

THE OBESOGENIC EFFECT OF HIGH-FAT DIETS ARE INFLUENCED BY BOTH PROTEIN SOURCE AND AMOUNT

SUSANNE BJELLAND

MASTER THESIS IN HUMAN NUTRITION



INSTITUTE OF MEDICINE, UNIVERSITY OF BERGEN (UIB)

NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH (NIFES)

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LIST OF ABBREVIATIONS

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
cDNA	Complementary deoxyribonucleic acid
CideA	Cell death-inducing DFFA-like factor A
CREB	cAMP response element-binding protein
DEXA	Dual-energy X-ray absorptiometry
Dio2	Deiodinase type-2
ELISA	Enzyme-linked immunsorbent assay
ETDA	Ethylenediaminetetraacetic acid
eWAT	Epididymal white adipose tissue
GTT	Glucose tolerance test
HF/HP	High fat and high protein
HF/HS	High fat and high sucrose
iBAT	Interscapular brown adipose tissue
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue
mTOR	Mmammalian target of rapamycin
MRI	Magnetic resonance imaging

NE	Norepinephrine
NPY	Neuropeptide Y
PB	Phosphate buffer
PET	Positron emission tomography
PKA	Protein kinase A
Pparg α	PPARgamma Coactivator 1 alpha
PPY	Peptid Y
prWAT	Perirenal white adipose tissue
PUFA	Polyunsaturated fatty acid
PVN	The paraventricular nucleus
RERs	Respiratory Exchange Ratios
ROS	Reactive oxygen species
RT	Reverse transcription
RT-qPCR	Real time quantitative polymerase chain reaction
SEM	Standard error of the mean
SNS	Sympathetic nervous system
TAG	Triacylglyceride
UCP1	Uncoupling protein-1
WAT	White adipose tissue
WHO	World Health organization
WHR	Waist-to-hip ratio

ABSTRACT

Although there has been a decline in the dietary fat intake over the last decade, the prevalence of obesity and type 2 diabetes is still rising. The contemporary Western diet provides an average of 49 % energy from carbohydrate, 35 % from fat and 16 % from protein. Earlier studies have demonstrated that increasing the dietary amount of protein at the expense of sucrose (i.e. increasing the protein:sucrose ratio) attenuates obesity development in mice fed high fat diets. Furthermore, an unpublished study in our group revealed that different protein sources have different (anti)-obesogenic properties when included in a high-protein high-fat diet. Interestingly, of all protein sources tested, casein was the only protein to attenuate body weight gain. We undertook this study to investigate the impact of protein:sucrose ratio in combination with other protein sources, such as cod and pork. Furthermore, we aimed to elucidate some of the underlying mechanisms by which different protein sources influence obesity development. Hence obesity prone C57BL/6J mice were fed either a high-sucrose or a high-protein diet containing casein, cod or pork as the protein source.

Our results demonstrated that increasing the protein:sucrose ratio markedly reduced feed efficiency and fat mass gain when mice were fed casein or pork protein. Interestingly, when mice were fed cod protein the protein:sucrose ratio was of no significant importance for either energy efficiency nor fat mass gain. Furthermore, in agreement with earlier studies, our results showed that mice fed casein was protected against high-fat induce obesity. Surprisingly, cod and pork fed mice not only gained more weight but also experienced a reduced glucose tolerance compared to casein fed mice. These findings indicate that both protein amount and protein source is of importance in the development of obesity and suggest that it may be beneficial to partially replace refined carbohydrate with carefully selected protein sources.

1 INTRODUCTION

1.1 OVERWEIGHT AND OBESITY

1.1.1 Definition and quantification of overweight and obesity

The World Health Organization (WHO) defines overweight and obesity as a state of “abnormal or excessive fat accumulation that presents a risk to health” [1]. The most widely used method to diagnose and classify overweight and obesity is determination of Body Mass Index (BMI). BMI is calculated by dividing body weight (BW) in kilograms by height in meters squared (kg/m^2). According to WHO an adult person with a BMI equal to or above 25 is classified as overweight, while a person with a BMI of 30 or more is considered obese [2]. BMI is a useful tool in monitoring an individual’s health status; however, it has some limitations. For example, BMI calculation is solely dependent on weight and height of the individual and does not account for differences in bone density and maybe more important, it does not distinguish fat mass from lean mass [3]. Waist circumference and waist-to-hip ratio (WHR) are also commonly used to identify overweight and obesity. Waist-to-hip ratio provides information about fat deposition in the upper body and, unlike BMI, it accounts for differences in body shape. This is of importance because individuals with increased visceral fat (apple shaped) are believed to have a higher risk of developing metabolic diseases, such as type 2 diabetes, compared to individuals with increased subcutaneous fat (pear shaped) [4]. Other more accurate measurements such as magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DEXA) also exist, however; these methods are comprehensive and expensive to perform.

1.1.2 Prevalence of obesity and overweight

Over the last decades the prevalence of overweight and obesity has become a major health problem and is now the fifth leading risk of global deaths [2]. Based on the most recent estimates by the WHO more than 1.4 billion adults (>20 yrs) worldwide are now considered overweight, and 500 millions of them are obese [2]. Childhood obesity is also an increasing problem, with numbers showing that more than 40 million children under the age of five are overweight [2]. One of the highest percentages of overweight is seen in the United States where about two-thirds of the population is classified as overweight or obese [5].

In Norway the average body weight has increased by 5-6 kg only the last 15 years and today more than half of the Norwegian population is overweight and 15-18 % is obese [6]. Overweight and obesity were once considered to be a problem related only to industrial countries, but are now also a rising challenge in low- and middle income countries. In fact, eight of the top ten countries in WHO's ranking list of prevalence of overweight are found in the Pacific region [7].

1.1.3 What causes obesity and overweight?

Overweight and obesity develops when there is a long-term imbalance between energy intake relative to energy expenditure. Thus excess energy consumption and physical inactivity is considered the main causative factors of obesity. After the agricultural revolution (10, 000 years ago) there has been a huge change in the human diet. During the last century there has been an increased consumption of processed carbohydrates, dietary omega-6 polyunsaturated fatty acids (n-6 PUFAs) and energy-dense food, as well as an increased intake of sugar-sweetened beverages [8]. Additionally, there has been a decrease in physical activity due to advances in technology and transportation [9]. However, attributing overweight and obesity solely to these factors would be an oversimplification. Other aspects such as genetic, environmental, economic, social, psychosocial and even political factors interact in varying degrees to promote the development of obesity [10]. Some individuals seem to be more susceptible to today's obesogenic environment and several twin studies have estimated that genes are responsible for 40-70 % of the phenotypic variance of obesity [11, 12]. The causes of overweight and obesity are clearly complex and several factors appear to contribute to its development.

1.1.4 Health consequences associated with overweight and obesity

The co-morbidities associated with obesity are of major public health concern and include development of insulin resistance and type 2 diabetes [13]. Obesity is also strongly associated with development of cardiovascular diseases such as heart diseases, stroke and atherosclerosis [13]. Furthermore, some types of cancer, including colon and breast cancer, have been linked to obesity [13]. Excess body fat is also a risk factor for complication such as osteoarthritis, as well as sleep apnea and respiratory problems due to extra weight placed

on joints and chest [13]. Consequently, overweight and obesity is not only a global health threat but also a huge economic burden for the society.

1.2 ADIPOSE TISSUE

The adipose organ is mainly made up of two types of tissue, white and brown adipose tissue. While white adipocytes store lipids which are used as fuel when needed, brown adipocytes have quite a different function; they oxidize lipids to produce heat.

1.2.1 White adipose tissue (WAT)

White adipose tissue mostly consists of white adipocytes. White adipocytes contain a single large lipid droplet which accounts for >90 % of the cell's volume. Additionally, white adipocytes have a peripheral nucleus and few mitochondria which are situated in the narrow space between the droplet and the membrane. Traditionally white adipose tissue was considered to be solely a fat storage for excess energy intake in the form of triacylglycerides (TAGs). However, after Friedman and colleagues discovered the secretion of leptin from white adipose tissue this traditional view was changed [14]. Later the list of protein signals and factors released from white adipocytes has grown, including angiotensinogen, adiponectin, acylation-stimulating protein, adiponectin, retinol-binding protein, tumour necrosis factor α , interleukin 6, and plasminogen activator inhibitor-1 [15]. Thus, white adipose tissue is now recognized to be a highly active endocrine organ. Some of the substances secreted from the adipose tissue organ are mediators in inflammatory processes, giving the adipose tissue an additional role as a regulator of the immune system [16]. In fact, extensive secretion of pro-inflammatory cytokines is believed to play a role in the development of several of the co-morbidities associated with obesity, including insulin resistance [17]

1.2.2 Brown adipose tissue (BAT)

In contrast to white adipocytes, brown adipocytes contain several smaller lipid droplets (multilocular). Brown adipocytes also have a much higher number of mitochondria and uniquely express uncoupling protein 1 (UCP1) [18]. UCP1 is localized in the inner mitochondrial membrane and acts to uncouple oxidative phosphorylation from ATP production, thereby releasing energy as heat (termed thermogenesis) [19]. The metabolic

activity of BAT is mainly regulated via input from the sympathetic nervous system (SNS). Norepinephrine (NE) released from axon terminals of sympathetic neurons binds to β -adrenergic receptors on the surface of brown adipocytes and stimulates cAMP production and protein kinase A (PKA) activation. PKA activates CREB which binds to certain DNA sequences and affect nuclear transcription of UCP1, resulting in increased heat production (Figure 1.1).

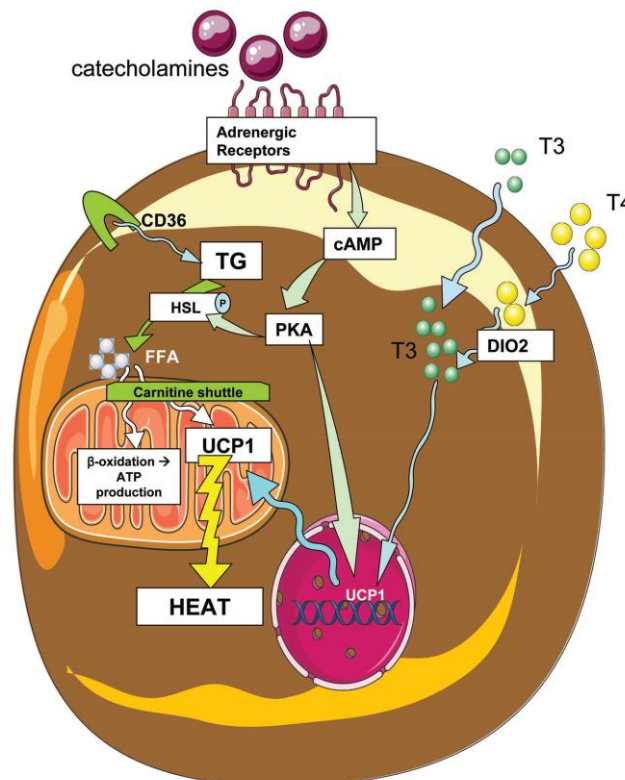


Figure 1.1: The adrenergic signaling pathway in mature brown adipocytes. From “Brown adipose tissue: Recent insight into development, metabolic function and therapeutic potential” [20].

Brown fat has long been known to exist in infants and in smaller animals such as mice where it plays an important role in regulating body temperature through non-shivering thermogenesis. Larger mammals often lose much of their brown fat depots after infancy and the role of BAT in adult humans has traditionally been considered absent. However, this view dramatically changed in 2009 when several studies demonstrated the occurrence of UCP1-positive brown fat in adult humans using positron emission tomography (PET) [21-24].

Brown adipocyte development

White and brown adipocytes have previously been assumed to be localized in distinct sites, but after the demonstration of inducible expression of UCP1 in WAT depots of cold-exposed or β -adrenoceptor agonist treated rodents, the two types of adipocytes were proposed to be intermingled in the adipose organ [25-27]. Supporting this hypothesis, Wu et al. managed to isolate so called “beige” cells from white adipose depots [28]. These “beige” cells resembled white fat cells in having an extremely low expression of UCP1 in the basal unstimulated state, but once stimulated these cells activate expression of UCP1 to levels similar to those of classical brown fat. While Cinti and colleagues have reported that browning of white fat in response to cold is mainly due to transdifferentiation of mature white adipocytes into brown adipocytes [18], recent studies have revealed that there are distinct progenitors that give rise to adipocytes located in different anatomic locations in rodents. The classical brown fat cells found in the interscapular region are thought to develop during the prenatal stage from Myf-5 positive myoblast precursors, resembling the gene signature of skeletal muscle cells [29]. Whereas “beige” adipocytes located within the white adipose tissue, also called “brite” or “brown-like” adipocytes, are believed to originate from a non-Myf-5 lineage [29]. However, the Myf-5 expressing progenitor cells first believed to only give rise to “classical” brown adipocytes and muscle cells have now also been identified in white adipose tissue where they are found to give rise to a subset of white adipocytes, suggesting that “beige” adipocytes may have multiple origins [30]. Hence, the cellular origin of “beige” adipocytes is not conclusive and is currently under debate.

1.2.3 Browning in white adipose tissue

Several studies have shown that obesity-resistant strains of mice, such as A/J and Sv129 mice, have higher amounts of “beige” adipocytes in white fat [31-33]. Furthermore, transgenic mice with increased amounts of UCP1-positive adipocytes in WAT are protected from high-fat diet-induced obesity [34]. Interestingly, the multilocular cells previously observed in humans are found to be more similar to “beige” fat cells rather than classical brown fat cells [28]. Consequently manipulation of inducible brown adipocytes in humans may be a potential target in the treatment and prevention of obesity and its related diseases. New data concerning BAT function in humans is still emerging, and one of the most

recent finding is that human infants have distinguishable interscapular BAT depots that consists of classical brown adipocytes, a cell type that has so far not been shown to exist in humans [35].

1.3 WEIGHT LOSS AND OBESITY PREVENTION

1.3.1 Macronutrient composition

Although obesity is known to be a disorder of energy balance, a true understanding of its causes and treatment remains elusive. The contemporary Western diet contains an average of 49 % energy from carbohydrate, 35 % from fat and 16 % from protein [36], which is an increase in the dietary level of carbohydrates at the expense of protein compared to Stone Age and Hunter-gatherer diets [8]. Since the adoption of the Western diet, the prevalence of obesity and type 2 diabetes has risen substantially. Therefore, it is plausible that changes in dietary macronutrient composition also play a role in the increasing incidence of obesity. Macronutrients not only supply calories but some components also directly or indirectly function as signaling molecules to affect appetite and metabolism [37]. The importance of the macronutrient composition of a diet in prevention and management of obesity is debated. However, despite the consistency among official recommendation, there has been a growing interest in alternative dietary approaches to reduce weight and fat mass. The ideal balance of macronutrients necessary to optimize weight loss and prevent obesity is an area of great controversy. Multiple strategies have been proposed, and in recent years low-carbohydrate and high-protein diets have attracted considerable attention as strategies for successful weight loss.

1.3.2 Low-carbohydrate diets

The Atkins Diet is an example of a low carbohydrate diet and involves limited consumption of carbohydrate (less than 20 grams per day) to switch the body's metabolism from metabolizing glucose as energy over to converting stored fat to energy. Moreover, because the body needs more than 20 grams of carbohydrates to cover its daily glucose requirements, low carbohydrate diets stimulate conversion of non-carbohydrate precursors to glucose, a process known as gluconeogenesis. Several trials have compared low-

carbohydrate vs. traditionally low-fat, high-carbohydrate diets and considerable evidence has demonstrated that reducing the carbohydrate content in the diet improves body weight loss [38-40]. A meta-analysis of randomized controlled trials concluded that low-carbohydrate, non-energy restricted diets are at least as effective as low-fat, high-carbohydrate diets in inducing weight loss [41]. Additionally, the low-carbohydrate diets were associated with favorably changes in triglyceride and high-density lipoprotein (HDL) values [41]. However, low-carbohydrate diets have also been associated with unfavorable changes in total cholesterol and low-density lipoprotein (LDL) and the long-term effect of such diets is still unknown [42].

The effect of carbohydrates on adipose tissue is not only determined by the amount of carbohydrate, but also the type of carbohydrate. Different types of carbohydrates have different effects on blood glucose levels and this knowledge has led to the term glycemic index (GI). High-GI carbohydrates such as pasta and white bread are rapidly digested and cause a high postprandial level of blood glucose, whereas whole grain carbohydrates give a steady rise in blood glucose levels and hence have a lower GI [43]. A study by Pawlak et al. demonstrated that rats and mice fed a low GI-diet gained less body fat compared to those fed a high-GI diet [44]. Of further interest, a recent study reported that subjects assigned to a low-GI diet in combination with a high protein content had higher rates of weight loss maintenance than subjects receiving a low-GI, low protein diet [45].

1.3.3 High-protein diets

In recent years, particularly after the low carbohydrate diet wave settled down, high-protein diets have become increasingly popular as an effective way to lose weight. There are no standard definitions of high-protein diets, but based on intervention studies a protein intake of 30% of total energy is generally considered as high [8]. One of the most popular high-protein diets are the “Zone diet”. The “Zone diet” centers on a 40:30:30 ratio of calories obtained from carbohydrates, proteins, and fats, respectively [46]. According to the Zone Diet doctrine a 0.75 protein to carbohydrate ratio will reduce insulin to glucagon ratio and allow excess body fat to be burned off and ultimately lead to weight loss. The efficiency of high-protein diets is not yet fully accepted by Health Authorities and the safety is debated as a high intake of proteins has been associated with potential dangers, such as bone mineral

loss [47] and kidney damage [48]. Yet, to this day there is not sufficient evidence to conclude that a high protein intake is dangerous for healthy individuals [49] and currently there is convincing evidence that protein-rich diets not only increases weight loss [50] but also attenuates loss of lean tissue [51] and improves glycemic control [52]. The mechanisms behind the weight reducing effect of high-protein diets are not yet understood but seem to include enhanced satiety and increased energy expenditure [53, 54] .

The effect of protein on satiety

Increased satiety and subsequently reduced energy intake have often been cited as a possible explanation for the reported success of high-protein diets. Studies have shown that under experimental conditions, subjects consumed less energy when given high-protein meals versus high-carbohydrate meals and consumed less energy at the subsequent meal [55, 56]. Various physiologic consequences of protein ingestion are likely to impact satiety. Proteins, unlike fat and carbohydrate stimulate the appetite suppressant gastrointestinal hormone cholecystikinin (CCK) [57]. CCK is released from the stomach and induces satiety by suppressing the NPY (neuropeptide Y) level in the doromedial hypothalamus [58]. In other words by stimulating CCK secretion dietary proteins may have a greater ability to induce satiety and reduce food consumption for a longer period of time compared to carbohydrate- or fat rich diets. High levels of protein have also been shown to stimulate the intestinal secretion of PPY (peptid YY) [8], a hormone which is believed to have anorectic effects and inhibit food intake [59]. Of further interest is the finding that specific amino acids have been shown to regulate appetite. Branched chain amino acids (BCAA), especially leucine (Leu), have been demonstrated to directly stimulate mTOR signaling in the hypothalamus and thereby decreasing food intake [60]. Leucine has also been reported to inhibit appetite and influence satiety by stimulating leptin secretion [61]. Another example of an amino acid believed to be involved in food intake regulations is tyrosine, which is a precursor of norepinephrine and dopamine. Norepinephrine has been found to stimulate eating, through activation of α 2-adrenergic receptors, and suppress appetite, through activation of α 1-adrenergic receptors in the paraventricular nucleus (PVN) of the hypothalamus [62]. Dopamine has also been shown to play a role in the motivation to eat, as studies on knockout mice found that the absence of dopamine production caused an inability to initiate feeding [63, 64]. Furthermore, the essential amino acid tryptophan has

been pointed out for its role in the synthesis of serotonin, a classical neurotransmitter which plays an important role in regulating food intake in mammals [65]. A study demonstrated that administration of 5-HT (serotonin) or its analogue reduced food intake by the generation of reactive oxygen species (ROS) in the hypothalamus through an NADPH oxidase-dependent pathway [66]. Together these findings suggest that the ability of high-protein diets to modulate body weight gain might, at least in part, be explained by their satiating effect.

The effect of protein on energy expenditure

Another potential mechanism by which high intake of proteins could promote weight loss is through their thermic effect and increased energy expenditure. The thermic effect can be defined as the energy required for digestion, absorption and disposal of an ingested nutrient [67]. Proteins have a relative high thermic effect (20-30 %) compared to carbohydrate (5-10 %) or fat (0-3 %) [67]. The higher thermic effect of proteins is partly explained by the fact that the body has no flexible storage capacity for excess intake of amino acids, which are therefore actively oxidized or eliminated [68]. The high cost of protein oxidation and urea synthesis, as well as the high ATP requirement of postabsorptive protein synthesis positively affect energy expenditure and likely account for some of the reduced feed efficiency of high-protein diets [68]. Of further interest is the finding that high-protein diets lead to a twofold higher meal-induced thermogenesis compared to high-carbohydrate diets in young women [69]. Moreover, studies have demonstrated an upregulation of UCP1 in inguinal fat pad of mice [70, 71], as well as in subcutaneous WAT of cattles fed a protein enriched diet [72]. The effect of high-protein diets on thermogenesis may partly be mediated through the amino acid tyrosin which is a precursor of norepinephrine. Through interaction with alpha and beta adrenergic receptors norepinephrine have been found to activate different signaling pathways in brown adipocytes resulting in increased cell proliferation and greater expression of *Ucp1* [19]. In summary, the increased energy expenditure from UCP1-dependent uncoupled oxidative thermogenesis in combination with the high energy cost from gluconeogenesis and ureagenesis may attribute to the reduced energy efficiency observed for high-protein diets in both mice [70] and men [69].

The effect of protein on insulin:glucagon ratio

Insulin is a powerful anabolic hormone and secretion of insulin stimulates glucose oxidation, glycogen synthesis, lipogenesis and protein synthesis. In other words insulin favors uptake and storage of all types of ingested nutrients, while it inhibits protein catabolism. Insulin has been found to play a vital role in development of obesity as it stimulates adipocyte differentiation and adipose tissue expansion. The importance of insulin signaling in adipocyte obesity development is underscored by the finding that mice lacking insulin receptor in adipose tissue [73] as well as mice lacking Ins2 gene expression in pancreas (i.e. have reduced insulin secretion) [74] are completely protected against high-fat diet-induced obesity. Glucagon has mainly opposing effect on the body and in contrast to insulin it causes an increase in glycogenolysis, gluconeogenesis, lipolysis and fatty acid oxidation. The insulin to glucagon ratio in healthy subjects is determined by the nutritional status. After a meal the insulin:glucagon ratio is high but when the nutritional status is low glucagon is secreted leading to a high glucagon:insulin ratio. The insulin:glucagon ratio can also be influenced by the macronutrient composition of the diet. Diets enriched in carbohydrates, in particular high-glycemic index carbohydrates, will lead to an enhanced insulin secretion and thereby a higher insulin:glucagon ratio [75], whereas high protein diets have been reported to reduce insulin:glucagon ratio after a meal [70, 76]. Additionally, the amino acid profile of the ingested protein itself may play a role in the control of insulin:glucagon ratio [77]. For example, amino acids such as alanin and arginine have been shown to stimulate glucagon release [78]. Thus, both the amount and the type of dietary proteins and carbohydrates may determine the insulin:glucagon ratio and thereby influence the adipogenic potential of a fat-containing diet.

1.4 INTRODUCTION TO THE STUDY

For decades, high-protein diets have been popular among bodybuilders and other athletes, as proteins are required to repair and rebuild muscles. More recently, high-protein diets have become increasingly popular in the general population as a tool in weight management. A number of studies have reported that a high dietary content of protein increases satiety and thermogenesis [54, 69], as well as reduces energy efficiency in men, and several studies have demonstrated the beneficial effects of high-protein diets in weight reduction in humans [53].

A number of animal studies have confirmed the ability of high-protein diets to attenuate feed efficiency and weight gain [70, 71, 75, 79, 80]. Earlier studies from our group demonstrated that a high level of dietary protein totally prevented high fat diet induced obesity in C57BL/6J mice [70]. Of note, the high-protein fed mice needed almost 7 times more calories to achieve a weight gain of 1 g than mice on the high-carbohydrate diet. Moreover, the high-fat diet in combination with protein translated into a high glucagon:insulin ratio leading to increased cAMP signalling. Thus, although the mice were in a fed state, molecular signaling and biochemical processes associated with fasting, such as lipolyses and fatty acid oxidation, were ongoing. Furthermore, the enhanced cAMP signaling was associated with increased UCP1 expression in inguinal white adipose tissue and presumably an increased number of brown adipocytes, aka beige, allowing energy to dissipate in form of heat. The finding that UCP1 expression was increased in inguinal white, but not in interscapular brown adipose tissue is of great interest as brown adipocytes in human adults are mainly found as islets within the white adipose tissue. Thus, if high-protein diets are able to increase thermogenesis by a similar mechanism in former white adipose tissue also in humans, this would provide an explanation, at least in part, to how high fat high protein diets, such as the Atkins diet can induce weight loss without a concomitant reduction in energy intake.

Casein is together with soy the most commonly used protein source in rodent studies, including ours. Animal proteins such as those from beef, pork or poultry, as well as fish proteins also play an important role in human nutrition worldwide. However, little data on the efficiency of different protein sources on obesity development exists [68]. Consequently,

a previous study by our group recently aimed to investigate whether intake of a high proportion of other protein sources than casein was able to reduce the adipogenic potential of high fat diets. Thus, obesity prone C57BL/6J mice were fed a high fat diet in combination with a high proportion of either milk casein, vegetable protein (soy), terrestrial animal proteins (beef, chicken and pork) or fish (cod) proteins. As references, a group of mice was given a low fat diet containing casein and another group was given a casein based high-fat high sucrose diet. In agreement with the earlier mentioned studies [70, 71, 75] a high content of casein in combination with a high fat diet totally prevented the high-fat diet – induced weight gain (Fig 1.1) Of note, mice fed a high proportion of cod, beef, chicken and pork, became as heavy as, or more heavy, than the high fat high sucrose fed mice. Of the protein sources tested, only casein was able to protect against diet-induced obesity.

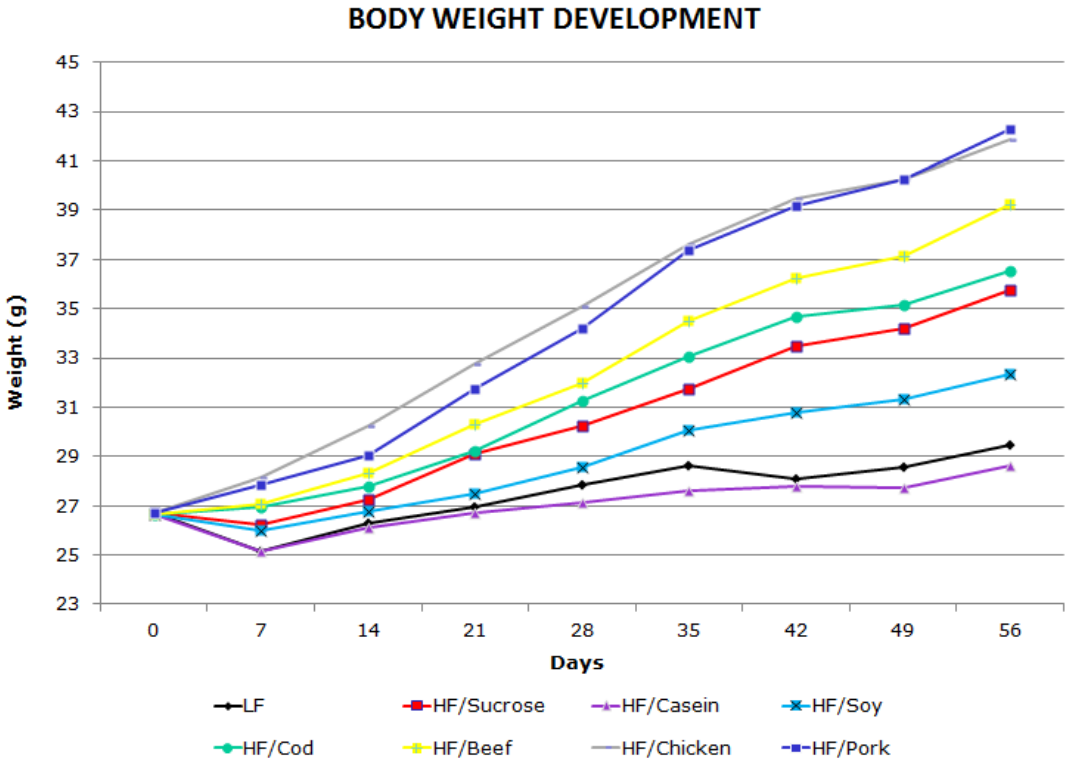


Figure 1.2: Results from a previous unpublished study performed in our group with C57BL/6J mice fed a high fat diet in combination with various protein sources.

1.5 AIM OF THE STUDY

The finding that only high proportion of casein, of the protein sources tested, were able to protect against diet-induced obesity demonstrate that different dietary proteins have different (anti)-obesogenic properties. Moreover, the finding that mice fed a high proportion of cod, beef, chicken and pork, became as heavy as, or more heavy than the mice fed a high-fat high-sucrose diet, challenging the earlier suggestion from our group that the protein:carbohydrate ratio determines the adipogenic potential of a high fat diet. This study aimed to investigate if the protein:carbohydrate ratio is of importance when other protein sources than casein is used. The possible relation between obesity development, energy expenditure and feed efficiency will be investigated. Furthermore, this study aimed to investigate if the obesogenic potential of the different diets was related to their capacity to stimulate insulin secretion.

2 MATERIALS AND METHODS

2.1 ANIMAL EXPERIMENT

THE ANIMAL MODEL

Seventy male mice of the inbred strain C57BL/6JBomTac were obtained at 8 weeks of age from Taconic, Denmark. The mice were placed in single cages (Techniplast 1291) and allowed to acclimatize to their surroundings for 1 week. C57BL/6J is one of the most commonly used mice strains and was particularly suitable for this experiment because of its ability to develop obesity, hyperglycemia and hyperinsulinemia when fed a high-fat diet [81].



Figure 2.1: Picture of a C57BL/6J mouse (Retrieved from <http://jaxmice.jax.org/strain/000664.html>).

ETHIC STATEMENT

The animal experiments were approved by the Norwegian Animal Health Authorities. Care and handling were in accordance with local institutional recommendations.

EXPERIMENTAL SET-UP

After one week of acclimatization the mice were scanned and weighted. Based on body weight and fat percentage the lower and upper extremes were removed, leaving a total of 63 mice for the experiment. The mice were then sorted into seven groups ($n=9$), making sure that the average body weight, fat mass, lean mass and fat percentage in each group were similar.

Housing: The mice were kept in single cages throughout the experiment to control the feed intake of each individual mouse. Each cage had standard wooden chips bedding (Scanbur Bedding Aspen, Norway) and nesting materials of shredded paper and cardboard. Tap water was constantly available. The animal room had artificial lighting with a twelve hour

light/dark cycle. Room temperature was kept between 28.5 and 30 degrees to ensure thermoneutrality and avoid cold stress. The humidity was between 39 and 55.

Food intake: The mice were fed three times a week for a total of 12 weeks. On the days of feeding, each mouse received a new food cup and the feed remnants from the previous food cup were weighed. Once a week the cages were changed and cleaned while the spillage was collected, weighed and counted for. From this data daily, weekly, and total caloric intake of each experimental group were calculated.

Body weight and composition: Body masses of all animals were measured before initiation of the feeding experiments and subsequently once per week on a Mettler Toledo Weight. In week 6 and week 10 of the experiment the mice were scanned in a Bruker Minispec LF50mq7.5 scanning apparatus and fat mass, lean mass and free water were measured. This apparatus uses a magnetic field to provide information about the animal's body compositions in order to determine whether the increased body weight is due to gained fat- or muscle mass, or both.

DIETS

The seven groups of mice received different diets. One group was given a low-fat (LF) control diet as a reference, while the six other experimental groups received either a high-fat high-sucrose (HF/HS) or a high-fat high-protein (HF/HP) diet containing different sources of protein. The sources of protein were casein, cod and pork. Casein powder (batch number BCBC3986V) was purchased from Sigma (batch number 080M0006) and cod fish powder was purchased from Seagarden AS. Pork sirloin was purchased from H. Brakstad AS, freeze dried and minced to powder at NIFES. The diets were prepared by weighing on a Mettler Toledo PG42002-S/PH weight and mixed in a Crypto Peerless EF20 blender. All diets were kept frozen throughout the experiment. The macronutrient distribution in the different diets is presented in figure 2.2. For a more detailed list of the composition of the diets see Appendix table A.1.

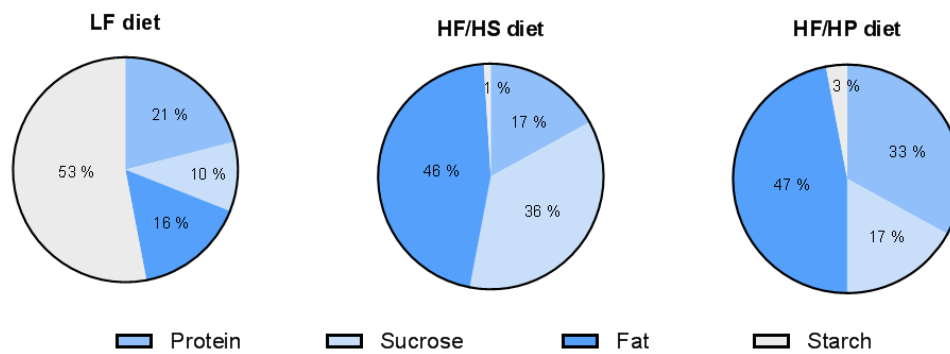


Figure 2.2: Distribution of protein, sucrose, fat and starch in the low fat control diet and the experimental diets.

COLLECTION OF FECES

In week 11 of the feeding trial the animals were moved to clean cages with less wooden bedding to allow collection of feces. After 4-6 days the feces were collected, weighted and placed in small tubes which were stored at -80°C until analysis could be performed.

TERMINATION

After 12 weeks of feeding the mice were terminated. Prior to the termination all animals were weighted and fasted for four hours to ensure that they were all in the same metabolic state. The mice were then anaesthetized with Isofluran (Isoba-vet, Schering Plough, Denmark) using the anesthesia apparatus Univentor 400 Anesthesia Unit (Univentor Limited, Sweden) and sacrificed with cardiac puncture.

Blood sample collection:

Blood samples were collected directly from the hearth with a syringe and separated into two tubes containing EDTA as anticoagulant. The samples were immediately centrifuged at 5000g in 4 degrees for 5 minutes, to separate plasma and red blood cells. The plasma was stored at -80°C until further analysis.

Organ collection:

During the termination three adipose depots (iWAT, eWAT and iBAT) were dissected out, weighted and freeze clamped in small plastic bags to ensure rapid freezing. The tibiales anterior muscle, pancreas, liver and kidneys were also excised, weighted and quickly frozen in liquid nitrogen. All tissues were temporarily put on dry ice and later stored at -80°C until further analysis.

In addition, a second set of tissue samples of eWAT, iWAT and iBAT from twenty-one randomly selected mice (three from each group) were fixed in 4 % formaldehyde and later prepared for histological examination. Also, photographs of one representative mouse from each group were taken during the dissection.

2.2 GLUCOSE TOLERANCE TEST (GTT)

After 10 weeks of feeding an intraperitoneal injected glucose tolerance test (*i.p* GTT) was performed to determine which, if any, of the animals had become glucose intolerant. The mice were fasted for 6 hours as overnight fasting can cause major metabolic stress on such small animals [82]. Prior to the test the animals were weighted and doses of glucose were calculated based on body weight (2 mg glucose/g body weight). Before glucose administration drops of blood were obtained by tail puncture in the upper part of the tail and fasted glucose concentrations were measured using an automatic glucometer (Ascensia, COUNTOUR, USA). Glucose was administered with an intraperitoneal (*i.p*) injection in the abdomen and blood glucose levels were again measured at 15, 30, 60 and 120 minutes after the injection. Additionally, 20 µl of blood from each mouse were collected at T0 and T15.

2.3 INSTULIN INTOLERANCE TEST (ITT)

After 11 weeks of feeding an intraperitoneal insulin tolerance test (*i.p* ITT) was performed to determine which, if any, of the animals had become insulin resistant. For this test the mice were not fasted but moved to clean cages with no access to food while the experiment was conducted. As described above blood glucose was measured at T0, followed by an intraperitoneal injection of insulin (Humulin-R) and then measured again after 15, 30, 45 and 60 minutes. Each mouse received 0.5 U insulin per kilo gram body weight.

2. 4 MEAL TOLERANCE TEST (MTT)

A meal tolerance test (MTT) was performed on a second set of mice at the University of Copenhagen. The procedure was based on the method described by [83].

Before testing mice were transferred into individual cages and fasted overnight (approximately 16 hours). The following morning, blood glucose concentrations were measured via tail vein using a handheld glycometer (Ascensia, COUNTOUR, USA). Additionally, 20 μ l of blood were collected from each mouse using micro capillary. The animals were then given access to a previously weighed amount of food corresponding to their specific diet group. The experimental diets tested were casein HF/HS, casein HF/HP, pork HF/HS and pork HF/HP, along with a LF control diet (n=5). Mice had free access to the food for a 30-minute period and then the remaining food as well as any food-spillage were removed and weighed. This amount was subtracted from the weight of the food given to calculate total food intake. Blood glucose concentrations were obtained from the tail vein immediately after the food was removed and then again 15, 30, 60 and 120 minutes after food ingestion. Additionally, 20 μ l of blood were collected from each mouse at T15 and T30. At completion of the MTT, mice were placed back into their original cages. The blood collected was later used to measure plasma insulin levels.

2. 5 INDIRECT CALORIMETRY

O₂ consumption and CO₂ production of the mice were measured in a PhenoMaster open-circuit indirect calorimetry system (TSE, Systems GmbH, Germany). The animals were first acclimated in the chambers on a LF diet for 5 day before they received either a high-sucrose or a high-protein diet supplemented with casein or pork as protein source. The indirect calorimetry provided Respiratory Exchange Ratios (RERs) which was used to determine the energy source being utilized by the animals. A RER of 0.70 indicates that fat is the main fuel source, while a value of 1.00 or above indicates that carbohydrate is the predominant fuel.

2.6 HISTOLOGY

2.6.1 Fixation with Paraformaldehyde and Phosphate Buffer (PB)

Immediately after dissection the cassettes with tissue samples (iWAT and iBAT) were fixated in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) to preserve its structure and protect it from degradation and autolysis. The 0.1 M PB was made by dissolving 3.68 g $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ and 16.82 g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ in 1000mL ddH₂O and adjusting pH to 7.4. The tissues were stored in the fixative overnight at 4°C. The next morning the tissues were washed once in 0.1 M PB and then left in the buffer until further treatment (approximately one week).

2.6.2 Dehydration with ethanol and xylene

To remove fixation solutes and water from the tissue the formaldehyde phosphate buffer was replaced with gradually increasing concentration of alcohol, following the time schedule given below (table 2.1). When the tissue was completely dehydrated in 100 % alcohol, the alcohol was replaced with Xylen. While alcohol is insoluble in paraffin, xylen is soluble in both alcohol and paraffin. The exchange of alcohol with xylene is therefore a necessary step before paraffin infiltration.

Table 2.1: List of reagents and time of each step in the dehydration process performed manually.

Reagent	Time
75 % Alcohol	45 min
95 % Alcohol	2 x 45min
100 % Alcohol	3 x 45 min
Xylene	2 x 45 min
Parafine	overnight
Parafine	2 x 15 min

2.6.3 Paraffin infiltration and embedding

The cassettes with the tissues were placed in liquid paraffin (Histowax, Histolab products AB, Sweden) holding a temperature of 59 °C, and stored overnight. Next day, the cassettes were put in a new bath of liquid paraffin for 30 minutes to remove all the remnants of xylene. Subsequently the tissues were embedded in paraffin using EC 350 Paraffin embedding center (Microtom International GmbH, Germany). First, a suitable metal mould had to be filled with small amount paraffin. Then, the tissue had to be placed in the mould and covered with the bottom of the cassette. Finally, the mould could be filled completely with paraffin and put on a cold board. When it was completely stiffened the block of paraffin could easily be removed from the mould and stored in the fridge.

2.6.4 Sectioning and staining

A microtome (Leica RM2165, Germany) was used to cut 3 µm thin sections of the embedded tissue. The slices were then carefully placed in dissected water heated to 35°C to help the slices stretch. Finally, the sections were placed on glass slides and left to dry.

In order to examine the slides with a microscope the section was stained with hematoxylin and eosin (table 2.2). Hematoxylin stains the nucleus of the cell, while eosin stains the cytoplasm. After staining, the slides were mounted with xylene based mounting medium (microscopy, Entellan, Germany).

Table 2.2: Overview of the rehydration, staining and 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	5 min
Hematoxylin	2 min
H2O	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

2.6.5 Microscopy

Cell size of eWAT and iWAT from the different groups was compared using a binocular microscope (Olympus BX5, system microscope, Japan) and representative parts were photographed using a camera (Olympus DP50 3.0) combined with the microscope.

2.7 REAL TIME qPCR

Small samples of the adipose tissues (iWAT and iBAT) collected during termination were extracted in Quiazol and isolated RNA was quality checked before transcribed into cDNA templates by reverse transcriptase. The cDNA templates were run in a real-time PCR instrument and relative mRNA expressions were measured.

2.7.1 RNA extraction with Qiazol

Principle:

The first step in RNA extraction is homogenization of the tissue in Triazol. Triazol is a monophasic reagent which contains phenol and guanidine salt and facilitates lysis of the tissue and inactivates RNases. After homogenization RNA is separated from DNA and proteins by adding chloroform. RNA is then extracted from the water phase by adding isopropanol. Precipitated RNA is washed in ethanol and finally dissolved in RNase free water.

Procedure:

Small tissue samples collected during termination were placed in small RNase free tubes with 1 mL of QIAzol and zirconium beads. The tubes were homogenized at 6000 rpm, 3 x 15 sec. in a homogenizer instrument (Precellys 24 lysis & homogenization instrument, Bertin Technologies, Franze). Afterwards, the homogenate were centrifuged at 1200 x g for 10 minutes at 4°C and incubated in room temperature for 5 min. Then 300 µl of chloroform was added to each tube and shaken manually for 15 seconds before incubated for another 2-3 minutes in room temperature. After incubation the samples were again centrifuged at 1200 rpm x g for 15 minutes at 4°C to separate the solution into three phases: a phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The upper layer was transferred to a new set of tubes and 500 µl of isopropanol was added to each tube. The samples were first incubated for 10 min in room temperature and then incubated another 10 min at 4°C. After incubation the samples were centrifuged at 1200xg for 20 minutes at 4°C. The supernatant was removed with a vacuum suction apparatus (IBS Integra Biosciences, Vacuboy, Switzerland). The pellet was washed twice with 1 mL 75 % ethanol in DEPC and once with 1 mL 100 % ethanol. Finally, 50 µl of ddH₂O were added to each tube to dissolve the pellet. All RNA samples were then frozen at -80°C until further analysis.

2.7.2 Measuring RNA quantity and quality on Nandrop ND-100

Principle:

The NanoDrop ND-1000 (Saveen Werner, Sweeden) is a spectrophotometer who enables highly accurate measurements of the absorbance of small samples of RNA. The instrument

measures absorbance at 230 nm, 260 nm and 280 nm and calculates both the A260/A280 ratio and the A260/A230 ratio which are indicators of RNA quality.

The optimal A260/A280 ratio is 1.8-2.1; a lower ratio might indicate that RNA has not completely been dissolved in the water or that there are protein remnants in the sample, a higher ratio on the other hand might indicate that there are phenol remnants in the samples. The A260/A230 ratio should not be below 1.8; a lower ratio might indicate that there are high salt content or other impurities in the sample. NanoDrop ND-1000 also measures sample concentration and it is preferable to have a RNA concentration of more than 150 µl when performing a PCR.

Procedure:

1.8 µl of the RNA sample was placed directly onto the lower pedestal on the Nanodrop instrument. The sampling arm was closed and measurement initiated using the software on the PC. When measurement was completed, the sample arm was opened and both the lower and upper pedestals were wiped using a soft tissue. Nanodrop measurements are listed in Appendix Table A.7.

2.7.3 Measuring RNA integrity on BioAnalyzer (RNA 6000 Nano)

Principle:

The RNA sample integrity was evaluated using a special RNA LabChip kit and a BioAnalyzer. This is a widely used instrument design to determine size and quality of RNA before running real-time PCR analyzes. The method uses a microfluidic-based platform to separate RNA fragments based on molecular weight and the BioAnalyzer detects the fragments by fluorescence. The results are shown as RNA integrity numbers (RIN), gel-like images (bands) and electropherograms (graphs). A high RIN number indicates high sample quality.

Procedure:

Twelve RNA samples were randomly selected and thawed on ice while kit reagents were allowed to reach room temperature before use. The selected RNA samples were measured on NanoDrop and diluted with RNase free water to concentrations between 25 and 500 ng/µl. A gel dye mix was prepared by adding 0.5 µl of dye concentrate to 32.5 µl filtered gel

matix followed by centrifugation for 10 min at 15 rcf. Then, 9 μ l of the prepared gel dye mix was placed on the RNA 6000 Nano chip in the well marked "G". The chip was placed in the chip priming station which was closed and reopened. Another 9 μ l of the prepared gel dye mix was placed in each of the wells marked "g". Additionally, 5 μ l of RNA 6000 Nano Marker was added to all the sample wells and 1 μ l of RNA ladder was transferred into the well marked with ladder symbol to serve as an external standard. The RNA samples were incubated for 2 minutes at 70°C before 1 μ l of each samples was loaded onto the chip. The chip was vortexed at 2400g for 1 minute and placed into the Bioanalyzer and analyzed. Before and after use the electrodes in the BioAnalyzer were carefully washed with water and RNaseZap. A more detailed list of chemicals and reagents, as well as the results are presented in Appendix Table A.3 and Figure A.1.

2.7.4 cDNA synthesis using reverse transcriptiprion (RT)

Principle:

Before the extracted single stranded RNA can be analyzed by quantitative real-time PCR it has to be converted to cDNA. The generation of cDNA from RNA requires the enzyme reverse transcriptase which originally is used by retroviruses to create DNA from viral RNA.

Procedure:

Frozen RNA samples were thawed and put on ice before a portion of each RNA sample were extracted and diluted with RNase free water until the concentration reached 50 ng/ μ l (+/- 5 %). Additionally, 1 μ l of each of the original RNA samples were mixed into a RNA pool and diluted to different concentrations to make a standard curve. The RT reaction mixture was prepared as described in table 2.3 and 40 μ l of this mix was added to each well in a 96-RT plate (Thermo Scientific, USA). Then, 10 μ l of the diluted RNA samples were placed into the wells in order. Two negative controls: non-amplification control (nac) lacking the multiscribe enzyme and non-template control (ntc) with no RNA template were also added to one well each for quality assessment. A clean plastic cover was placed on top of the plate and the plate was centrifuged for 1 min at 50 g. Finally the plate was placed in the GeneAm.PCR system 9700 PCR machine (Applied Biosystems, USA) and a specified thermal cycle program was run. The finish RT plate was stored in – 20°C until further use.

Table 2.3: *Ingredients for the RT reaction mix.*

Reagent	Amount (μl)
ddH ₂ O	890
10 x TagMan RT buffer	500
25 mM MgCl ₂	1100
10 mM dNTP mix	100
Oligo D	250
RNase Inhibitor	100
Multiscribe rev. T	167

2.7.5 Real Time quantitative PCR

Principle:

Real-time quantitative polymerase chain reaction (qPCR) is a method used to amplify and quantify small sequences of cDNA. The reaction is run in a real-time PCR instrument with thermal cycling and fluorescence detection capabilities. Sequence specific primers are used to allow amplification of particular genes of interest while a fluorogenic DNA-binding dye makes it possible to monitor the magnification process. Every target molecule is copied once each cycle and data are captured throughout the thermal cycling. The speed of which the fluorescent signal reaches a threshold correlates with the amount of original gene expressed in the sample.

Procedure:

The prepared cDNA (see section 2.7.4) was thawed on ice and then vortexed at 1100 rpm for 3 minutes. Subsequently, the plate was diluted adding 50 μ l ddH₂O and centrifuged for 1 minute at 1000g. The real-time reaction mix was made by mixing 5.7 μ l of the both forward and reverse primer selected to detect the gene of interest with 570 μ l Cyber Green and 331 μ l ddH₂O. 8 μ l of the reaction mix and 2 μ l of the cDNA were transferred to a 384-well real-time PCR plate and mixed using pipette robot (Biome 3000 Laboratory Automation

Workstation, Beckman Coulter, USA). The PCR plate was then coated with an optical cover and centrifuged at 1500g for 2 minutes. Finally, the PCR plate was placed in a Light Cycler 480 machine and a real-time PCR reaction was run. The different primers used and their sequences are shown in Appendix A.6.

2.8 ELISA INSULIN KIT

Quantitative levels of plasma insulin were measured using an ELISA kit for mouse (DRG Instruments, GmbH, Germany). The ELISA kit reagents are listed in Appendix Table A.8.

Frozen plasma samples were thawed on ice and ELISA kit reagents were allowed to reach room temperature. Then, 25 μ l of different calibrators and plasma samples were placed in appropriate anti-insulin antibody-coated wells. 100 μ l of Enzym Conjugat containing peroxidase-conjugated anti-insulin antibodies was also added to each well. Subsequently, the plate was covered with plastic and incubated on a shaker for 2 hours. The incubation period enables insulin to react with both the enzyme-linked antibodies and the antibodies on the coated wells. After incubation the plate was washed 6 times in a washing buffer, using an automatic plate washer, to remove unbound conjugate. Then 200 μ l TMB substrate solution was pipette into each well followed by 15 minutes incubation. During the second incubation period the colorless TMB solution was converted into a colored product by the antibody-bound enzymes. After the incubation 50 μ l of Stop Solution was added in each well and the plate was put on a shaker for 10 minutes. Finally, the plate was placed in a spectropotometric plate reader (iEMS Reader MF, Labsystems, Helsinki) and optical density was measured at 450 nm and 620 nm. The intensity of the produced color is proportional to the amount of insulin in the plasma sample.

2.9 STATISTICAL ANALYSES

2.9.1 Microsoft Excel 2007

Microsoft Excel 2007 was used for data preparation and to calculate standard error of the mean (SEM). All data are presented as mean +/- SEM.

2.9.2 Graph Pad Prism 5

Graph Pad Prism 5 was used to perform an unpaired t-test with Welch's correction to test for differences among the means between the high-protein and high-sucrose groups within individual protein sources. All data were initially tested for normality using Shapiro Wilk normality test and D`Agostino-Pearson normality test. Results were considered significant different with P-values < 0.05.

2.9.3 STATISTICA 9.0

The treatment effects of protein:sucrose ratio and different protein sources was analyzed with STATISTICA 9.0 using a factorial ANOVA test with protein amount and protein source as categorical predictors. All data were initially tested for homogeneity and normality using the Levene`s Test for Homogeneity of Variance and P-plot, respectively. Data with heterogeneous variance were log-transformed before statistical analyses. A value of P < 0.05 was considered as statistical significant. The mice fed the LF diet were used as a reference group only and data from these mice was not included in the statistical analyses.

3 RESULTS

3.1 BODY WEIGHT GAIN AND DEVELOPMENT OF OBESITY

3.1.1 Body weight gain

It has earlier been demonstrated that increasing the protein:sucrose ratio in a high-fat diet reduced body weight gain when casein was used as protein source [70, 75, 84]. To assess the importance of protein:sucrose ratio in diet-induced obesity when other protein sources are ingested, obesity-prone C57BL/6J mice were fed the experimental diets shown in table A.1 for 12 weeks. The body weight gain of the different groups is shown in figure 3.1.

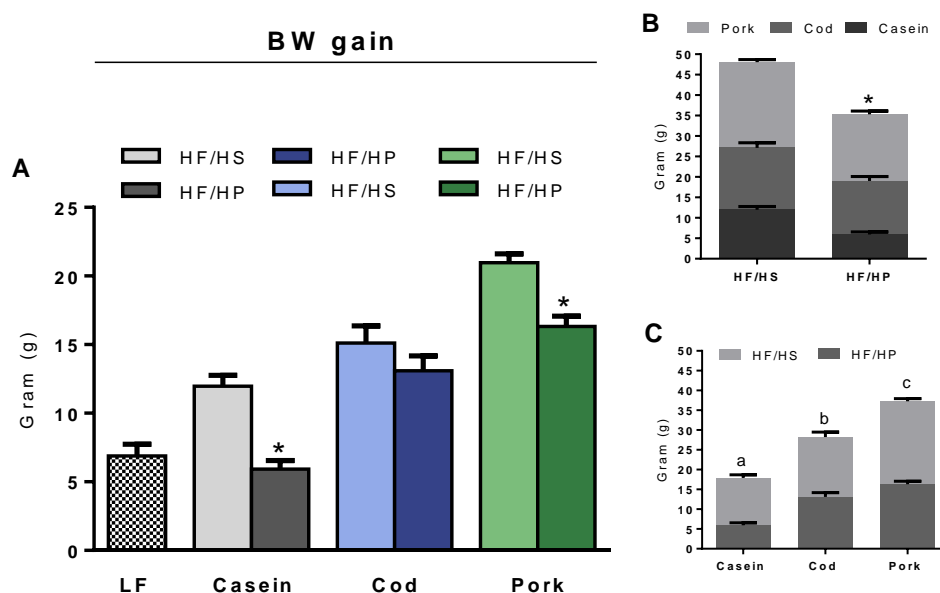


Figure 3.1: Body weight gain in C57BL/6J mice fed high-fat high-sucrose (HF/HS) or high-fat high-protein (HF/HP) diets with casein, cod or pork as protein source. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

In agreement with the earlier published results, when casein was used as protein source mice fed the HF/HS diet gained significantly more body weight than mice fed the HF/HP diet (Fig 3.1 A). Also when pork was used as protein source the HF/HS group gained significantly more weight than the HF/HP group. However, when cod was the protein source, the

difference in weight gain between the HF/HS and HF/HP group did not reach statistical significance.

To evaluate the impact of protein:sucrose ratio independent of protein source in the diet, the data was analyzed using a 2-way ANOVA test with protein amount and protein source as categorical predictors. As illustrated in figure 3.1 B, mice fed HF/HS diets gained significantly more body weight compared to mice fed HF/HP diets, independent of protein source in the diet.

Furthermore, to evaluate the impact of protein source in the diet, independent of protein:sucrose ratio, the data was once more analyzed using a 2-way ANOVA test. As illustrated in figure 3.1 C, casein-fed mice gained significantly less weight compared to mice receiving cod or pork as protein source. There was also a significant difference in weight gain between cod and pork, with pork fed mice gaining most body weight. Thus, both the type and amount of dietary protein impacted on body weight gain.

3.1.2 MRI scanning

To determine if the observed weight gain was due to increased fat mass and/or lean mass the mice were MRI scanned prior to the start of the experiment and after 10 weeks on experimental diets. The results from the MRI scan performed at week 10 are shown in figure 3.2.

When casein and pork were used as protein source, mice fed the HF/HS diet had significantly more body fat mass than mice fed the HF/HP diet (Fig 3.2 A). However, there was no significant difference in fat mass between the HF/HP and HF/HS group when cod was used as protein source. Further analyses of the data from the MRI scan showed that independent of protein source; mice fed HF/HS diets had significantly more fat mass compared to mice fed HF/HP diets (Fig 3.2 B). Furthermore, evaluating the impact of different protein sources showed that independent of protein:sucrose ratio, mice given casein had significantly less fat mass compared to both cod and pork fed mice (Fig 3.2 C). Moreover, mice fed pork had significantly more fat mass than mice fed cod.

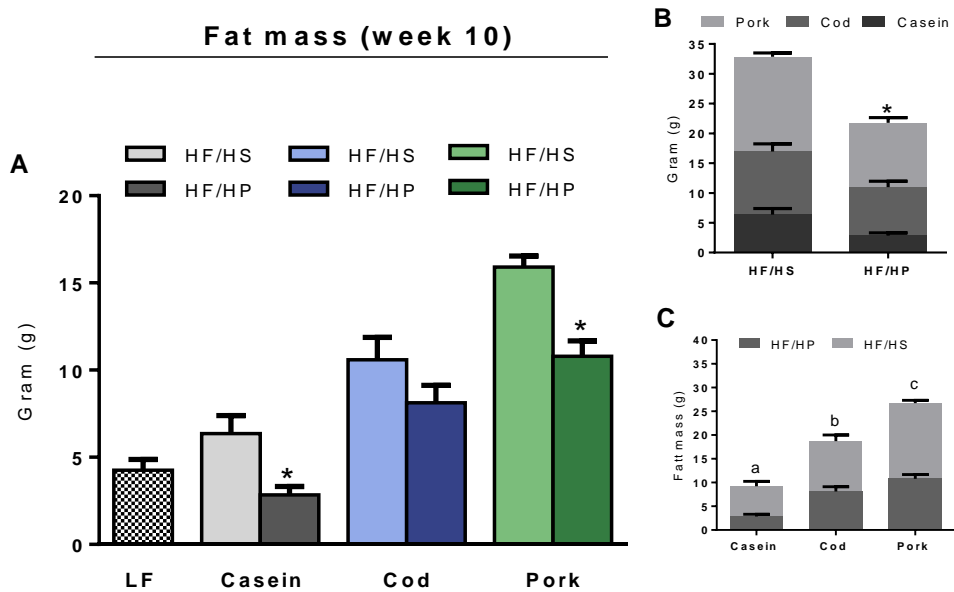


Figure 3.2: Fat mass after 10 weeks on experimental diets. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

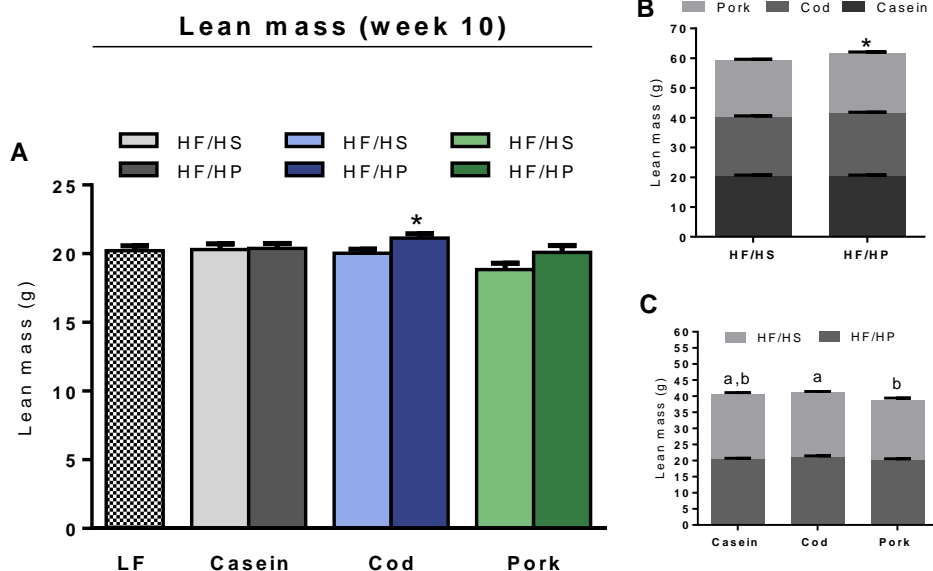


Figure 3.3: Lean mass after 10 weeks on experimental diets. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of

protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

There were no significant differences in lean mass between the HF/HS and HF/HP group when mice were fed casein and pork as protein source. However, when cod was the protein source mice fed the HF/HP diet had significantly more lean mass than mice fed the HF/HS diet (Fig 3.3 A). Despite few differences between the HF/HS and HF/HP group of the individual protein sources, a 2-way ANOVA test showed independent of protein source mice fed HF/HS diets had significantly less lean mass than mice fed HF/HP diets (Fig 3.3 B). Furthermore, evaluating the impact of different protein sources showed that cod fed mice had significantly more lean mass compared to pork fed mice (Fig 3.3 C).

Although there were some differences in lean mass after 10 weeks of feeding, the observed body weight variations between the different experimental groups were most likely due to differences in adipose tissue composition and not differences in lean body mass or free water.

3.1.2 Adipose tissue depots

To further verify differences in fat mass, one representative mouse from each group was photographed at the termination before dissection and the adipose tissue depots were weighted and their masses documented.

These pictures illustrate the differences found from statistical analysis of adipose tissue masses. As expected from the MRI scan, the adipose depots were more pronounced in mice fed HF/HS diets compared to mice fed HF/HP diets. Moreover, mice given casein as the protein source appeared to have smaller adipose tissue depots than cod and pork fed mice.



Figure 3.4: Picture of one representative mouse from each group after 12 week on experimental diet.

eWAT, iWAT and prWAT masses:

Mice fed the HF/HP casein diet had significantly less eWAT, iWAT and prWAT masses than mice fed the HF/HS casein diet (Fig 3.5 A). Also when mice received cod as protein source the eWAT and iWAT masses were significantly lower in the HF/HP group compared to the HF/HS group, but, there were no differences in prWAT mass between these two groups. When mice were fed pork there was only a significant difference in iWAT mass, and the HF/HS group had more iWAT mass than the HF/HP group. Further statistical analyses demonstrated that independent of protein source mice fed HF/HS diets had significantly more eWAT, iWAT and prWAT masses than mice fed HF/HP diets (Fig 3.5 B). Furthermore, analyses of the impact of protein source demonstrated that independent of protein:sucrose ratio casein fed mice had significantly less eWAT, iWAT and prWAT masses than mice fed cod and pork (Fig 3.5 C). There were also significant differences in eWAT, iWAT and prWAT masses between mice given cod and pork, with pork having the largest WAT depots. Thus, both the amount and source of dietary protein influenced the development of obesity.

WAT depots

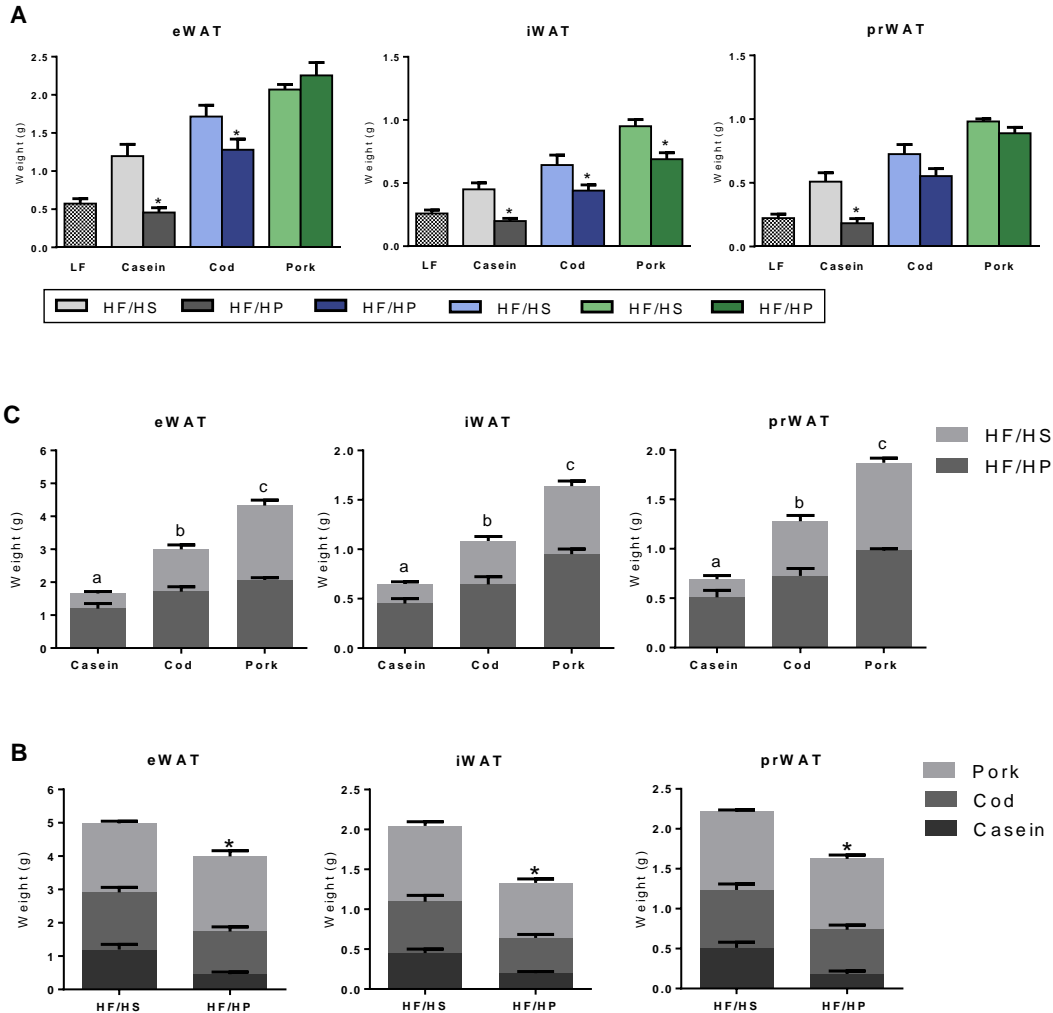


Figure 3.5: Mass of various white adipose depots. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source ($p < 0.05$). **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

BAT mass:

The iBAT mass were significantly higher in the HF/HS group compared to its respective HF/HP group (Fig 3.6 A) for all protein sources. Thus, increasing the protein amount reduced iBAT mass irrespective of protein source (Fig 3.6 B). Further analyses demonstrated that casein fed mice had developed significantly less iBAT mass than cod and pork fed mice (Fig 3.6 C). Additionally, there was a significant difference in iBAT development between cod and pork fed mice, with pork fed mice having the highest iBAT masses.

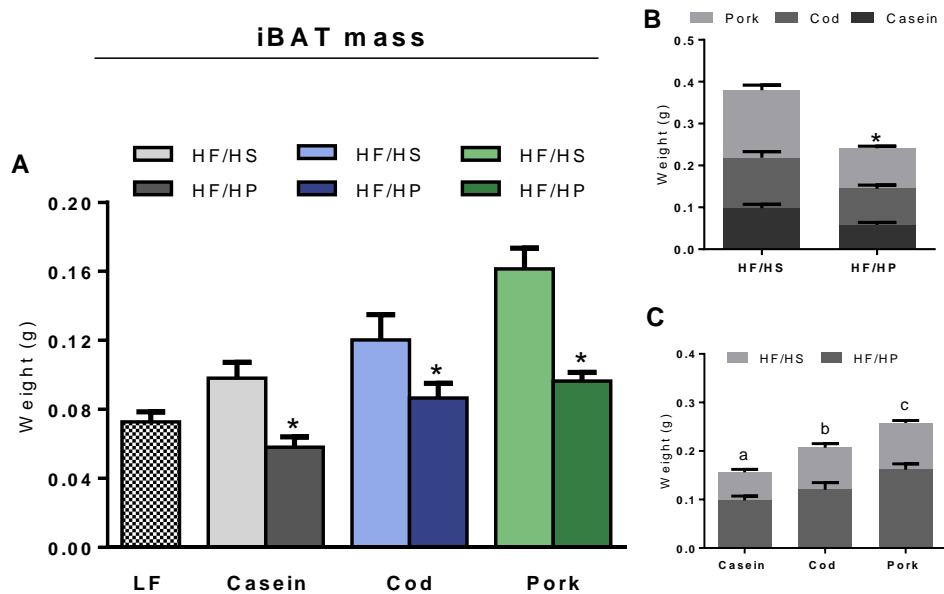


Figure 3.6: iBAT mass. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

3.1.3 Adipocyte size

Adipose tissue (iWAT) collected during termination was stained and representative parts were photographed to evaluate differences in adipocyte size.

Histology of iWAT samples illustrated that adipocyte size of iWAT in mice fed HF/HS diets was enlarged compared to mice fed HF/HP diets. Furthermore, there were visible differences in adipocytes size between mice fed casein and pork, with larger cells in the pork groups. The adipocytes from the low fat group had a size somewhere in-between the casein HF/HS and casein HF/HP group.

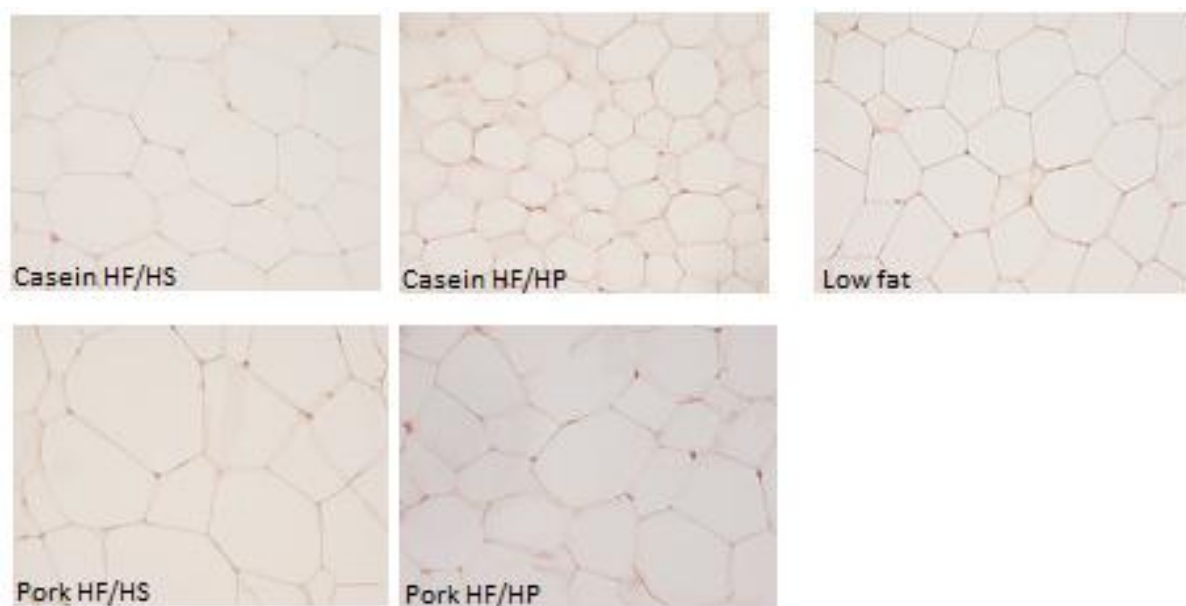


Figure 3.7: Adipocyte morphometry. Representative parts of iWAT (magnified to 400x).

3.1.4 Other organ masses

To investigate if the diets influenced on other organ masses, liver, muscle, pancreas and kidney were dissected out and weighed.

When casein and pork were used as protein source, the liver masses were significantly higher in HF/HS fed mice than in HF/HP fed mice (Fig 3.8 A). However, when cod was the protein source there was no significant difference in liver mass between the HF/HS and HF/HP group. Still, as illustrated in figure 3.8 B, independent of protein source mice fed HF/HP diets had significantly higher liver masses compared to mice fed HF/HP diets. Of

further interest, mice fed casein as protein source had significantly lower liver masses compared to both cod and pork fed mice (Fig 3.8 C).

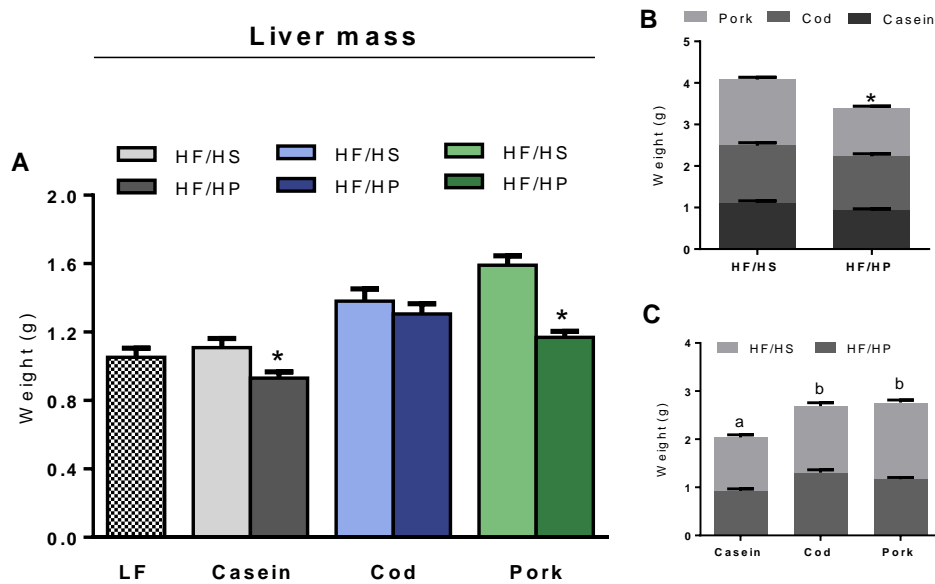


Figure 3.8: Liver mass. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

When the HF/HS and HF/HP group within the same protein group was analysed separately, no significant differences in kidney mass were found (Fig 3.9 A). However, when analysed independent of protein type, kidney masses were significantly lower in mice fed HF/HS diets compared to HF/HP diets (Fig 3.8 B). Furthermore, kidney masses were significantly lower in casein fed mice than in cod and pork fed mice (Fig 3.9 C).

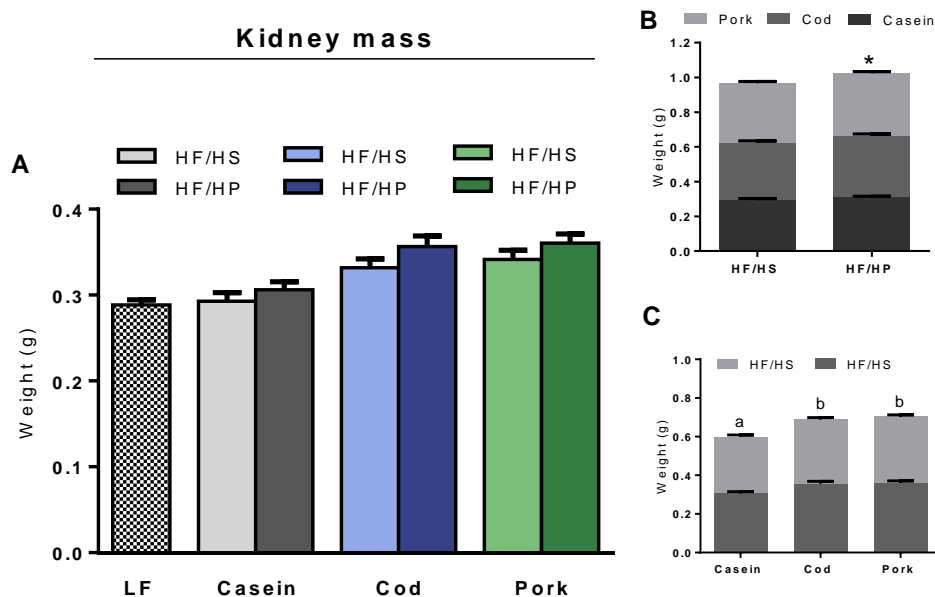


Figure 3.9: Kidney mass. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

Neither pancreas nor Musculus Tibiales masses were influenced by the experimental diets (Fig A.2 and A.3). Despite the small variations in kidney and liver weight between some of the experimental groups, differences in body mass after 12 weeks of feeding seemed to be caused by difference in adipose tissue mass.

3.2 GLUCOSE TOLERANCE AND INSULIN SENSITIVITY

3.2.1 Glucose tolerance test (GTT)

To investigate the impact of dietary protein amount and source on glucose tolerance mice were subjected to an intraperitoneal glucose tolerance test (*i.p* GTT) after 10 weeks of experimental feeding. The results of the GTT are shown in figure 3.10.

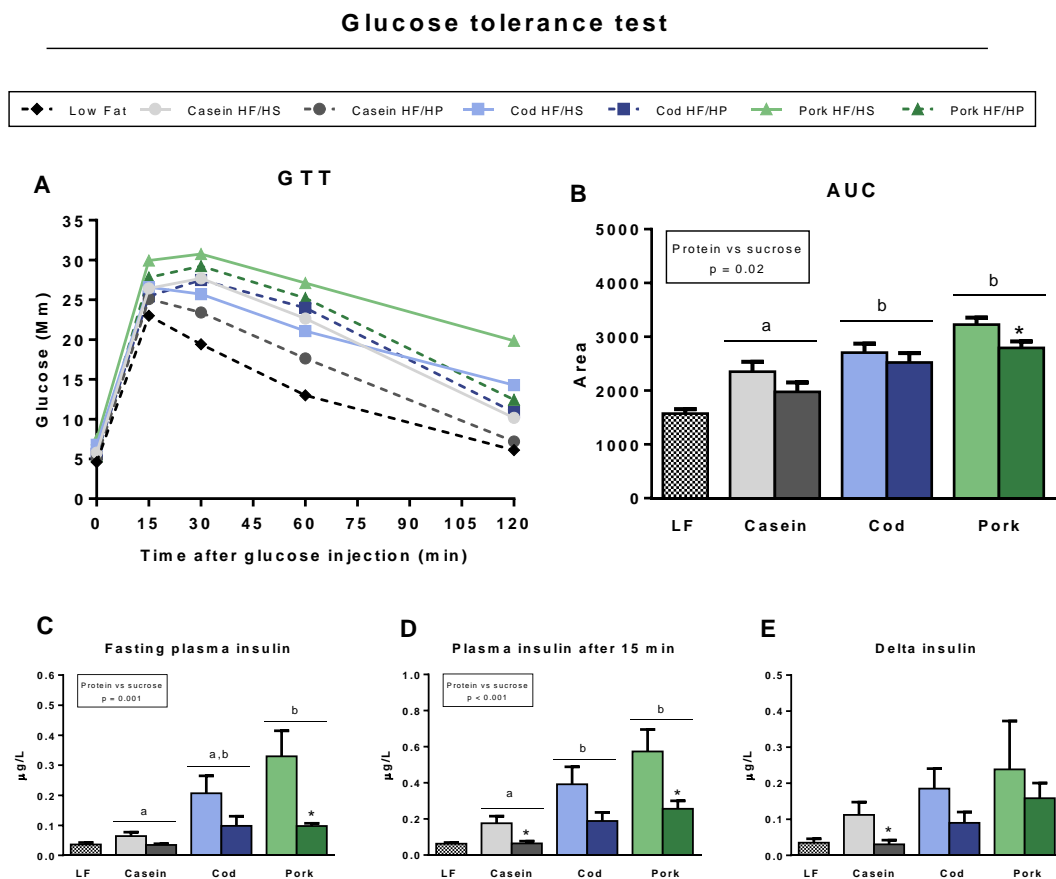


Figure 3.10: Intra-peritoneal glucose tolerance test performed after 10 weeks on experimental diets. **Figure 3.10 A.** The modification in glucose level following an injection of glucose. **Figure 3.10 B.** Area under curve (AUC). **Figure 3.10 C.** Fasting plasma insulin. **Figure 3.10 D.** Glucose-stimulated insulin secretion (GSIS). **Figure 3.10 E.** Delta insulin. **B-E:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source, p value denotes statistical significance between HF/HS independent of protein source ($p < 0.05$), different letters denotes statistical significance between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

From figure 3.10 A it appears that glucose tolerance is inversely correlated with body weight. The basal blood glucose concentrations at time 0 were approximately 5 (mmol/L) and did not differ among the treatment groups. In response to injected glucose, blood

concentrations of glucose peaked after 15-30 minutes in all the mice. Fifteen to 30 minutes postadministration of glucose, blood glucose concentrations were significantly higher in all the treatment groups when compared to the LF reference group, except for the HF/HP casein group. The same tendency was seen 60 minutes after glucose injection. Additionally, at this time point the HF/HP casein fed mice had a significantly lower blood glucose concentration than the HF/HP cod and HF/HP pork fed mice. 120 minutes after glucose injection, blood glucose concentrations in the HF/HS pork group were significantly higher than all of the other groups, except from the HF/HS cod group. Moreover, at this time point the glucose concentrations in the LF and HF/HP casein group had decreased back to basal levels, whereas the blood glucose concentration in the other groups remained elevated.

Analysis of the glucose AUC showed that when mice were fed casein or cod as protein source, there were no significant differences in glucose clearance between the HF/HP and the HF/HS group (Fig 3.10 B). However, pork fed mice in the HF/HS group had a significantly higher glucose AUC compared to mice in the HF/HP group. Evaluating the effect of protein amount, independent of protein source, showed that mice fed HF/HP diets had a significantly better glucose clearance than mice fed HF/HS diets. Furthermore, analyzing the AUC of the different protein sources showed that casein fed mice had a significantly lower glucose AUC compared to mice given cod and pork.

Glucose stimulated insulin secretion (GSIS):

As shown in figure 3.10 C fasting plasma insulin levels tended to be higher in the HF/HS group compared to the HF/HP group when mice received casein as protein source ($p=0.058$). When pork was the protein source differences between the HF/HS and HF/HP group reached statistical significance and mice in the HF/HS group had higher insulin levels compared to the HF/HP group. However, when mice were fed cod there were no differences in fasting plasma insulin levels between the HF/HS and HF/HP group. Still, a 2-way ANOVA test showed that independent of protein source, HF/HS diets lead to higher fasting insulin levels than HF/HP diets. Furthermore, mice fed casein had significantly lower fasting insulin levels than pork fed mice. Plasma levels 15 minutes after glucose injection were significantly higher in the HF/HS than the HF/HP group when casein and pork was used as protein source (Fig 3.10 D). However, when cod was the protein source there were no differences in plasma levels of

insulin between the HF/HS and HF/HP group. Anyhow, 2-way ANOVA analyses showed that independent of protein source, mice fed HF/HS diets had higher glucose-stimulated insulin levels than mice fed HF/HP diets. Furthermore, casein fed mice had significantly lower insulin levels than both cod and pork fed mice. Calculations of delta insulin (changes in insulin from 0 to 15 minutes) showed that there was only a significant difference in delta insulin between the HF/HS and HF/HP group in mice fed casein, with higher delta insulin values for the HF/HS group (Fig 3.10 E).

3.2.3 Insulin tolerance test (ITT)

To explore the role of dietary protein on development of insulin-resistance, an insulin tolerance test was performed after 11 weeks of experimental feeding. The blood glucose levels during the ITT and AUC calculations are presented in figure 3.11.

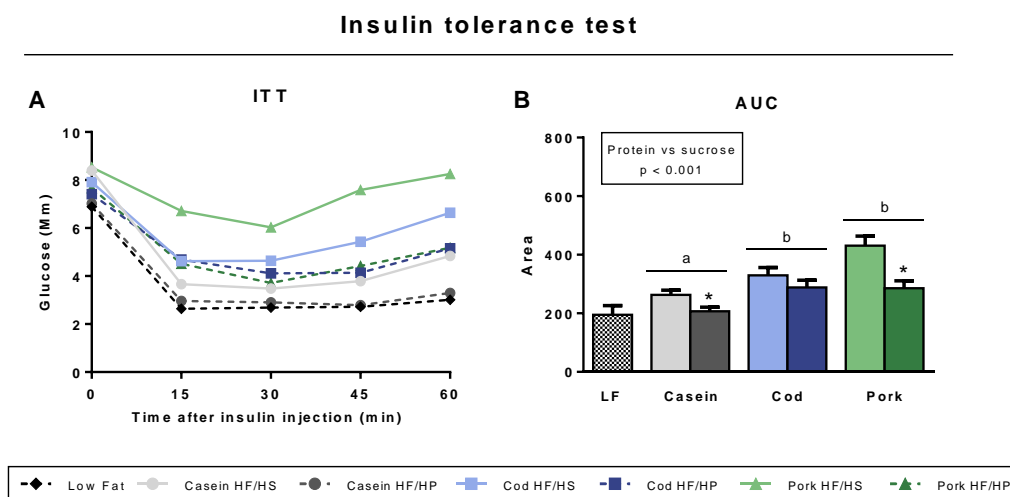


Figure 3.11: Insulin tolerance test performed after 11 weeks on experimental diets. **Figure 3.11 A.** The modification in glucose level following an injection of glucose. **Figure 3.11 B.** Area under curve (AUC): * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source, p value denotes statistical significance between HF/HS independent of protein source ($p < 0.05$), different letters denotes statistical significance between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

From figure 3.11 A it appears that insulin sensitivity is reduced with increasing adiposity of the animals. At time 0 there were no significant differences in the blood glucose levels of the treatment groups. Fifteen minutes after insulin administration all groups had a decline in plasma glucose, however glucose levels remained significantly higher in the pork HF/HS

group compared to the LF group and both casein groups at all time-points up to 60 minutes. Furthermore, at time 45 and 60, plasma glucose was significantly higher in the pork HF/HS group compared to the other experimental groups. Hence, the pork HF/HS group was not able to take up glucose in response to insulin at the same rate as the other groups. Of further interest, 60 minutes after insulin injection all treatment groups had a significantly elevated plasma glucose concentration compared to the LF control group, except for the casein HF/HP group.

Analysis of the insulin AUC values (Fig 3.11 B) showed that there were significant differences in insulin response when mice were fed casein or pork with greater insulin AUC in the HF/HS group compared to the HF/HP group. However, when mice were fed cod as the protein source no significant differences were observed between the HF/HS and HF/HP group. Furthermore, comparing the HF/HS diets versus the HF/HP diets showed that independently of protein source mice given HF/HP diets had a significantly better uptake of glucose in response to insulin compared to mice receiving HF/HS diets. Of further interest, mice fed casein had significantly lower insulin AUC compared to mice given cod and pork.

3.3 FEED EFFICIENCY AND DIGESTIBILITY

3.3.1 Energy intake

To exclude the possibility that the observed differences in weight gain was simply not due to variations in energy intake, feed consumption and total caloric intake was calculated. The total energy intake of the different groups is presented in figure 3.12.

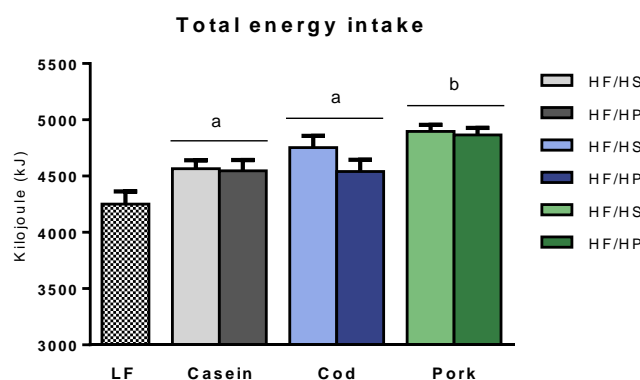


Figure 3.12: Total energy intake. Different letters denotes statistical significance between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

No differences in energy intake were observed between the HF/HS group and HF/HP group within the individual protein sources. Thus, increasing the protein amount did not have any effect on total energy intake (Fig 3.12 B). However, analyses of protein source demonstrated that independent of protein:sucrose ratio in the diet, mice fed pork consumed significantly more energy than both casein and cod fed mice.

3.3.2 Feed efficiency

To investigate if the type and amount of dietary protein had any impact on energy efficiency body weight gain from each individual mouse was divided by kilocalories eaten during the same period.

In agreement with earlier studies energy efficiency was significantly lower in the HF/HP group compared with the HF/HS group when casein was used as the protein source (Figure 3.13 A). In the high protein group of casein 184 kcal were needed to produce a weight gain of 1 g, whereas the high sucrose group only needed half as much calories to produce the same weight gain. A similar result was seen when pork was used as a protein source. However, when cod was used as protein source no significant difference in energy efficiency between the HF/HS group and HF/HP group was seen. As illustrated in figure 3.13 B, independently of protein source, mice fed HF/HP diets had markedly lower energy efficiency than mice fed HF/HS diets. Furthermore, independent of protein:sucrose ratio in the diet, mice fed casein had a significantly lower feed efficiency compared to cod and pork fed mice (Figure 3.13 C). There were also differences in energy efficiency between mice receiving cod and pork as a protein source with a significantly lower feed efficiency for cod fed mice.

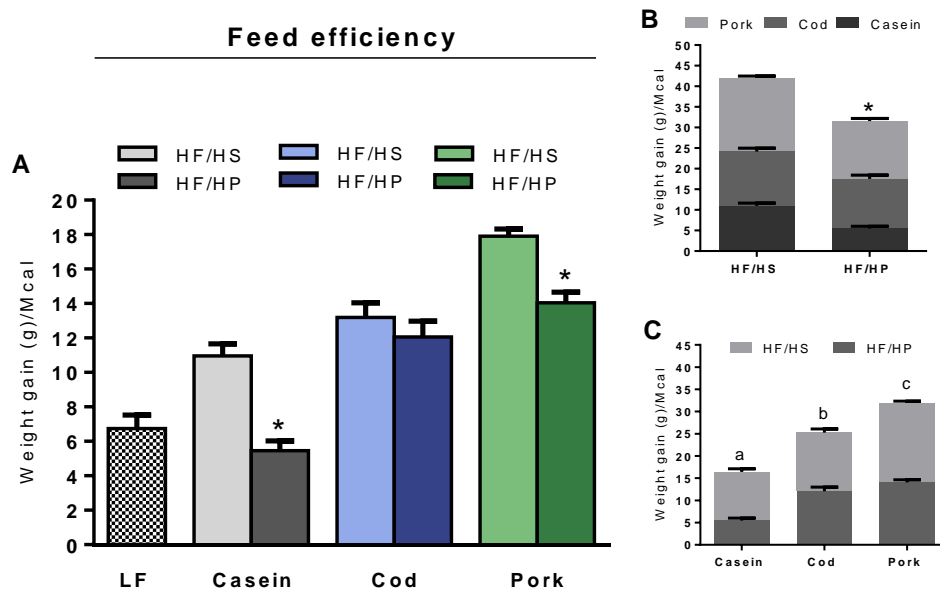


Figure 3.13: Energy efficiency. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

3.3.3 Digestibility

To investigate if the differences in energy efficiency were simply due to differences in energy absorption feces were collected, weighed and analyzed for total fat content. The amount of fat ingested during the same time period was calculated, and thus the Apparent Feed Digestibility (AFD) could be calculated using the formula:

$$\text{AFD} = ((\text{amount of fat eaten} - \text{amount of feces excreted}) / \text{amount of fat eaten}) \times 100\%.$$

Analysis of the AFD showed that when mice were given casein as the protein source, fat absorption was significantly lower in the HF/HS group compared to the HF/HP group. In contrast, no significant differences in fat digestibility were found between the HF/HS and HF/HP group when mice were given cod or pork. However, independent of protein source, mice given HF/HS diets had a lower fat absorption than mice fed HF/HP diets. Furthermore, evaluating the apparent fat digestibility in mice given different protein sources showed that mice fed casein had a significantly lower fat absorption compared to cod and pork fed mice.

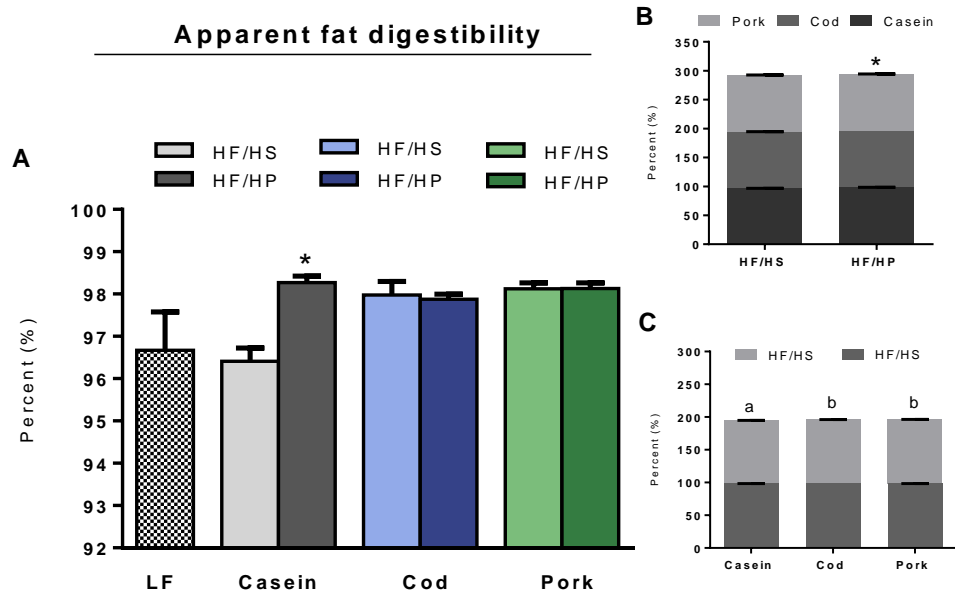


Figure 3.14: Apparent fat digestibility. **A:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source. **B and C:** Data from the HF/HS and HF/HP groups were analyzed using a 2-way ANOVA test with protein amount (B) and protein source (C) as categorical predictors. **B:** * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP diets independent of protein source. **C:** different letters denotes statistical significance ($p < 0.05$) between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

3.4 GENE EXPRESSION

Increasing the amount of casein in a high-fat diet has earlier been demonstrated to dose-dependently increase the expression of *Ucp1* and other markers for brown adipocytes in iWAT, but not in iBAT [70]. Increased *Ucp1* expression might allow energy to dissipate in form of heat and thereby protect against diet-induced obesity. To investigate if the protein:sucrose ratio was of importance in energy dissipation also when other protein sources than casein is used, expression levels of *Ucp1* and other markers for brown adipocytes were measured in both iBAT and iWAT.

Gene expression in iWAT:

Gene expression levels of *Ucp1* in iWAT, as well as other adipocyte selective genes such as *Dio2* and *Ppargca* were analyzed, but no significant differences were detected. The expression of *CideA* was significantly higher in mice fed HF/HP diets compared to HF/HS diets when analyzed independent of protein source.

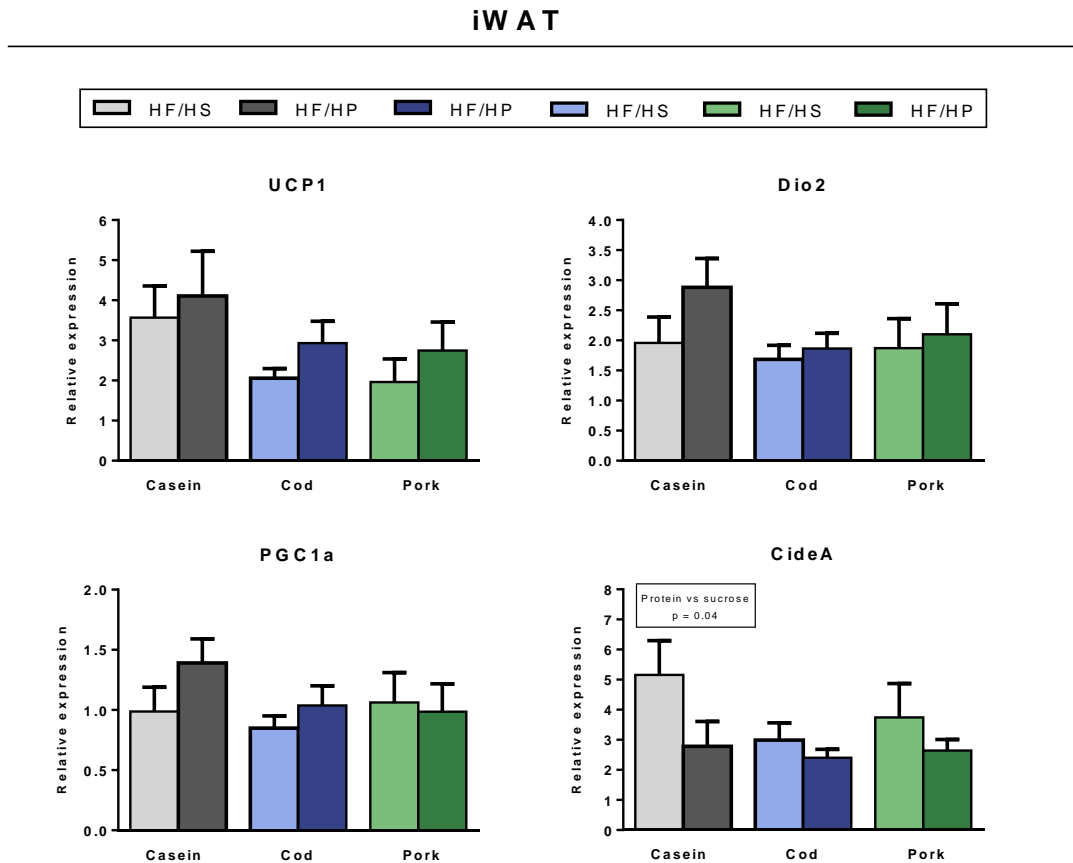


Figure 3.15: Relative gene expression of BAT-specific genes in iWAT. * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source, p value denotes statistical significance between HF/HS and HF/HP diets independent of protein source ($p < 0.05$), different letters denotes statistical significance between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

Gene expression in iBAT:

The *Ucp1* expression levels in iBAT were only significantly higher in the HF/HS group compared to the HF/HP in mice given pork as protein source. No differences were found in expression levels of *Dio2*, *Ppargα* or *CideA* between any of the groups.

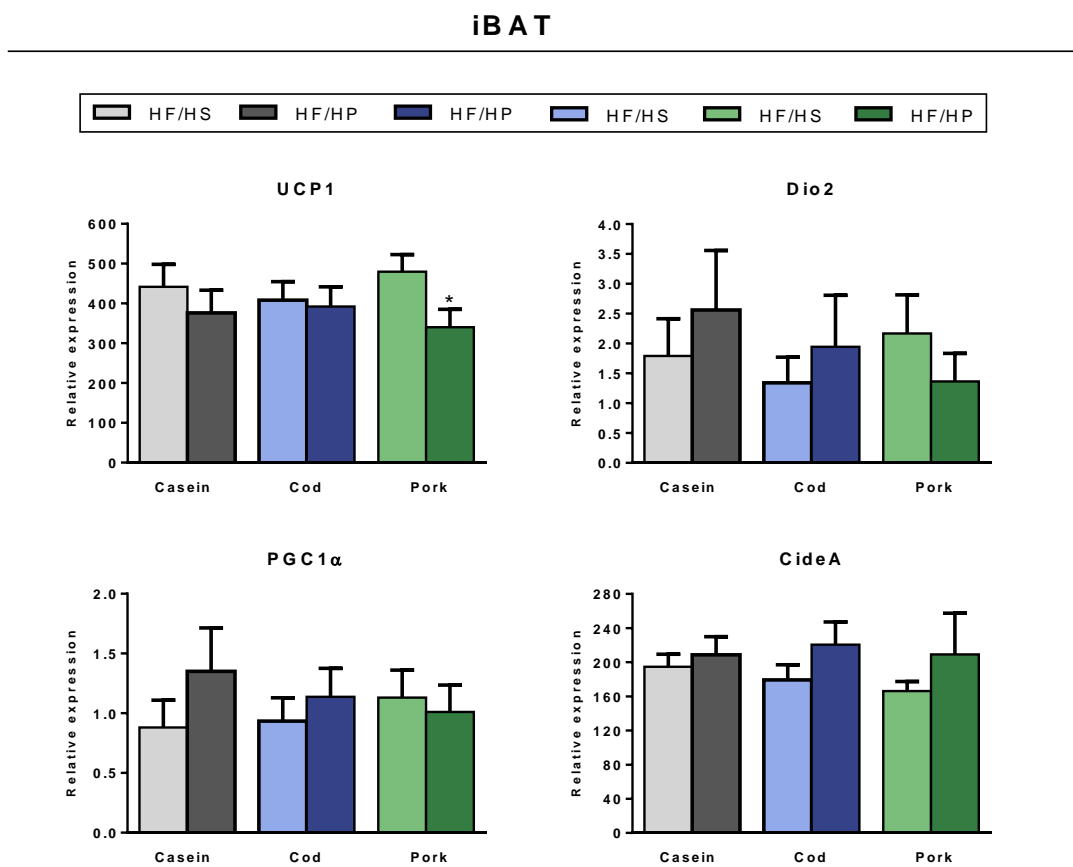


Figure 3.16: Relative gene expression in iBAT. * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source, p value denotes statistical significance between HF/HS and HF/HP diets independent of protein source ($p < 0.05$), different letters denotes statistical significance between casein, cod and pork, independent of protein amount. The results are presented as mean \pm SEM.

3.5 INDIRECT CALORIMETRY

To investigate if the experimental diets have any impact on energy expenditure a second set of mice were placed in metabolic cages and CO₂ production and O₂ consumption was measured. From these measurements the respiratory exchange ratio (RER) was calculated by dividing CO₂ produced on O₂ consumed. The RER of the different groups is shown in figure 3.17.

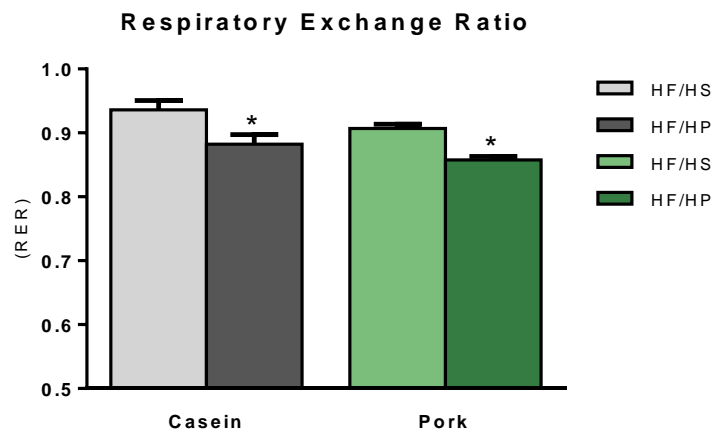


Figure 3.17: Respiratory Exchange Ratio (RER). * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups within the same protein source. The results are presented as mean \pm SEM.

The RER (the ratio of carbon dioxide produced to oxygen consumed) was significantly higher in the HF/HS group compared to the HF/HP group of both casein and pork fed mice, thus, indicating a lower rate of fatty acid oxidation in mice fed the HF/HS diets.

3.6 MEAL TOLERANCE TEST (MTT)

To further investigate by which mechanisms the different experimental diets influenced the feed efficiency and body mass development a meal tolerance tests was performed. The results of the MTT are presented in figure 3.18.

As expected, glucose AUC was significantly higher in mice given HF/HS diets than HF/HP diet when both casein and pork were used as protein source. Thus, a higher amount of sucrose in the diet significantly increased AUC compared to diets high in protein ($p < 0.001$). However, there were no significant differences in AUC between the casein and pork diet, when analyzed independent of protein:sucrose ratio.

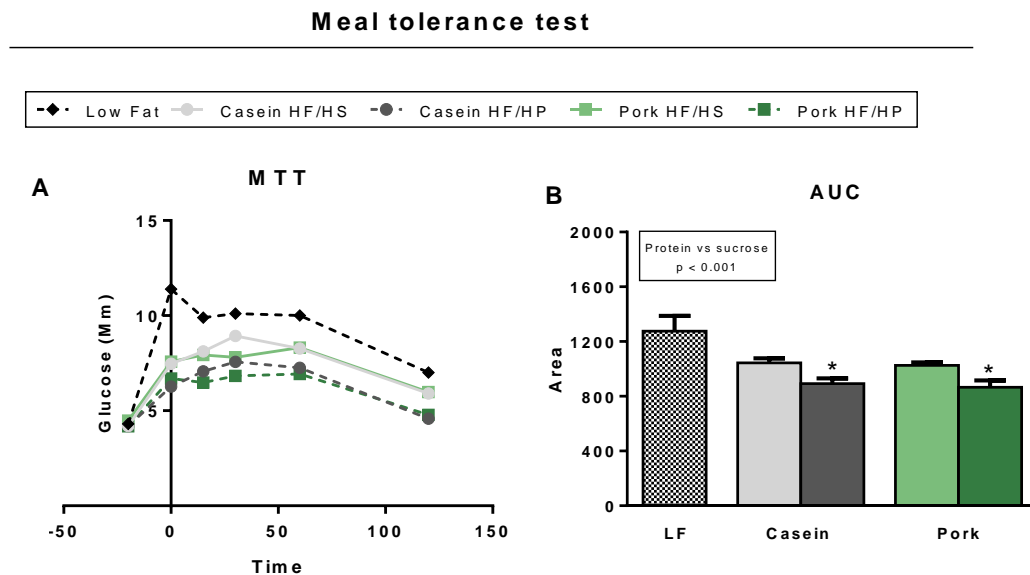


Figure 3.18: Meal tolerance test (MTT). **Figure 3.18 A.** Plasma glucose response for meal tolerance test (MTT) by dietary treatment. **Figure 3.18 B.** Area under curve (AUC). **Figure 3.18 C.** Fasting plasma insulin. * denotes statistical significance ($p < 0.05$) between HF/HS and HF/HP groups of the same protein source, p value denotes statistical significance between the HF/HS and HF/HP diets independent of protein source ($p < 0.05$). The results are presented as mean \pm SEM.

4 DISCUSSION

A number of rodent studies have demonstrated that the balance between carbohydrates and casein in the feed is a powerful regulator of the obesogenic effect of fat [70, 71, 75, 79, 80]. In fact, increasing the dietary casein:sucrose ratio strongly protects against high fat diet induced obesity (Madsen et al., 2008, Ma et al., 2011, Hao et al., 2012.) However, a recent unpublished study in our group, aiming to investigate whether intake of a high proportion of other protein sources also was able to reduce the adipogenic potential of dietary fat, demonstrated that of the protein sources tested, only a high proportion of casein were able to protect against diet-induced obesity. Terrestrial animal proteins, such as pork actually stimulated obesity whereas fish protein (cod) had an intermediate effect. Based on these previous findings we considered it important to determine if the dietary protein:sucrose ratio also is a regulator of obesity development when other sources of dietary proteins, such as pork and cod, are used as protein source. Here we demonstrate that although the type of proteins is of pivotal importance, increasing the protein:sucrose ratio suppresses obesity development in general. Thus, both dietary protein source and amount are of importance in obesity development. The mechanisms by which protein amount and source influence obesity development are not yet elucidated, but appear to involve satiety, energy expenditure and fat absorption, as well as insulin secretion.

4.1 Increasing the dietary protein:sucrose ratio attenuates obesity development

The present study demonstrated that increasing the protein:sucrose ratio attenuated body weight gain and obesity development when mice were fed casein and pork as protein source. Surprisingly, this ratio was of no significant importance when cod were used as the protein source. However, although the protein:sucrose ratio did not affect body weight gain when cod was the protein source, the 2-way ANOVA analyses revealed that independent of protein source a high dietary amount of protein reduced weight gain and obesity development. Thus, the amount of dietary protein is of pivotal importance in obesity development and several possible explanations that may explain this exists.

4.1.1 The effect of protein:sucrose ratio on satiety

Enhanced satiety and subsequent reduced energy consumption has often been cited reasons for the weight reducing effect of high-protein diets. Several studies have demonstrated that protein is more satiating than carbohydrate and fat [56, 85]. Moreover, ingestion of high-protein meals have been shown to have a stronger appetite suppressive effect than normal protein meals, both in short term and long term studies [50, 86]. The mechanisms behind the appetite suppressive effect of high-protein diets are not fully understood, but stimulation and secretion of various anorexic hormones are believed to play a role. For instance, higher plasma levels of PYY and GLP-1 in subjects consuming high-protein meals have been linked to satiety [68, 87]. Stimulation of CCK have also been linked to the satiating effect of high protein diets as proteins, but not carbohydrate and fat, stimulate secretion this peptide hormone. [58]. However, variability in protein amount, duration, meal size and subjects (normal weigh or overweight) in studies regarding this topic, makes it difficult to draw clear conclusion on the contribution of various hormones in protein-induces satiety. More research is therefore needed before nutrient-induced secretion of anorexigenic hormones can be related to satiety. Another theory implies that the satiating effect of high-protein diets are associated with elevated blood concentration of amino acids. This is termed the aminostatic hypothesis and suggests that there is a "satiety center" in the brain which are sensitive to serum amino acids levels, and once levels reaches a certain point, hunger is induced [88]. Increased energy expenditure has also been related to the appetite suppressing effect of high protein diets. For instance, Westerp-Plantenga observed that differences in dietary induced thermogenesis over a 24-hour period were significantly correlated with differences in satiety [89]. In contrast to the convincing evidence that high protein diets enhances satiety, our study did not demonstrate any differences in energy intake and hence no differences in the satiating effect of the high-protein and high-sucrose diets. This contradicting finding may be explained by the fact that although the sucrose:protein ratio in the experimental diets varied, the level of protein was relatively high in all the diets. Hence, we were not comparing normal protein diets versus high-protein diets, but rather moderate (17 E%) versus high (33E %) protein diets, indicating that all the diets had a satiating effect compared to diets with less than 20 E % protein. Satiety is clearly

influenced by a wide variety of factors and the mechanisms behind the satiating effect of high protein diets require further investigation.

4.1.2 The effect of protein:sucrose ratio on energy expenditure

Since no differences were observed in energy intake between mice fed high-sucrose and high-protein diets, other mechanisms must underlie the diets diverse obesogenic effects. An interesting finding of this study is the notable differences in feed efficiency. For example, in the HF/HP group of casein 184 kcal were needed to produce a weight gain of 1 g, whereas the HF/HS group of casein only needed half as much calories to produce the same weight gain. The finding that mice fed HF/HP diets had a significantly lower feed efficiency immediately raised the question of where the energy was dissipated and we speculated if energy expenditure or energy wasting may be increased in mice fed the high-protein diets.

Earlier studies by our group have reported that feeding mice high-fat high-casein diets increased cAMP-signaling and expression of *Ucp1* in inguinal white adipose tissue compared to high fat high sucrose diets [70, 84]. An upregulation of *Ucp1* has also been found in subcutaneous WAT of cattles fed protein-enriched diets [72]. Increased *Ucp1* expression in white adipose tissue might allow energy to dissipate in form of heat and thereby protect against diet induced obesity. In fact, reduced adiposity in transgenic mice expressing *Ucp1* has been associated with increased energy dissipation in white, but not interscapular brown adipose tissue [90]. Collectively these findings indicate that high protein diets might increase thermogenesis and dissipate energy as heat through upregulation of *Ucp1*. However, in contrast to the above mentioned studies this present study did not detected any significant differences in expression of *Ucp1* or other brown adipocyte markers in inguinal white adipose tissue. The reason for this discrepancy results is not clear, but differences in dietary levels of protein may be an explanation. Whereas the high-protein diet in our study comprised 47 E%, 17 E% and 33 E% from fat, sucrose and protein respectively, the above mentioned studies had the same percentage of fat but a higher percentage of protein (45 E%) at the expense of sucrose (8 E%). However, it should be noted that there was a pattern toward a higher expression of *Ucp1* and other brown adipocyte markers in mice fed high-protein diets compared to high-sucrose diets.

Since diet-induced thermogenesis could not explain the low feed efficiency and reduced obesity development in mice fed high-protein diets, we speculate if other energy-demanding processes might have been stimulated by the high intake of protein. In contrast to carbohydrate and fat, excess intake of protein cannot be stored in the body and needs to be processed or eliminated immediately. The high energy cost of peptide bond syntheses, as well as urea production and de novo synthesis of glucose from amino acids (gluconeogenesis) positively affect energy expenditure and might be likely contributors to the reduced feed efficiency in the high protein fed mice. A previous study by our group demonstrated that gluconeogenesis was markedly induced in mice fed high-protein diets compared to mice fed high-sucrose diets [84]. This finding was verified by measuring expression levels of enzymes involved in amino acid degradation and gluconeogenesis, as well as measuring the rise in blood glucose after injection of pyruvate. Unfortunately, we did not measure any of these parameters. The same study also reported that mice fed the high-sucrose diets had a higher CO₂ production than the high-protein fed mice and consequently had a significant higher RER. This finding is consistent with the results from our study showing that RER was significantly higher in the HF/HS groups compared to the HF/HP groups, indicating a lower rate of fatty acid oxidation in mice fed high-sucrose diets. In summary, it is reasonable to believe that increased energy expenditure, due to increased protein synthesis, ureagenesis and gluconeogenesis, together with increased fatty acid oxidation accounted for some of the reduction in feed efficiency in mice fed high-protein diets.

4.1.3 The effect of protein:sucrose ratio on fat absorption

Another potential explanation for the anti-obesogenic effect of high-protein diets is reduced digestibility and/or increased secretion of fat. Calculation of apparent fat digestibility (AFD) showed that mice fed high-protein diets had a significantly lower fat absorption than mice fed high-sucrose diets. The amount of energy or fat that is taken up from the intestinal lumen has been linked to gut bacteria composition [91]. Although time series data indicates that the composition of gut bacteria is relative stable in healthy individuals over time [92], diet-induced changes have been demonstrated to occur [93]. Interestingly, Trunbaugh et al. reported that a shift from a low-fat, plant polysaccharide-rich diet to a high-fat high sugar “Western” diet changed the microbiota in mice within a day [94]. Based on these findings it

is tempting to speculate if the experimental diets in our study may have influenced gut bacteria and thereby increased or decreased the capacity to absorb dietary fat. To date, we are not aware of any studies where possible changes in gut bacteria in response to different protein sources in mice are studied. However, a study on intestinal microbiota of adult cats demonstrated that a high dietary protein concentration resulted in a shift in the microbiota composition [95]. In conclusion, reduced fat absorption via modification of gut bacteria may also be a possible mechanism by which an increase in dietary protein:sucrose ratio reduced feed efficiency and suppressed obesity development.

4.2 Substituting casein with cod or pork protein promotes obesity development

A novel and exciting finding of this study is that different dietary proteins have different (anti)-obesogenic properties. In agreement with earlier studies by our group we found that increasing the amount of casein in the diet at the expense of sucrose protected against high-fat diet induced obesity. Surprisingly, obesity development was not prevented when the amount of cod and pork was increased. In fact, both cod and pork fed mice developed significantly more body fat than mice fed casein. Of note, mice fed pork even more body mass than mice fed cod. Thus, we demonstrate that the type of dietary protein is of pivotal importance.

4.2.1 The effect of different protein sources on satiety

Some studies have reported that different types of protein appear to exert differential effects on satiety, but little evidence exist regarding which type of protein are the most satiating. In this study, we demonstrate that mice fed pork consumed significantly more energy than mice fed cod and casein, indicating that pork may have a lower satiating effect compared to the two other protein sources. This is in agreement with an earlier study showing that a meal composed of beef or chicken induced a lower satiety than a fish meal [96]. Another study confirmed the satiating effect of fish protein, and additionally found that whey proteins had even greater effect on appetite compared to turkey and egg proteins [97]. Differences in the satiating efficacies of various proteins have been related to their specific amino acids patterns and changes in hormone concentrations. Particularly, leucine has been pointed out for its suppressing effect on appetite through its stimulation of leptin

secretion and mTOR signaling in the hypothalamus [60]. Interestingly, milk proteins contain a high proportion of BCAA, especially leucine. Thus a higher content of BCAA could, at least in part, be responsible for the lower energy intake in mice fed casein compared to mice given pork. However this would not explain the differences in energy intake between cod and pork, as these two proteins contain the same amount of BCAA. Protein-induced satiety might also be mediated through the involvement of CCK; an hormone which induces satiety by suppressing NPY levels in the hypothalamus [58]. Diepvens et al. observed that milk proteins elevated CCK more than whey proteins and pea protein; however they did not relate it to any satiating effect [98]. Another interesting finding is that incomplete protein, which lack one or more essential amino acids lack, are more satiating than complete proteins, which have adequate amounts of the essential amino acids [98-100]. The proposed mechanism behind this finding is that consumption of incomplete protein disrupts the protein synthesis process and leads to a higher concentration of circulating amino acids, which thereby function as a satiety signal [88]. Another mechanism to explain the appetite suppressive effect of incomplete protein involves recognition of protein sources low in EAA and rejection of such EAA deficient food due to adaptive behavior [101]. Casein protein, which is low in cysteine and glycine, might have a similar effect and thereby induce a stronger satiating signal than cod and pork protein. In summary, increased feed intake due to a less satiating effect of pork might, at least in part, explain why mice fed pork protein gained more weight than mice fed casein and cod. However, it is more likely that other factor such as taste, smell and texture accounted for the differences in energy intake as these factors varied greatly between the different feeds.

4.2.2 The effect of different protein sources on energy expenditure

Although pork fed mice had a higher energy intake compared to mice fed casein and cod, the most remarkable differences between the three protein sources were observed in their effect on feed efficiency. Independent of protein:sucrose ratio in the diet, mice fed casein had a significantly lower feed efficiency compared to cod and pork fed mice. There were also differences in energy efficiency between mice receiving cod and pork, with a significantly lower feed efficiency in cod fed mice. These differences in feed efficiency might be related to

the ability of the protein sources to affect energy expenditure or energy wasting. Since high protein and high sucrose diets have been shown to trigger *Ucp1* expression in white adipose tissue differently, there might be a possibility that various proteins sources also trigger expression of *Ucp1* in different degrees. Increased energy expenditure due to diet induced thermogenesis could therefore be a potential explanation for the reduced feed efficiency of casein fed mice. However, no differences in expression levels of *Ucp1* or other brown markers were found between any of the experimental groups. We therefore speculate if the reduced feed efficiency and adipose tissue mass of mice fed casein were due to other means of increased energy expenditure such as increased protein synthesis, urea production and gluconeogenesis. A study by Mikkelsen et al. demonstrated that during a 24-h stay in a respiratory chamber subjects consuming pork meals had higher energy expenditure than those consuming soy meals [102]. Tan et al. on the other hand did not find any significant differences in the effect of meat protein rich meals and dairy or soy protein meals on energy expenditure and fat oxidation [103]. At the moment the literature contains little information about this topic and no clear evidence showing that different protein sources affect energy expenditure differently exist. More studies comparing the effect of different protein sources on energy expenditure is therefore needed before any conclusions can be made.

4.2.3 The effect of different protein sources on fat absorption

Another potential explanation for the reduced feed efficiency and body fat mass in casein fed mice is reduced digestibility and/or increased secretion of fat. Indeed, calculations of Apparent Feed Digestibility (AFD) showed that casein significantly reduced fat absorption compared to cod and pork. This is consistent with previous work showing that mice fed a very high-fat diet in combination with casein extracted a much higher percentage of fat in feces than mice receiving a very high-fat diet with salmon as protein source [104]. Furthermore, a study by Lorenzen et al. reported that subjects consuming a high amount of calcium from dairy products had a lower postprandial lipidemia [105]. This suggests that the reduced fat absorption in mice fed casein might be related to the calcium content. Several studies, both in animals and humans, have shown that calcium intake increases the fecal excretion of fat, presumably via formation of insoluble calcium fatty acid soaps or by binding of bile acids [106, 107]. However, the fact that we used casein sodium salt from bovine milk (Sigma, batch number 080M0006) which is relatively low in calcium excludes dietary calcium

content as an explanation for the reduced fat absorption in casein fed mice. However, we cannot exclude the possibility that the amino-acid composition of the different protein sources may influence on the absorption of fat. In contrast to cod and pork protein, casein has a relatively low content of the sulfur amino acids cysteine and glycine, which both are known to play a role in bile secretion [108]. It is a possibility that the low content of cysteine and glycine in casein reduces bile secretion and thereby decreases fat absorption and increases faecal fat extraction. However, at the moment the literature contains no information to support or discard this theory and more research is needed in order to reveal the mechanism behind the differential effect of various proteins on postprandial fat absorption. Taken together, our finding that casein protein reduced fat absorption indicates that increased faecal secretion of fat might be a contributing factor to the lower feed efficiency in mice given casein as protein source. However, apparent fat digestibility fails to explain differences in feed efficiency between cod and pork as there were no differences in fat absorption between these two protein groups.

4.3 The effect of protein amount and protein source on glucose tolerance and insulin sensitivity

Obesity and glucose intolerance often co-occur. Obesity is also frequently associated with insulin resistance and is a major risk factor for non-insulin-dependent diabetes type 2 [13]. The mechanisms linking obesity to insulin resistance and diabetes in humans is not well understood but is believed to involve production of various secretory products from the adipose tissue [17]. The results from the GTT demonstrated that mice fed high-protein diets had a significantly lower glucose AUC than mice given high-sucrose diets. Additionally, the GTT showed that casein fed mice had a significantly lower glucose AUC than mice fed cod or pork. Furthermore, fasting plasma insulin levels were significantly higher in mice fed the high-sucrose diets compared to mice fed the high-protein diets. The higher fasting insulin level suggests that the pancreas already was trying to compensate for a lower glucose tolerance with increased secretion of insulin. To investigate if the lower levels of blood glucose were due to increased secretion of insulin, plasma insulin levels fifteen minutes after glucose administration were also measured. The insulin levels fifteen minutes after

administration of glucose increased in all experimental groups but calculation of delta insulin revealed a significant difference between the HF/HS and HF/HP only in mice fed casein. This indicates that the low levels of glucose seen in mice fed the high-proteins diets compared to the high-sucrose diets, and casein fed mice compared to cod and pork fed mice, most likely were not due to increased secretion of insulin from pancreas. However, since the administered glucose dose was based on body weight (2 mg glucose/g body weight) we speculate if the differences in glucose levels simply just reflect differences in dose of glucose given.

To evaluate insulin sensitivity, an insulin tolerance test where mice received 0.5 U insulin per kilogram body weight was performed. The results from the ITT showed that although the high-sucrose fed mice received a greater dose of insulin, they still had an increased level of blood glucose (higher glucose AUC) compared to the high-protein fed mice. Furthermore, the results from the ITT demonstrated that mice receiving casein as the protein source had a significantly lower glucose AUC compared to mice given cod or pork, despite the fact that they were given a lower dose of insulin. This indicates that mice given high-sucrose diets had a reduced glucose tolerance compared to mice fed high-protein diets. Furthermore, it indicates that mice receiving casein had a better glucose tolerance than cod and pork fed mice. Notably, the HF/HP casein group had glucose levels comparable with the LF control group. We further speculate if some of the groups might have developed insulin resistance. The high levels of blood glucose despite administration of a high dose of insulin, as well as the great fat mass gain of the HF/HS pork group suggests that mice in this group might have become insulin resistant.

Another important finding from the GTT and ITT is that whereas the blood glucose concentration and insulin secretion were higher in the HF/HS group than the HF/HS group of mice fed casein or pork as protein source, there were no differences between the HF/HS and HF/HP group when mice were fed cod. These findings are in line with body mass gain data, suggesting that difference in body mass development may be related to insulin secretion. Insulin is known to be a strong adipogenic hormone which stimulates adipocyte differentiation and adipose tissue expansion. The crucial role of insulin in obesity development has been confirmed by Bluher et al. who demonstrated that transgenic mice

lacking insulin receptors in adipose tissue were completely protected against high-fat diet-induced obesity [73]. As pointed out earlier, delta insulin calculation revealed that injection of glucose stimulated a significantly higher secretion of insulin in mice fed casein HF/HS diets compared to mice fed HF/HP diets. However, although there was a pattern towards a higher insulin secretion in the HF/HS group for mice fed cod and pork, the differences did not reach statistical significance due to the large individual differences within each group. Yet, we cannot exclude the possibility that a statistical significance would be obtained by including a higher number of mice in each group. Therefore, it is reasonable to suggest that the beneficial effect of high-protein diets on adipose tissue development to some degree are derived from a lower carbohydrate content resulting in lower postprandial increase in blood glucose and lower insulin response. To confirm this hypothesis we conducted a meal tolerance test. The animals received either a high-sucrose or a high-protein diet supplemented with casein or pork as protein source. These two protein sources were chosen as they represent the lower and upper extreme regarding body fat mass. As anticipated, the MTT results demonstrated that high-sucrose diets lead to a higher blood glucose concentration compared to high-protein diets. Unfortunately, due to complications with the deliverance of ELISA-kits, plasma insulin levels have not yet been measured. However, in keeping with the ability of sucrose to stimulate insulin secretion via its glucose moiety, we predict that plasma levels of insulin will be increased in mice given high-sucrose diets.

4.4 THE ANIMAL MODEL AND RELEVANCE TO HUMANS

Mice are a broadly used animal model in nutritional research because of their remarkable genetic as well as physiological and metabolic similarities to humans. However, precautions must be taken when interpreting the human relevance of mice studies as experimental mice often are inbred strains with a less genetic diversity than humans. Additionally, it is important to keep in mind that particular mice strains have specific features and therefore not necessarily assume that an observed effect is a general phenomenon. The feeding experiment in this study was performed on C57BL/6J mice which has a unique ability to develop obesity, along with hyperinsulinemia and hyperglycemia in response to high-fat diets. Consequently, this mice model provides a great model to study the pathophysiology of an obesity syndrome quite similar to human obesity.

A great deal of the research regarding overweight and obesity has focused on the effect of different types of dietary fat (n-6 and n-3 PUFAs) and carbohydrate (high- and low GI). Here we demonstrate that also the type of dietary protein is of importance in obesity development. Interestingly, our results showed that casein protein clearly attenuated obesity development, while pork protein had the opposite effect and promoted fat mass gain. If similar effects are found in humans, this is of great concern as pork is a major component in our diet and is recognized as a healthy alternative to red meat, while cheese on the other hand, along with other dairy products, have been incriminated as a source of unhealthy saturated fatty acids. Interestingly, researchers from the University Catholique de Louvain in Belgium found that treating obese and type 2 diabetic mice with ripened cheese improved glucose tolerance and adipose tissue oxidative stress [109]. Of note, high cheese consumption has also been suggested as an explanation of the “French paradox” [110]. Furthermore, some researcher at the University of Oslo reported in an epidemiologic study that the frequency of cheese intake dose-dependently counteracted the association between soft drink intake and risk of developing metabolic syndrome [111]. This is of importance as today’s high intake of soft drinks, due to their sugar content, often is linked to the persistent epidemic rate of obesity. It is tempting to draw a connection between these finding and the result of our study showing that increasing the protein:sucrose ratio in the diet (“high cheese:soda ratio”) attenuated obesity development and reduced the incidence of glucose intolerance. However, if our results have human relevance and if cheese or other types of dairy products have potential health benefits remains to be investigated.

4.5 FUTURE PERSPECTIVES

The contemporary Western diet contains an average of 49 % energy from carbohydrate, 35 % from fat and 16 % from protein. Thus, it would have been interesting to look at the effects of casein and other protein sources when included in a typical Western diet. Since a high protein:sucrose ratio appeared to be of no importance when cod was used as the protein source, further investigation using cod might be of particular interest. Additionally, since high- and low-GI diets will affect insulin secretion differently, it would be interesting to combine different protein sources with high- and low GI carbohydrates.

To further investigate if increased thermogenesis is a potential mechanism by which high protein diets reduce feed efficiency and obesity development, we could have performed a Western blot or/and an immunohistochemical staining of the white adipose tissue to detect UCP1 protein levels rather than *Ucp1* mRNA levels. More investigation regarding energy expenditure will also be required in the nearest future. The data from the metabolic cage experience in Copenhagen needs to be further analyzed to check for differences in eating pattern, heat production and activity.

This experiment was carried out to investigate if increasing the protein:sucrose ratio could attenuate obesity development. However, our results do not predict if increasing the protein:sucrose ratio can reduce already developed overweight or obesity. Therefore, it would be exciting to investigate if any of the different experimental diets could promote weight loss in animals that already were obese.

Furthermore, it would be of great importance to investigate if our results can be linked to human nutrition as our findings provide novel information regarding the role of amount and type of proteins on obesity development. Similar findings in human studies might be useful in both prevention and treatment of obesity. In order to elucidate if the demonstrated result also concerns human nutrition, human intervention trials comparing the effect of different protein sources and amount on energy expenditure, conducted in a standardized context over a longer period of time, are necessary. Moreover, since humans consume proteins as a part of a meal, that also contains other macronutrients; the metabolic effect of the different experimental diets should be evaluated as part of composite meals.

5 CONCLUSION

The current study presents two main findings. Firstly, we report that increasing the dietary protein:sucrose ratio in general attenuates obesity development in C57BL/6J mice. Secondly, we demonstrate that casein protein protects against high-fat diet-induced obesity in C57BL/6J mice, whereas cod, and pork protein in particular, promotes obesity development. The beneficial effects of the high-protein diets containing casein as protein source appear to involve multiple mechanisms, including increased energy expenditure, reduced fat absorption and potentially decreased insulin secretion. Although the results cannot be directly interpolated to human nutrition due to the experimental model used, our findings suggest that in dietary practice it may be beneficial to partially replace refined carbohydrate with carefully selected protein sources. More research is needed in order to elucidate the specific mechanisms underlying our unique findings and to further establish if the results have human relevance.

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APPENDIX

Appendix I – Diets

Table A.1: Components of the different experimental diets (given in g/kg).

Group	A	B	C	D	E	F	G
(g/kg)	Low fat	High fat					
Ingredients	Casein	High sucrose Casein	High protein Casein	High sucrose Cod	High protein Cod	High sucrose Swine	High protein Swine
Casein	207	207	414	–	–	–	–
Cod	–	–	–	209	419	–	–
Swine	–	–	–	–	–	237	474
L-Cystine	3	3	3	3	3	3	3
Sucrose	100	440	210	440	210	440	210
Corn oil	69	249	248	244	238	233	217
Cellulose	50	50	50	50	50	50	50
t-Butylhydroquinone	0,014	0,014	0,014	0,014	0,014	0,014	0,014
Min.mix	35	35	35	35	35	35	35
Vit.mix	10	10	10	10	10	10	10
Choline Bitartrate	2,5	2,5	2,5	2,5	2,5	2,5	2,5
Potato starch (Dextrin)	523,71	3,71	27,93	6,02	32,56	-10,73	-0,95
Fat (from protein powder)	1,12	1,12	2,23	5,84	11,68	16,63	33,25
Total	1000	1000	1000	1000	1000	1000	1000

Appendix II – Reagents used in RealTime qPCR

Table A.2: Reagents used in RNA extraction.

Product	Vender
QIAzol reagent	QIAgen, Germany
Chloroform	Merch, Germany
Isopropanol	Arcus kjemi, Norway
Ethanol	Arcus kjemi, Norway
DEPC	Sigma, USA
RNase free ddH2O	MiliQ Millipore, USA

Table A.3: Reagents used in RNA qualification in Bioanalyzer.

Product	Vender
Rnase free ddH2O	MiliQ Millipore, USA
RNA 6000 Nano LabChip Kit	Agilent Technologies
RNA 6000 Nano Ladder	Ambion, USA

Table A.4: Reagents in RT reaction mix.

Product	Vender
RNase free ddH ₂ O	MiliQ Millipore, USA
TagMan RT buffer 10x	Applied Biosystems
25 mM magnesium chlorid	Applied Biosystems
10 mM DeoxyNTPs	Applied Biosystems
50 µM Oligo d(T) 16 primer	Applied Biosystems
RNase Inhibitor (20 U/µl)	Applied Biosystems
Multiscribe Reverse Transcriptase	Applied Biosystems

Table A.5: Reagents used in Quantitative Real-Time qPCR

Product	Vender
RNase free ddH ₂ O	MiliQ Millipore, USA
SYBR GREEN Master	Roche, Norway
Primer (see table XX)	Invitrogen, UK

Table A.6: List of primers used in Real-Time qPCR (obtained from Invitrogen, UK).

Housekeeping gen	Sequence 5' → 3'	
TBP	Forward	ACC CTT CAC CAA TGA CTC CTA TG
	Reverse	ATG ATG ACT GCA GCA AAT CGC
β-actin	Forward	ATG GGT CAG AAG GAC TCC TAG G
	Reverse	AGT GGT ACG ACC AGA GGC ATA C

Primer	Sequence 5' → 3'	
UCP-1	Forward	AGC CGG CTT AAT GAC TGG AG
	Reverse	TCT GTA GGC TGC CCA ATG AAC
Dio2	Forward	GCC CAG CAA ATG TAG AC
	Reverse	TGG CAA TAA GGA GCT AGA A
PGC1α	Forward	CAT TTG ATG CAC TGA CAG ATG GA
	Reverse	CCG TCA GGC ATG GAG GAA
Ap2	Forward	ACA GGA AGG TGA AGA GCA TC
	Reverse	CCT TTG GCT CAT GCC CTT TC
CideA	Forward	TGC TCT TCT GTA TCG CCC AGT
	Reverse	GCC GTG TTA AGG AAR CTG CTG

Appendix III – Nano Drop

Table A.7: Nano Drop measurements of RNA concentration and A260/A280 and A260/A230.

iWAT - NanoDrop measurements				iBAT - NanoDrop measurements			
Mouse no.	[RNA] (ng/uL)	260/280	260/230	Mouse no.	[RNA] (ng/uL)	260/280	260/230
1	474,1	1,91	2,24	1	931,6	2,01	2,31
2	387,3	1,83	2,19	2	484,2	1,95	2,6
3	419,9	1,84	2,28	3	407,8	1,94	2,26
4	68,7	1,74	2,17	4	578,1	1,98	2,29
5	244,7	1,85	2,27	5	530,3	1,95	2,03
6	367,0	1,85	2,29	6	1596,9	1,98	2,28
7	430,9	1,90	2,35	7	1178,1	1,96	2,27
8	517,6	1,91	2,35	8	629,2	1,96	1,90
9	317,8	1,87	2,18	9	1047,4	1,96	2,21
10	364,3	1,84	2,17	10	1041,9	1,97	1,88
11	407,4	1,84	2,32	11	737,9	1,95	1,57
12	358,1	1,88	2,24	12	617,0	1,94	1,67
13	333,0	1,85	2,26	13	675,3	1,94	2,28
14	449,6	1,84	2,21	14	981,0	1,96	2,31
15	425,8	1,91	2,35	15	659,7	1,97	2,24
16	343,0	1,91	2,17	16	558,3	1,92	2,34
17	254,2	1,86	2,31	17	679,2	1,94	1,98
18	493,4	1,86	2,24	18	557,5	1,92	2,23
19	466,7	1,87	2,24	19	441,3	1,84	1,25
20	423,8	1,86	2,32	20	481,9	1,81	2,14
21	220,1	1,82	2,14	21	460,5	1,85	1,95
22	443,4	1,87	2,27	22	586,6	1,94	2,12
23	351,7	1,83	2,17	23	443,3	1,86	2,31
24	443,2	1,87	2,31	24	655,6	1,96	2,21
25	150,4	1,80	2,09	25	411,3	1,85	2,27
26	172,9	1,82	2,25	26	500,3	1,87	2,3
27	355,6	1,81	2,21	27	313,5	1,94	2,43
28	272,6	1,86	2,16	28	547,5	2,00	2,39
29	384,7	1,90	2,20	29	650,2	2,01	2,38
30	101,6	1,80	1,90	30	499,4	2,03	2,39
31	214,7	1,87	2,45	31	710,4	2,05	2,41
32	102,2	1,81	2,47	32	336,9	1,98	2,34
33	244,8	1,84	1,85	33	433,2	1,95	1,99
34	220,6	1,81	2,21	34	543,8	1,96	2,28
35	242,1	1,83	2,17	35	650,5	1,98	2,32
36	210,4	1,84	2,14	36	949,7	2,03	2,29
37	358,0	1,90	2,35	37	597,8	1,98	2,31
38	342,3	1,86	2,18	38	256,7	1,89	2,23
39	222,0	1,85	2,12	39	692,9	2,03	2,34
40	481,0	1,90	2,21	40	671,7	2,04	2,35

41	260,5	1,86	2,22	41	499,4	1,96	2,36
42	284,0	1,84	2,23	42	674,7	2,04	2,34
43	199,5	1,84	2,27	43	1096,9	2,01	2,32
44	260,9	1,80	2,28	44	NaN	NaN	NaN
45	340,2	1,86	2,36	45	962,4	2,01	2,23
46	385,5	1,87	2,16	46	718,9	1,99	2,26
47	116,7	1,73	2,03	47	613,3	1,96	2,34
48	189,5	1,75	2,02	48	891,8	1,99	2,37
49	199,5	1,83	2,09	49	906,6	2,01	1,7
50	-	-	-	50	-	-	-
51	275,3	1,88	2,11	51	932,3	2,00	2,32
52	162,1	1,83	1,92	52	820,0	1,99	2,33
53	109,9	1,81	2,10	53	984,1	1,99	2,35
54	242,6	1,82	2,29	54	974,5	1,98	2,36
55	309,5	1,86	2,16	55	667,2	1,97	2,36
56	452,5	1,86	2,10	56	948,1	1,99	234
57	222,2	1,82	2,46	57	968,2	1,99	2,33
58	81,5	1,81	2,03	58	1096,1	1,98	2,33
59	132,2	1,82	2,10	59	1178,0	1,98	2,34
60	171,8	1,81	2,04	60	1030,4	1,96	1,95
61	196,7	1,87	2,14	61	974,6	2,00	2,32
62	251,1	1,81	2,34	62	480,9	1,95	2,38
63	372,1	1,89	2,31	63	603,1	1,94	2,38

Appendix VI: ELISA Kit

Table A.8: Reagents in Insulin Mouse Ultrasensitive Elisa Kit.

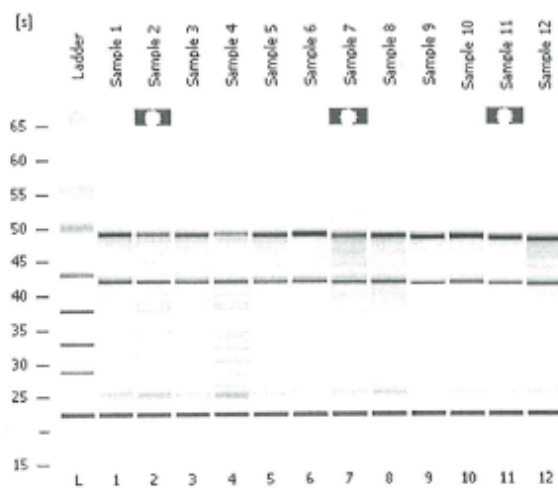
Product	Vender
Insulin Mouse Ultrasensitive ELISA Kit	DRG Instruments GmbH, Germany
Coated plate	
Calibrator 0 (1 vial)	
Calibrator 1,2,3,4,5 (5 vials)	
Enzyme Conjugate 11X (1 vial)	
Enzyme Conjugate buffer (1 vial)	
Wash buffer (1 bottle)	
Substrate TMB (1 bottle)	
Stop solution (1 vial)	

Appendix IV – Bioanalyzer

Assay Class: EukaryoteTotal RNA Nano
 Data Path: C:\... EukaryoteTotal RNA Nano DE34903690 2013-02-14 11-51-31.xad

Created: 2/14/2013 11:51:31 AM
 Modified: 2/14/2013 12:15:37 PM

Electrophoresis File Run Summary



Instrument Information:

Instrument Name: DE34903690 Firmware: C.01.069
 Serial#: DE34903690 Type: G2938C

Assay Information:

Assay Origin Path: C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy
 Title: Eukaryote Total RNA Nano Series II
 Version: 2.5
 Assay Comments: Copyright © 2003-2006 Agilent Technologies

Chip Information:

Chip Lot:
 Reagent Kit Lot:
 Chip Comments:

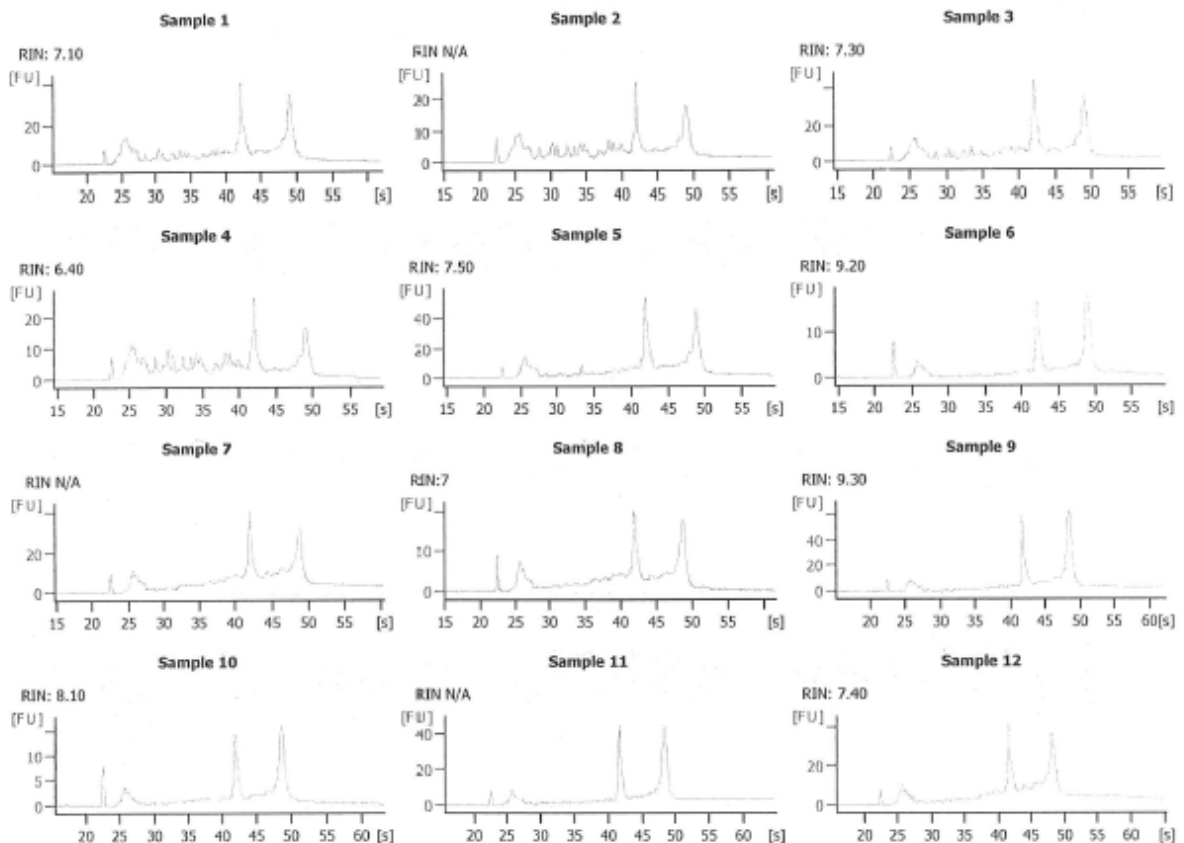


Figure A.1: Results from BioAnalyzer, resented as gel-like pictures and electrophrograms.

Appendix V: Histological methods

Table A.9: Chemicals and reagents used in fixation, dehydration, embedding, sectioning and staining.

Product	Vender
4 % formaldehyd	Merck, Germany
NaH ₂ PO ₄ x H ₂ O	Merck, Germany
Na ₂ HPO ₄ x H ₂ O	Merck, Germany
Ethanol	Arcus, Norway
Rectified Alcohol	Arcus, Norway
Xylene	Prolabo
Parafin	Histovax, OneMed
Hematoxylin	EMS
Eosin Y	Sigma, USA
Entellan	Merck, Germany

Appendix VII: Organ weights

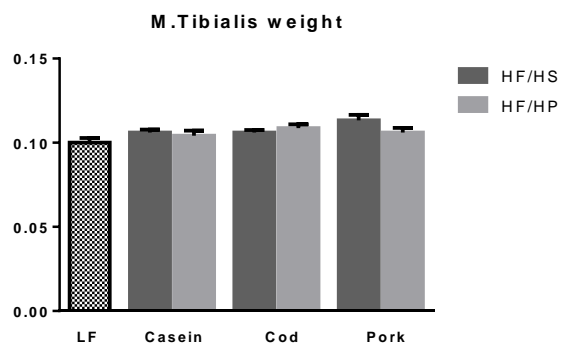


Figure A.2: Weight of M.Tibialis.

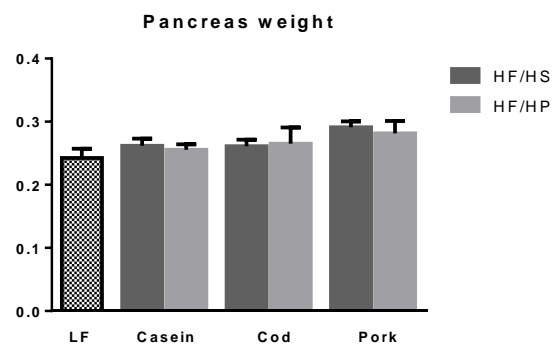


Figure A.3: Weight of pancreas.