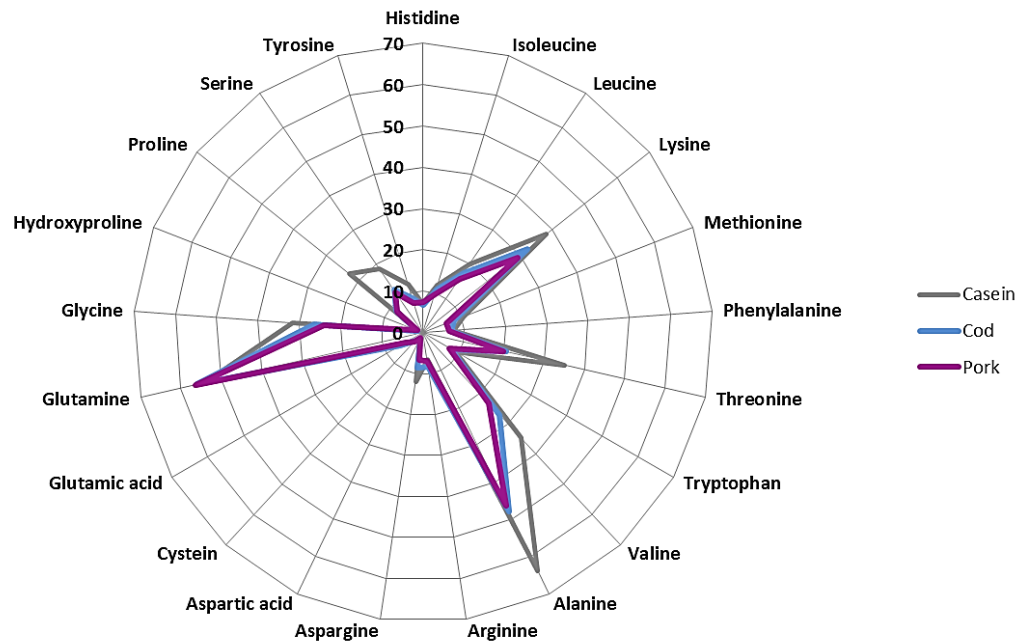
Figure 3.12: Free amino acids and in non-fasting mouse plasma (µmolAA/100 ml plasma) in the HF/HP casein, cod and pork fed groups.

As presented in figure 3.12 the total amount of free amino acids (AAs) was highest in the group fed casein, including the branched-chained amino acids (BCAAs). The cod and pork groups had a quite similar content of free AAs, but lower values of the BCAAs and several non-essential AAs compared to casein. For a more detailed overview, the AA values in plasma are listed in appendix V table A.6. Additional analyses of AAs in the experimental diets and protein sources are presented in appendix V figure A.7 and A.8, respectively.

Urea and taurine levels in plasma

As presented in figure 3.13 A, the group fed casein had a significantly higher amount of urea in plasma compared to the group fed cod and the pork group. When cod was used as a control group, ANOVA analysis showed a significantly higher level of taurine in the plasma of cod-fed mice compared to the other groups (Fig 3.13 B).
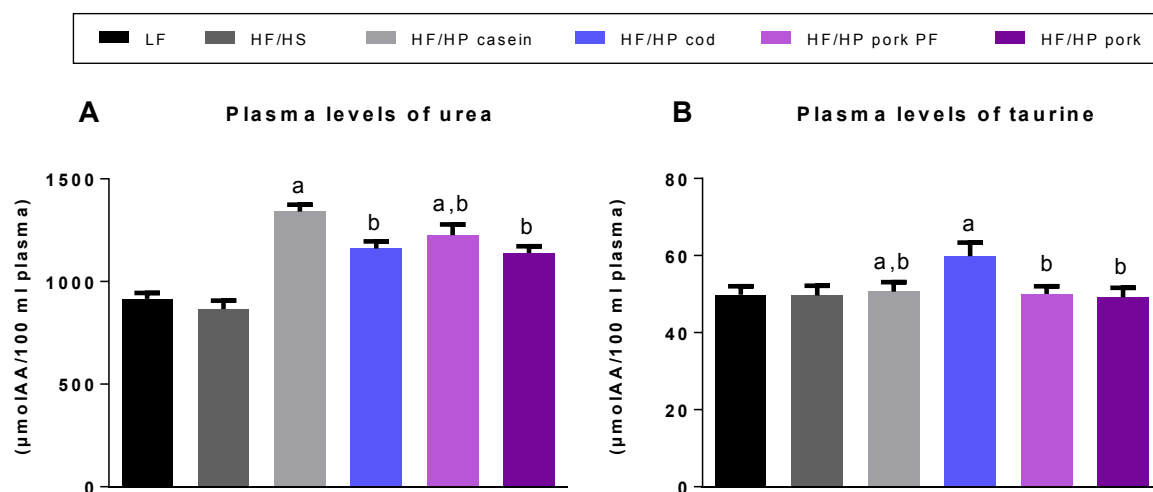
Figure 3.13: Plasma levels of urea (A) and taurine (B) in non-fasting mouse plasma (μmolAA/100 ml plasma).**A:** Differences in plasma levels of urea were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. **B:** Differences in plasma levels of taurine in the experimental groups were analyzed using a one-way ANOVA test with the cod as the control group. Different letters denote statistical significance ($p < 0.05$) compared to the cod group. The results are presented as mean ± SEM.

3.2 FEEDING EXPERIMENT 2

To evaluate the effect of different proteins on weight reduction, diet induced obese (DIO) C57BL/6J mice were given calorie restricted (30%) low fat diets with casein, cod or pork as the protein source. Results from this experiment are also presented in Nina Norberg's master thesis.

3.2.1 Body mass development and weight reduction

The body mass development and body mass reduction after 5 weeks of experimental feeding is presented in figure 3.14 as means ± SEM.
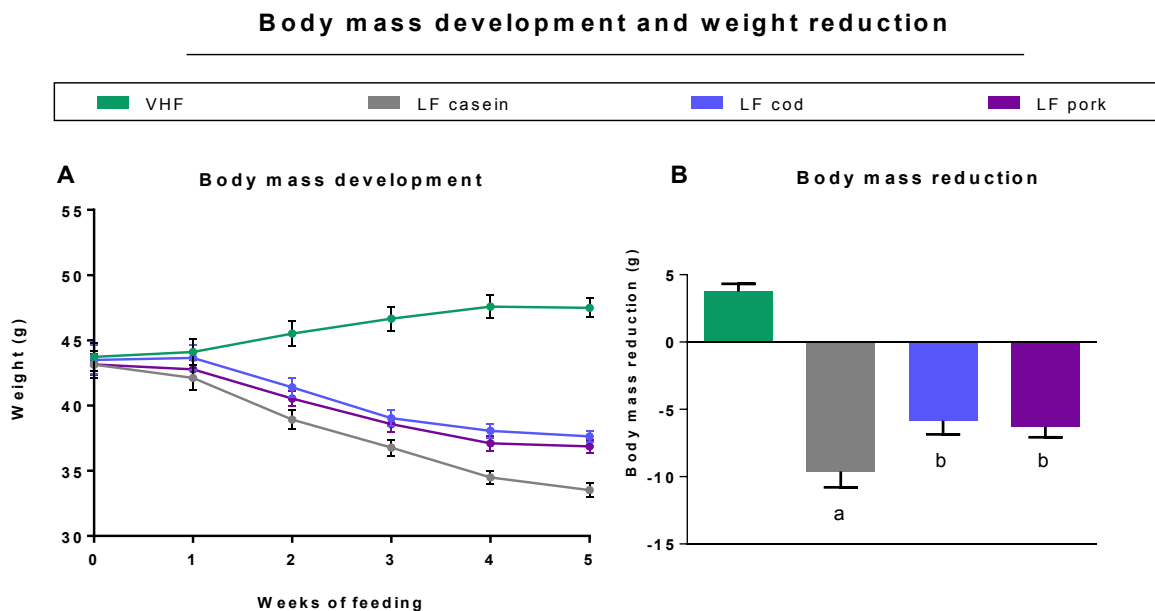
Figure 3.14: Body mass development (A) and body weight reduction (B) in groups (n=13) of C57BL/6J mice fed LF diets with casein, cod or pork as the protein source for 5 weeks. **B:** Differences in body weight reduction were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

As presented in figure 3.14 A, the proteins exerted various effects on body mass development. After five weeks of experimental feeding, the casein-fed group had lost significantly more body mass then the other experimental groups (Fig 3.14 B).

MRI scans of fat and lean mass

To determine if the weight loss was due to a decreased amount of lean- or fat mass, the mice were MRI scanned prior to the diet intervention and after 5 weeks of experimental feeding. The differences in lean-and fat mass from week 0-5 are presented in figure 3.15 as means ± SEM.
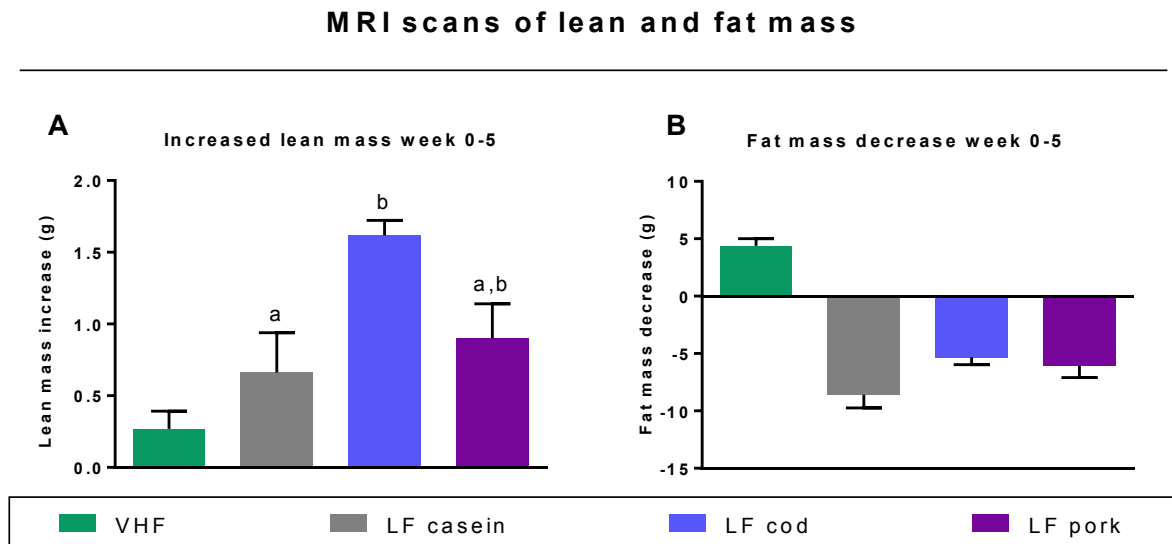
Figure 3.15: Changes in lean- (A) and fat mass (B) from week 0-5.**A and B:** Differences in lean-and fat mass were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p<0.05$) between the mean of the groups. The results are presented as mean ± SEM.

As illustrated in figure 3.15 A, the cod-fed mice had a significant increase in lean mass compared to casein after 5 weeks of experimental feeding. The casein-fed group had the greatest reduction in fat mass after 5 weeks of experimental feeding, but differences between the groups did not reach statistical significance (Fig 3.15 B).

3.2.2 Glucose tolerance and insulin sensitivity

Oral glucose tolerance test (OGTT)

To evaluate if the different proteins improved glucose tolerance, fasting mice were subjected to an oral glucose test (OGTT) after 5 weeks of experimental feeding. The results from the OGTT are presented in figure 3.16 as means ± SEM.
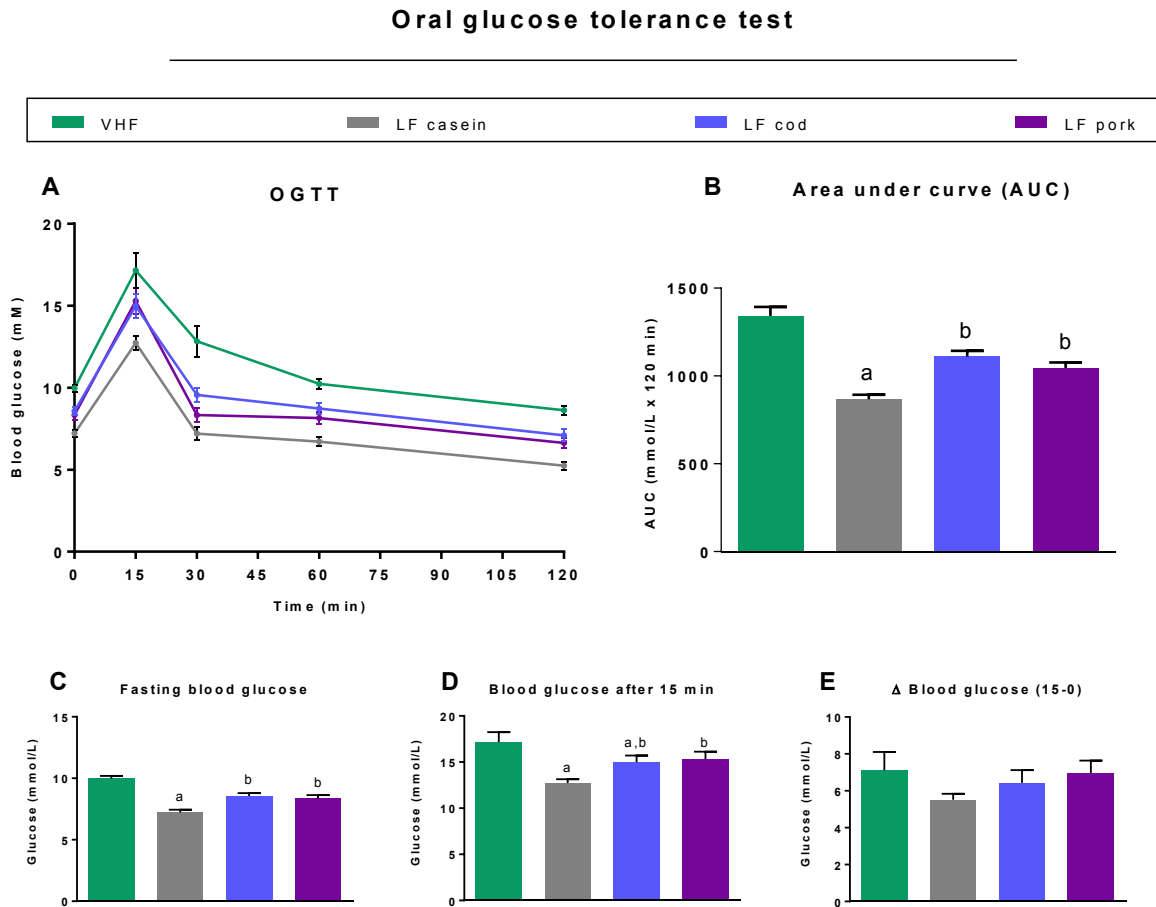
Figure 3.16: Oral glucose tolerance performed on fasting mice (6 h) after 5 weeks of experimental feeding. **A**: Blood glucose levels during the OGTT. **B:** Calculated area under curve. **C:** Fasting blood glucose. **D:** Blood glucose after 15 minutes **E**: Delta blood glucose. Differences in blood glucose levels and AUC were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each experimental group. Different letters denote statistical significance ($p<0.05$) between the mean of the groups. The results are presented as mean ± SEM.

According to the OGTT results, the amount of weight loss appears to correlate with an improved glucose tolerance (Fig 3.16). Fasting blood glucose levels were significantly lower in the group fed casein compared to the other LF groups (Fig 3.16 C), and measurements after 15 minutes were significantly lower in the casein-fed mice compared to the group fed pork (Fig 3.16 D).

The delta blood glucose revealed no significant variations (Fig 3.16 E), while the calculated AUC was significantly lower in the casein group compared to the other experimental groups (Fig 3.16 B). Notably, all experimental groups had an improved glucose tolerance after 5 weeks of experimental feeding.

Insulin tolerance test (ITT)

To evaluate if the different protein sources improved insulin sensitivity, the mice were subjected to an insulin tolerance test after 4 weeks of experimental feeding. The results from the ITT are presented in figure 3.17 as means ± SEM.
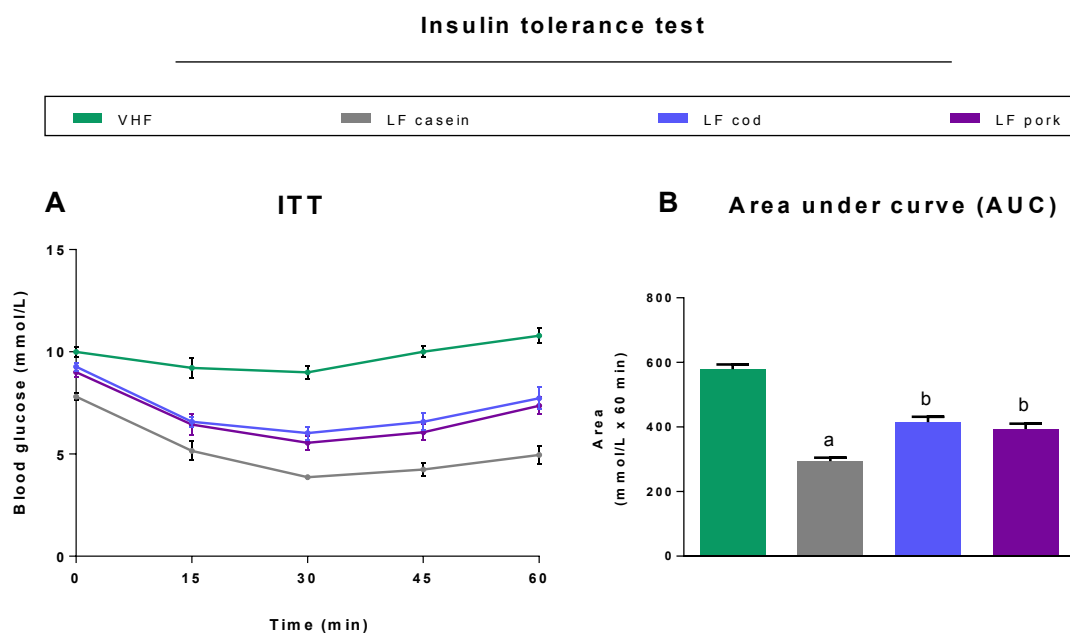
Figure 3.17: Insulin tolerance test (ITT) performed after 4 weeks of experimental feeding. **A:** Blood glucose levels during the ITT. **B:** Calculated AUC. Differences in AUC were analyzed using a one-way ANOVA test with multiple comparisons of the mean of each group. Different letters denote statistical significance (p<0.05) between the mean of the groups. The results are presented as mean ± SEM.

As presented in figure 3.17, the amount of weight loss correlated with an improved insulin sensitivity. The calculated AUC was significantly lower in the casein-fed mice compared to the other LF groups. In summary, all the experimental groups had become more insulin-sensitive after 4 weeks of feeding.

4. DISCUSSION

Numerous animal studies have demonstrated that a high proportion of dietary casein abrogates the obesogenic effect of fat (Madsen, Pedersen et al. 2008; Ma, Liaset et al. 2011; Lillefosse, Tastesen et al. 2013). An unpublished study from our group, which aimed to investigate if a high amount of other protein sources could induce the same weight-regulating effect as casein in a HF/HP diet, discovered that neither of the other proteins tested were able to protect against diet-induced obesity. In fact, proteins from pork promoted obesity development while proteins from cod had an intermediate effect. Unpublished studies from our group have also registered a lower feed intake in mice fed cod protein compared to mice fed pork, which raises a question whether cod promotes satiety in a higher degree than pork. Based on these observations we found it interesting to investigate if the higher satiating effect of cod protein affected energy intake, and then eliminate the eventual satiating variations by pair-feeding mice fed cod and mice fed pork. Furthermore, we found it compelling to explore the weight-reducing effects of the same protein sources on diet induced obese (DIO) mice. Our present studies demonstrate that the dietary protein source is an important factor in both high-and regular-protein diets.

4.1 A HIGH PROPORTION OF DIETARY CASEIN ATTENUATES THE OBESOGENIC EFFECT OF A HIGH-FAT DIET

High protein diets have been presented as a beneficiary method for maintaining a stable body weight and attenuate obesity development, but the focus on different dietary protein sources have been scarce. Here we report how various dietary proteins exert different effects on body mass and metabolism in mice fed a HF/HP diet. In concurrence with earlier publications from our group, we found that casein was the only protein source that attenuated obesity development in a HF/HP diet. Furthermore, the mice that were fed diets with pork as the protein source developed obesity and gained a significant amount of fat mass, while cod protein exercised an intermediate obesogenic effect. Hence, we demonstrate that the source of dietary protein is of great importance.

4.1.1 The effect of different protein sources on satiety

High-protein diets have been known to induce a greater appetite suppressing effect than diets high in fat or carbohydrates, but there is little evidence regarding the satiating effects of specific proteins. In the present study we investigated if the increased weight gain observed in mice fed pork was due to a lowered satiety and thereby increased energy intake, by pair-feeding the group fed cod and one of the groups fed pork. A former study has demonstrated that fish proteins have a higher satiating effect than beef and chicken, while our group previously observed a higher energy intake in mice fed pork compared to mice fed cod (Uhe, Collier et al. 1992). In contrast to these observations we found that the pair-fed pork group needed almost no energy restriction to match the energy intake in the group fed cod. Additionally, we observed that the *ad libitum* fed pork group only consumed 30 kcal more than the group fed cod during the 12 weeks of feeding. These findings are in agreement with studies showing that the satiating effects of different proteins may be superseded in high protein diets, due to the high satiating power of a large protein intake (Bowen, Noakes et al. 2006; Bowen, Noakes et al. 2006). Thus, our results imply that the obesogenic effect of pork protein in this study cannot be attributed to a lower satiating effect.

Another interesting observation in our study was that the mice fed casein had the highest energy intake of all the experimental groups, despite having the lowest body weight. By analyzing non-fasting plasma samples we observed that cod- and pork-fed mice had a similar amount of free AAs in plasma, whilst the casein fed mice had higher amounts of circulating AAs, including the BCAAs. Circulating levels of AAs, especially the BCAA leucine, has been described as a potent satiety stimulator via its regulatory effects on leptin secretion and hypothalamic mTOR signaling (Cota, Proulx et al. 2006). However, these mechanisms did not seem to reduce the energy intake in our casein-fed mice. A plausible explanation for the variations in energy intake observed in our group may be the inconsistent use of protein sources and the different textures of the experimental diets. While we used minced cod fillet in the present study, previous studies have used cod fish powder. These are factors that influence taste and texture, and quite possibly the energy intake.

4.1.2 The effect of different protein sources on energy expenditure

Although we observed no significant differences in energy intake between the experimental groups, the differences in feed efficiency were very pronounced. Interestingly, we discovered that the mice fed casein had a significantly lower feed efficiency than the other groups as well as the highest energy intake. Additionally, we observed a significantly lower energy efficiency in the group fed cod compared to the pork-fed group. These differences in in feed efficiency may be related to the impact of different proteins on energy expenditure through various metabolic processes.

Casein differs from the other protein sources with its high content of the BCAA leucine, which is known to exert an anabolic effect on skeletal muscle and thereby increase protein turnover (Norton, Layman et al. 2009). The significantly higher amount of lean mass in our casein-fed mice imply that they have an elevated protein turnover compared to the other experimental groups, which can be related to their higher energy intake. Furthermore, by analyzing non-fasting plasma samples we detected significantly higher levels of urea in plasma from mice fed casein, which might suggest that they have a more effective nitrogen excretion. An elevated ureagenesis can be advantageous as it leads to an increased amount of carbon skeletons available for *de novo* synthesis of glucose via gluconeogenesis, which in turn promotes glucose homeostasis. In addition to the BCAAs and urea, we found the highest level of tyrosine in the plasma of casein-fed mice. Tyrosine is known as a precursor to norepinephrine, which may enhance energy expenditure through β-adrenergic stimulation and expression of the *Ucp1* gene in WAT (Cannon and Nedergaard 2004). Unfortunately we did not get to measure mRNA levels of *Ucp1* in our study due to unforeseen complications, but earlier studies from our group have detected increased expression of *Ucp1* in iWAT of mice fed HF/HP diets (Madsen, Pedersen et al. 2008; Ma, Liaset et al. 2011).

The difference in energy efficiency between cod -and pork-fed groups cannot be explained by the BCAA or urea content, but we speculate if the higher levels of arginine found in plasma of mice fed cod could partly account for their lower body weight. In support of this argument, two studies have demonstrated that dietary supplementation of arginine successfully reduced body weight and fat mass in rats (Fu, Haynes et al. 2005; Jobgen, Meininger et al. 2009). Additionally, a recent study showed that 4 weeks of fish protein supplementation increased lean mass and reduced fat mass in human subjects (Vikoren, Nygard et al. 2013). Nevertheless, the effect of specific proteins and AAs on energy expenditure is a field that requires more research, and we can merely speculate if their properties can exert any significant effects.

Another factor that may influence energy expenditure is the possibility of obesity-induced whitening in BAT by adipocyte transdifferentiation or *de novo* synthesis of precursor cells. Sectioning and staining of BAT revealed that the casein-fed mice had a normal BAT morphology mostly consisting of multilocular adipocytes, while the BAT in the pork group had the same appearance as WAT. The BAT from mice fed cod had an intermediate morphology between that of BAT and WAT, mostly consisting of unilocular adipocytes. We found these variations surprising, considering that our animal experiment was carried out in thermoneutral surroundings. Studies have shown that a positive energy balance causes an expansion of the existing WAT which promotes either a direct transformation of heat-producing brown adipocytes into white adipocytes (Cinti, Frederich et al. 1997) or synthesis of white adipocytes from precursor cells (Bachman, Dhillon et al. 2002). However, the literature is mainly focused on induced browning in WAT, while little emphasis is put on the mechanisms leading to the whitening of BAT in mice exempt from thermal stress. One study addressed this topic and demonstrated that *Ucp1* ablation solely induced obesity development and abolished DIT in mice housed in a thermoneutral environment (Feldmann, Golozoubova et al. 2009). The underlying mechanisms of BAT-whitening may be partly elucidated by the lowered sympathetic activity observed in obese men and mice, but this topic is clearly in need of further exploration. In conclusion, we cannot be certain if the whitening of BAT had any negative influence on energy expenditure in the current study, but we observed a pattern between increased adiposity and the degree of whitening in BAT.

4.1.3 The effect of different protein sources on fat absorption

An additional factor that may account for some of the differences in feed efficiency is the percentage of digested and excreted fat. Our calculations of apparent fat digestibility (AFD) showed that the group fed cod had digested a significantly lower amount of fat than the group fed casein. The lowered fat digestibility in cod was not significantly different compared to the pork PF group, but an increased fat excretion may partly account for their differences in body weight. It has been hypothesized that the different AA compositions of the protein sources may influence fat absorption, but the literature presents meager evidence. A study by Murakami and colleagues proposed that a long-term supplementation of taurine increased bile secretion and thereby fat absorption in C57BL/6J mice (Murakami, Kondo et al. 2000), while human studies have observed a decreased fatty acid excretion in children with cystic fibrosis given taurine supplements (Smith, Lacaille et al. 1991). These findings are clearly contradicting to our observations, where the cod-fed mice had the lowest fat absorption in addition to the highest levels of taurine. In conclusion, the lower AFD in mice fed cod may partly explain the differences in energy efficiency observed between them and the groups fed pork, whereas the higher AFD in mice fed casein appears to be contradicting.

4.1.4 The effect of different protein sources on glucose tolerance and insulin sensitivity

Glucose intolerance, insulin resistance and obesity is so closely related that researchers in the 1970s invented the term "diabesity" (Sims, Danforth et al. 1973). In concurrence with this term, we observed that an impaired glucose tolerance and reduced insulin sensitivity was directly related to increased adiposity. The results from the OGTT demonstrated that the mice fed casein had a significantly lower AUC than the pork group, as well as a significantly lower blood glucose 60 minutes after glucose administration. Additionally, fasting insulin levels were significantly lower in the group fed casein compared to the pork PF group. This implies that the mice fed pork have an impaired glucose tolerance, and that the lower blood glucose levels observed in casein-fed mice is not due to an increased insulin-secretion. In the present study, we based the glucose dosage on lean mass weight instead of BW, given that lean mass is the main target for glucose uptake. This method is believed to provide more robust results, especially when there are great group variations in lean mass.

High blood glucose levels are often related to high insulin levels. To explore if this connection was relatable to our study, an ITT where the mice were given 1.0 U/kg lean mass was performed. In direct relation to the OGTT, we found that the casein-fed group had a significantly lower AUC than the other groups. Furthermore, calculation of DAUC illustrated that the mice fed casein had a significantly better glucose uptake in response to insulin than the pork PF group, while the cod-fed group and pork PF group had intermediate responses. The results from the OGTT and ITT could then be translated into a HOMA-IR index, which was significantly higher in the pork PF group compared to the group fed casein.

To further evaluate the insulinotropic properties of the different protein sources, we performed an MTT on young normal-weight mice who had been fasted overnight. Unfortunately, the MTT did not produce any significant results. A study by Andrikopoulos and colleagues evaluated the glucose tolerance test in mice, and demonstrated that a low glucose dosage (0.5-1 g/kg) generated an insufficient response in blood glucose levels (Andrikopoulos, Blair et al. 2008). We therefore speculate if the amount of food given at the test (0.15 g) was too low to produce an adequate response.

In addition to the effects of excess fat mass, certain AAs have been known to exert various insulinotropic effects. We contemplate if the higher levels of the BCAAs in non-fasting plasma may be related to the insulin-sensitive properties observed in the mice fed casein. Both human and rat studies have demonstrated that leucine and isoleucine improve blood glucose clearance and uptake in skeletal muscle in acute-response studies (Doi, Yamaoka et al. 2007; Kalogeropoulou, Lafave et al. 2008). While others argue that leucine and the BCAAs actually promote insulin resistance and hyperinsulinemia, this theory is based on fasting levels of BCAAs in plasma. In relation to our study, we only observed that higher levels of BCAAs in non-fasting plasma improved glucose tolerance. In relation to the differences in glucose tolerance we observed in the mice fed cod and pork, we contemplate if the organic acid taurine may exert any regulatory effects. There is a possible connection between the significantly higher levels of taurine in the plasma of mice fed cod and an improved insulin-sensitivity. Previous rat studies have demonstrated a positive correlation between taurine and an improvement of diabetes (Nakaya, Minami et al. 2000; Nandhini, Thirunavukkarasu et al. 2005).

Collectively, the data indicate that the mice fed casein have a normal response to insulin and glucose, while the mice fed pork have an impaired glucose tolerance. Furthermore, these results are consistent with body mass development and the morphological differences we observed in the adipose tissue.

4.2 CASEIN PROMOTES WEIGHT LOSS IN OBESE MICE FED A LOW FAT DIET

LF diets have been a popular dietary method for reducing calorie intake, and thereby reduce body weight. Several studies have reported positive effects on weight loss and glucose tolerance when diet-induced obese (DIO) mice are switched from a HF to a calorie restricted LF diet, but the role of dietary protein source has not been sufficiently explored (Parekh, Petro et al. 1998; Shi, Akunuru et al. 2009). Here we report how various proteins exert different effects on weight loss and glucose tolerance in DIO mice.

We found that proteins from casein produced a significantly greater body mass reduction compared to pork and cod proteins, and it was the most efficient protein source in terms of fat-mass reduction. However, a 30% calorie restriction was necessary to promote an adequate weight loss. When the mice were fed LF diets *ad libitum* in the first week of the trial, we observed a marginal weight reduction and differences between the groups were undefined. This observation can be related to the more gluttonous behavior observed in animals fed diets with a moderate protein content, which emphasizes the necessity of calorie restriction in the present study (Stock 1999).

Interestingly, we observed that mice fed the LF cod diet had a significantly higher increase in lean mass (fat-free body mass) then the casein-fed mice after 5 weeks of experimental feeding. An increase in lean mass after fish protein supplementation was also observed in a human study with overweight candidates ; however, calorie restriction was not enforced (Vikoren, Nygard et al. 2013). Another interesting discovery was that all the groups fed LF diets gained more lean mass compared to the VHF reference group, despite having a much lower dietary energy- and protein content.

Amelioration of glucose tolerance and insulin sensitivity

The DIO mice were subjected to an OGTT and ITT before and after the diet intervention to examine the dietary effect on glucose tolerance and insulin sensitivity. We found that the DIO mice had a lowered glucose tolerance before the intervention, in addition to insulin-resistance. Remarkably, these parameters were radically altered after 4 weeks of experimental feeding. The casein fed mice had a significantly lower glucose and insulin AUC compared to the other groups, which could be mediated by the hypothesized insulin-sensitizing effect of the BCAAs. In support of this finding, a study by Kawaguchi and colleagues demonstrated that daily supplementation of BCAAs improved insulin resistance in men with chronic liver disease (Kawaguchi, Nagao et al. 2008). Compared to our previous HF/HP study the mice fed LF casein also had the best glucose tolerance, despite being fed a lower amount of dietary protein. Surprisingly, the group fed cod had the highest AUC in both the ITT and OGTT tests in the current study, suggesting that a lower amount of cod protein might not produce the same effects as the high amount in the HP diet. In conclusion, the positive effects of casein might be less dose-dependent than the effects of cod protein.

4.3 THE ANIMAL MODEL AND RELAVANCE TO HUMANS

Mice are the most commonly used animal models in nutritional research, due to their genetic and physiological similarities to humans. However, it is essential to consider that mice used for research purposes mostly are inbred strains with less genetic variance than humans. Hence, it is not reasonable to interpret effects in a specific strain as general, as it may be a unique feature for the actual strain. The C57BL/6J mice model used in these feeding experiments is characterized by its ability to develop DIO, hyperinsulinemia and hyperglycemia when fed a HF diet. Thus, this specific strain provides an excellent model to study the pathophysiology of an obesity syndrome quite similar to human obesity.

The Norwegian health authorities give specific advice on the most beneficial types of fat and carbohydrates to consume, but their focus on protein sources has been limited. In the present study we have demonstrated that the choice of dietary protein source also is an important factor in the development of obesity. The obesogenic effect of pork protein is thought provoking, as it constitutes a large component of a typical Western diet. Fortunately, the Norwegian health authorities "degraded" pork from white to red meat in 2013, which may imply that more specific advice on protein sources is emerging. While whole-fat dairy products have been branded as a source of unhealthy saturated and trans-fatty acids for humans, we only observed positive effects of a HF/HP casein diet in our primary trial. Additionally, we observed the positive effects of casein in the LF-trial, indicating that low-fat dairy products also are beneficiary. However, we may not state anything as definite when we compare the monotonous mouse diets with a normal and varied food intake. Conclusively, it remains to be seen if our results have human relevance.

4.4 FUTURE PERSPECTIVES

The present study demonstrated that casein attenuates obesity development in a HF/HP diet. In future studies, it would be interesting to investigate the effects of different types of fish and seafood in HP diets. Since cod protein produced intermediate results in our current study, it would be exciting to see if other marine proteins could exert different effects.

To further elucidate the underlying mechanisms of thermogenesis and energy expenditure in HP diets, we could have performed a qRT-PCR analysis of *Ucp1* levels in BAT. Additionally, we could have measured protein levels of UCP1 in BAT and WAT with immunohistochemical staining or a western blot. These analyses would be especially interesting to perform on the varied morphologies we observed in BAT. Additionally, it would be interesting to study the effect of β-adrenergic stimuli on UCP1 expression.

Furthermore, it would be interesting to explore the effects of gut bacteria on nutrient absorption and excretion in diets with different macronutrient compositions. Other interesting analyses would be to measure and compare AA levels of mice or humans in a fed and fasted state.

Our second feeding experiment was conducted to investigate the weight reducing effects of different proteins on DIO mice fed a LF diet. In future studies it would be interesting to observe if HP and Western diets with different protein sources could promote weight loss in DIO mice.

Lastly, it would be of great importance to explore if the effects we observed in mice could be mimicked in humans. To gain a deeper understanding of the therapeutic effects of proteins in human nutrition, it is necessary to conduct more human intervention trials with different proteins sources and elucidate the effects on energy expenditure and other parameters.

5. CONCLUSION

The current study presents one main finding, and two secondary findings:

- Primarily, we report that a high amount of dietary casein attenuates obesity development in C57BL/6J mice fed a high-fat high-protein diet. The beneficial effects of the high-protein diet with casein as the protein source appears to involve several metabolic mechanisms, including increased energy expenditure and a decreased dietary-induced insulin secretion.
- Secondly, we report that there were no differences in the satiating effects of cod and pork proteins.
- Lastly, we report that a low fat diet with casein as the protein source produces the greatest weight-loss in obese mice.

Allthough the results obtained from the animal experiments cannot be directly linked to human nutrition, our findings suggests that it may be beneficial to partially replace pork with casein and moderate amounts of cod. Further research on this field is paramount in order to elucidate the underlying mechanisms of obesity and explore their potential role in human nutrition.

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APPENDIX

Appendix I – Diets

Table A.1: Diet compositions and analyzed nutrients in animal experiment 1 (g/kg).

Group	1	2	3	4	5
(g/kg)	Low fat	High fat			
		Sucrose	Protein	Protein	Protein
Ingredients	Casein	Casein	Casein	Cod	Pork
Casein	206	206	413	-	-
Cod	-	-	-	400	-
Pork	-	-	-	-	438
L-Cystine	3	3	3	3	3
Sucrose	100	440	210	210	210
Corn oil	69	249	248	247	205
Cellulose	50	50	50	50	50
t-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Min.mix: SDS, AIN93Gminmix	35	35	35	35	35
Vit.mix: SDS, AIN93VX NCR95compliant	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
Potato starch (Dextrin)	524	4	29	42	46
Sum	1000	1000	1000	1000	1000
Analyzed					
Crude fat	71	250	250	240	250
Crude protein	200	190	380	400	390
Cholesterol	0.043	0.024	0.111	1.013	0.741
Energy (kJ/g)	18.2	22.9	24.0	23.6	24.2

Fat from the casein powder contributed with 1.14 g/kg in the LF and HF/HS groups and 2.27 g/kg in the HF/HP Casein group. Fat from the cod fillet and pork sirloin contributed with 2.64 and 22.38 g/kg fat, respectively.

Table A.2: Diet compositions and analyzed nutrients in animal experiment 2 (g/kg).

Group	1	2	3
(g/kg)		Low fat	
Ingredients	Casein	Cod	Pork
Casein	206	-	-
Cod	-	200	-
Pork	-	-	218
L-Cystine	3	3	3
Sucrose	91.8	91.8	91.8
Corn oil	68.8	68.6	47.6
Cellulose	50	50	50
t-Butylhydroquinone	0.01	0.01	0.01
Min.mix: SDS, AIN93Gminmix	35	35	35
Vit.mix: SDS, AIN93VX NCR95compliant	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Dextrin from Melis	1.84	1.84	1.84
Potato starch (Dextrin)	532	538	541
Sum	1000.00	1000.00	1000.00
		Analyzed	
Crude fat	71	74	72
Crude protein	190	200	200
Energy (kJ/g)	18.2	18.1	18.2

Fat from the casein, cod and pork proteins contributed with 1.14, 1.32 and 22.38 g/kg fat, respectively.

Appendix II – ELISA insulin kit

Table A.3: Reagents in the Insulin Mouse Ultrasensitive Elisa Kit.

Product	Vender
Insulin Mouse Ultrasensitive ELISA Kit	DRG Instruments GmbH, Germany
Coated plate	
Calibrator 0 (1 vial)	
Calibrator 1,2,3,4 and 5 (5 vials)	
Enzyme Conjugate 11X (1 vial)	
Enzyme Conjugate buffer (1 vial)	
Wash buffer (1 bottle)	
Substrate TMB (1 bottle)	
Stop solution (1 vial)	

Appendix III – Histological methods

Table A.4: Chemicals and reagents used for fixation, dehydration, embedding, sectioning and staining.

Product	Vender
4 % formaldehyde	Merck, Germany
NaH2PO4 x H2O	Merck, Germany
Na2HPO4 x H2O	Merck, Germany
Ethanol	Arcus, Norway
Rectified Alcohol	Arcus, Norway
Xylene	Prolabo
Paraffin	Histovax, OneMed
Hematoxylin	EMS
Eosin Y	Sigma, USA
Mounting medium	Merck, Germany

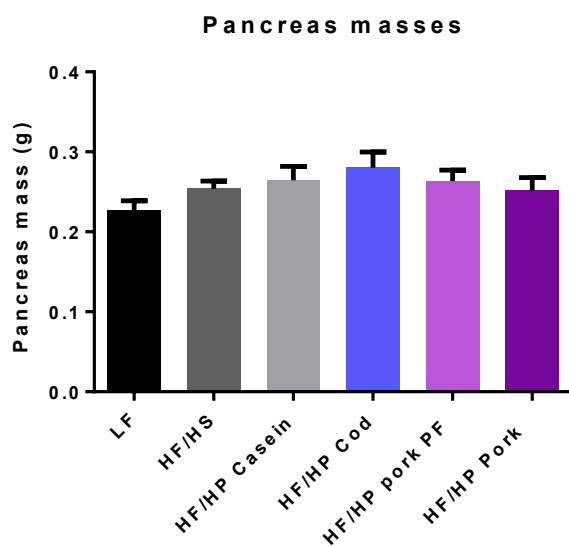## Appendix IV – Pancreas mass

Figure A.5: Pancreas masses in the different groups. The results are presented as means ± SEM.

Appendix V – Amino acid analyses

Table A.6: Free amino acids in non-fasting mouse plasma (μmol aa/ 100 ml plasma).

Free amino acids in plasma						
μmol aa/ 100 ml plasma	LF	HF/HS	Casein	Cod	Pork PF	Pork
Histidine	8.4	7.9	7	6.6	7.4	7.3
Isoleucine	10.9	9.8	11.9	11	10.5	9.6
Leucine	18.5	16.9	20	17.3	16.7	15.7
Lysine	47.5	46	38.1	32.4	33	29.1
Methionine	11.7	10.8	10.5	8	7.3	6.2
Phenylalanine	7.4	7.4	7.4	6.7	6.5	6.3
Threonine	38	31.8	35.1	20.6	23.3	20
Tryptophan	10.2	10.1	9.1	7.9	7.6	7.4
Valine	30.8	28.1	34.7	27.2	25.4	23.3
Alanine	81.9	73.4	63.7	48	52.1	46.4
Arginine	7.1	7.6	6.6	8.4	8	6.9
Aspargine	11.8	12.7	11.9	8.4	7.5	6.6
Aspartic acid	2	1.9	1.8	1.7	1.6	1.5
Cystein	2.6	2.7	2.9	2.6	3.1	3
Glutamic acid	6	6.1	5.4	6.3	5.2	5
Glutamine	59.4	61.7	54.2	56.2	55.7	56.5
Glycine	29.9	26	31.6	26.3	24.8	24
Hydroxyproline	2.2	1.6	1.4	1.4	1.8	1.6
Proline	20.3	19.2	22.8	8.3	8.7	8.0
Serine	22.6	19.7	18.8	12.5	12.6	11.9
Tyrosine	11.8	12.7	12.2	8.7	7.9	7.5
Sum essential AAs	183.3	168.8	173.8	137.8	137.7	124.9
Sum non-essential AAs	175.7	245.3	233.2	189	188.9	178.6
Sum BCAAs	37.8	34.6	38.9	34.9	34.6	32.6
Total	359	414.1	407	326.8	326.6	303.6
Taurine *	49.8	49.6	50.6	59.8	50	49.2

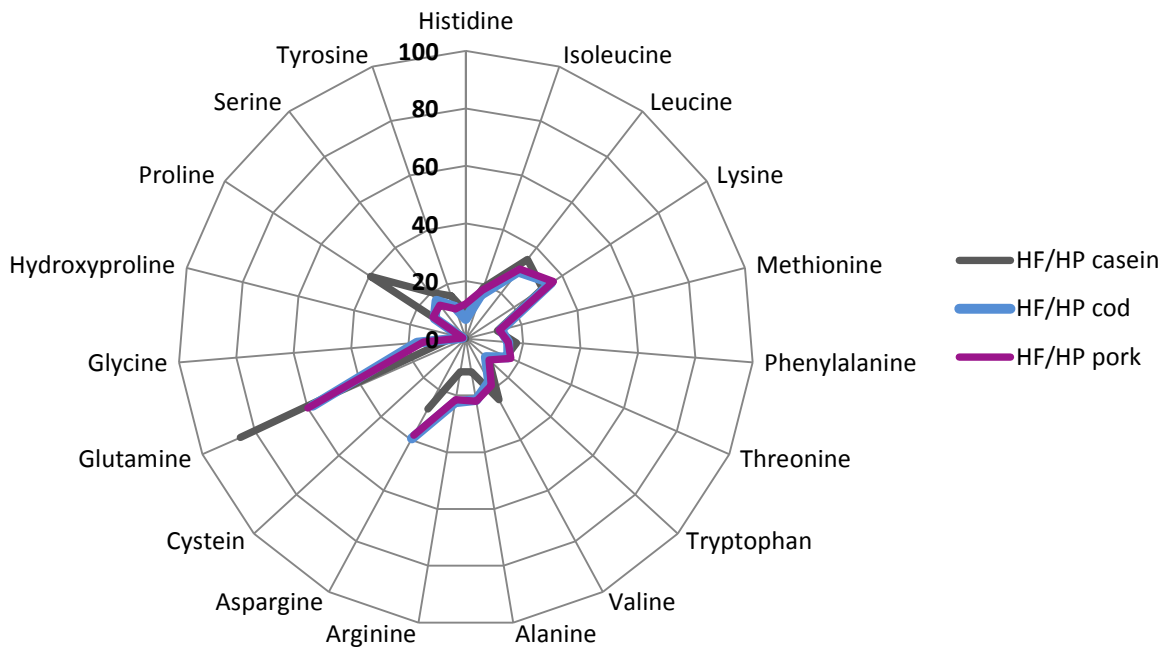## Amino acid composition of the experimental diets (mg/g)

Figure A.7: Amino acid composition of the experimental diets (mg/g).

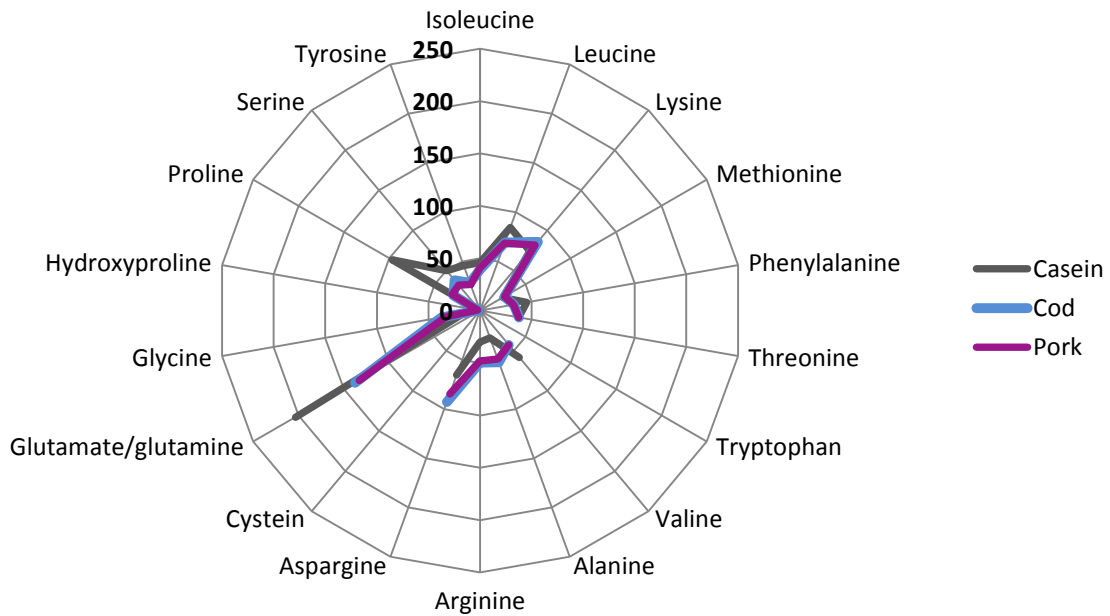## Amino acid composition of the protein sources (mg/g)

Figure A.8: Amino acid composition of the protein sources (mg/g).