DIETARY CASEIN HAS A HIGHER POTENTIAL THAN COD AND PORK TO INDUCE WEIGHT LOSS IN OBESE C57BL/6J MICE

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MASTER THESIS IN HUMAN NUTRITION

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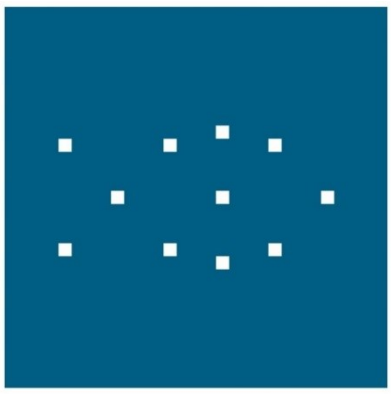

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Bergen, May 2015

Mona Søgnen

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Appendix:

LIST OF ABBREVIATIONS

2 – AG	2-Arachidonoylglycerol
AA	Amino Acid
ABC	Avidin – Biotin complex
AEA	Anandamide/ N-arachidonoylethanolamine
AMPK	Adenosine monophosphate – activated protein kinase
ANOVA	Analysis of variance
ARA	Arachidonic acid (20:4 ω-6)
ATP	Adenosine triphosphate
AUC	Area under curve
β3 – AR	Beta 3 adrenoreceptor
BAT	Brown Adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CCK	Cholecystokinin
DAUC	Decremental area under curve
CD36	Cluster of differentiation 36 (fatty acid translocase)
cDNA	Complementary Deoxyribonucleic acid
CideA	Cell death – inducing DNA fragmentation factor, alpha subunit–like effector A
Cox8b	Cytochrome c oxidase subunit VIIIb
DAB	3,3'-diaminobenzidine
DHA	Docosahexaenoic acid (22:6 ω-3)
DIO	Diet induced obesity
Dio2	Deiodinase type 2
DIT	Diet induced thermogenesis
DNA	Deoxyribonucleic acid

EDTA	Ethylene Diamine-Tetra-acetic Acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid (20:5 ω-3)
ER	Energy restriction
eWAT	Epididymal white adpiose tissue
FDG	^{18}F-fluorodeoxyglucose
FFA	Free fatty acid
Fmo1	Flavin containing mooxygenase 1
GLUT	Glucose transporter
Gpx3	Glutathione peroxidase
HC	High carbohyrdate
HDL	High-density lipoprotein
HF	High fat
HP	High protein
iBAT	Interscapular brown adipose tissue
iWAT	Inginual white adipose tissue
IL – 6	Interleukin - 6
IR	Insulin resistance
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
LA	Linoleic acid (18:2 ω6)
LDL	Low-density lipoprotein
LNAA	Large neutral amino acids
LSD	Least significant difference
MAGL	Monoglyceride lipase
MJ	Megajoule
MRI	Magnetic resonance imaging
Mt1	Metallothionein 1
mTOR	Mammalian target of rapamycin

MUFA	Monosaturated fatty acid
NapePLD	N – acyl phosphatidylethanolamine phospholipase
NE	Norepinephrine
NPY	Neuropeptide Y
ω -3	Omega 3
ω -6	Omega 6
OGTT	Oral glucose tolerance test
PET	Positron emission tomography
Ppargclα	Peroxisome proliferator – activated receptor alpha
PUFA	Polyunsaturated fatty acid
RIN	RNA integrity number
RNA	Ribo nucleic acid
ROUT	Robust regression and Outlier removal
RT	Reverse Transcriptase
rWAT	Retroperitoneal white adipose tissue
SEM	Standard error of the mean
SFA	Saturated fatty acids
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglyceride
TBP	TATA box binding protein
TNF – α	Tumor necrosis factor alpha
UCP1	Uncoupling protein 1
VHF	Very high fat
WAT	White adipose tissue
WHO	World Health Organization
qPCR	Quantitative polymerase chain reaction

ABSTRACT

OBJECTIVE: In the western world, a high energy intake combined with limited physical activity have resulted in an obesity epidemic. Challenges associated with this is an altered glucose homeostasis, increased risk of several diseases and economical concerns. A high proportion of dietary protein is known to induce weight loss, but little attention is given to effects of different protein sources. We aimed to investigate the weight reducing effect of low-fat diets enriched with the different protein sources casein, cod and pork.

RESEARCH DESIGN AND METHODS: An animal study was conducted, using C57BL/6J mice. Obesity was induced, by giving the animals a very high fat (VHF) diet. Three groups (n=10) were given a low fat diet (15 E% from fat, 16 E% from protein, 57 E% from carbohydrates, 12 E% from sucrose) and one group continued on a VHF control diet (52 E% from fat, 16 E% from protein and 32 E% from carbohydrates). Feed intake was recorded and an OGTT and ITT was performed after 3 weeks of experimental feeding. Plasma insulin levels were measured using ELISA, and mRNA levels of genes were measured using qPCR. Histological sections were exposed to both H&E and immunohistochemical staining. Data from an earlier completed experiment was included, following the same design, only with 30 % energy restriction.

RESULTS: Mice fed casein had the greatest loss of body weight and fat mass, but the cod –fed mice obtained the highest lean mass. Protein from casein also seem to improve glucose tolerance and insulin sensitivity in the mice, compared to cod and pork. However, when fed *ad libitum* a modest change was seen. Furthermore, our results show a significantly lower energy intake in the cod–fed mice. Sections of interscapular brown adipose tissue (iBAT) from the different groups indicate a higher degree of multilocular cells in iBAT from casein–fed mice in addition to a higher *Ucp1* expression. Possible mechanisms to elucidate the findings are explored and discussed.

CONCLUSIONS: The results herein indicate that feeding obese mice a diet with casein decreases body weight and improves glucose and insulin homeostasis to a greater extent than a diet with cod or pork. Research suggests that casein can mimic the beneficial effects of a high-protein diet, compared to other protein sources. Further research is necessary to demonstrate whether these findings are of relevance to human nutrition.

1.0 INTRODUCTION

1.1 Obesity and overweight

1.1.1 Prevalence and cause

Overweight and obesity is a problem on the rise throughout the entire world, and recent estimations reveal that at least 3.4 million adults succumb to diseases related to obesity each year (WHO, 2000). These so–called non-communicable diseases can be cardiovascular diseases such as heart attack, stroke, type 2 diabetes mellitus (T2DM) or cancer. Obesity is defined as an abnormal or excessive accumulation of fat that increase the risk of severe health issues. A general classification of overweight and obesity is the body mass index (BMI), where one is considered obese with a BMI >30, and overweight with a BMI >25 (WHO, 2000). Abdominal fat accumulation appears to have the strongest association for impaired health (ORahilly, 1997). The cause of obesity is multifactorial, with genetic, environmental and nutritional factors having important roles in its development. The accumulation of body fat from an excess energy intake, combined with a low physical activity is a crucial cause to the obesity epidemic (Vieira et al., 2009, ORahilly, 1997).

1.2 The adipose organ

The adipose organ consists of two different types of coexisting adipocytes: white and brown. White adipose tissue (WAT) is involved in energy storage while brown adipose tissue (BAT) in energy expenditure and regulation of body temperature (Fu et al., 2014). The composition of these types within the adipose organ depends on several factors, such as age, environmental and nutritional circumstances (Cinti, 2005).

1.2.1 White adipose tissue

The white adipose tissue (WAT) consists of unilocular adipocytes, each containing one large lipid droplet, with few mitochondria (Townsend and Tseng, 2012, Cinti, 2005). In a healthy individual, the WAT represents about 10% of the bodyweight, but can increase substantially when the body is in a long–term positive energy balance (Seale et al., 2009). In an obese state, the adipose organ increases its white adipocyte component and the cells become hypertrophic. This increment is facilitated by allowing synthesis of triacylglyceride (TAG) from glucose, and accumulate TAG in lipid vesicles within the cell, adopting the tissue's extensive storage-capacity (Cinti, 2005).

In addition to functioning as an energy storage, the WAT also has an endocrine secretory function. Among the signal factors secreted is the hormone Leptin, exerting a satiety signal to prevent overeating. However, excess food intake can lead to a hypersecretion and a counterproductive effect of leptin, leading to leptin resistance, decreased satiety signaling and consequently obesity (Ronghua and Barouch, 2007). Furthermore, obesity increases the secretion of pro–inflammatory cytokines from the adipose tissue, and causes an inflammatory state that can participate in development of several obesity – related challenges such as an impaired glucose homeostasis (Trayhurn and Beattie, 2001).

1.2.2 Brown adipose tissue

Localization and function

The brown adipocytes consist of many mitochondria and contain a considerable amount of lipid droplets. The BAT is heavily innervated by nerves and blood vessels, compared to WAT (Cinti, 2005). One of the hallmarks of BAT is the expression of the uncoupling protein 1 (UCP1) (Townsend and Tseng, 2012), representing 10 % of all the inner mitochondrial membrane proteins (Kozak and Anunciado-Koza, 2008). Its role is to uncouple protons from oxidative phosphorylation and adenosine triphosphate (ATP)-production, orchestrating the dissipation of energy as heat (Townsend and Tseng, 2012). This phenomenon is referred to as non–shivering thermogenesis. The brown adipocytes express the specific adrenergic receptor β3 (β3–AR) and their function rely on norepinephrine (NE) stimulation via the sympathetic nervous system (Cinti, 2005). During cold exposure, NE from the posterior pituitary gland will bind to G-protein coupled receptors in BAT, and lipolysis is stimulated after a cascade of reactions involving Cyclic adenosine monophosphate (cAMP)-activated protein kinase. Free fatty acids (FFAs) from this reaction stimulate formation of new mitochondria in the brown adipocyte. The FFAs are subjected to β-oxidation, and the protons in the respiratory chain and formation of ATP is uncoupled by UCP1, and heat is dissipated (Roman et al., 2015).

BAT serves as a regulator of body temperature in infants and small mammals, and for years it was assumed not to be present or have any significant effects in the adult the human body (Townsend and Tseng, 2012). However, the presence of a metabolically active BAT has been located in the shoulder and thoracic spine area of adult humans. With the use of a positron emission tomography (PET), an increased uptake of ^{18}F fluorodeoxyglucose (FDG) was

observed in areas believed to contain BAT. Increased uptake of this FDG marker indicates the amount of tissue uptake of glucose. Because this uptake increase with cold–exposure and treatment with β–adrenergic blockers, it can be associated with increased amounts of BAT (Saito et al., 2009).

Browning of white adipocytes

Cold-exposure has proven to be the most efficient way to activate BAT in humans (Roman et al., 2015). In addition, supporting studies performed with mice show that cold-exposure and the following β3 adrenergic (β3-AR) activation can generate a recruitment of UCP1-containing adipocytes in the classical WAT. When brown adipocytes are identified in areas predominant with WAT, this is referred to as "browning of white adipocytes", beige or "brite" (brown in white) adipocytes (Barbatelli et al., 2010, Cousin et al., 1992). The beige adipocytes contain fewer mitochondria than the classical brown adipocytes, yet more than the white does. Classical brown and white adipocytes are believed to arise from different cell lineages. The cell progenitors expressing *Myf5* have shown be committed brown adipocyte precursors (Fu et al., 2014), although some suggest that *Myf5* positive precursors also differentiate into a type of white adipocytes (Sanchez-Gurmaches et al., 2012). Furthermore, the literature proposes two ways in which the formation of beige adipocytes in WAT is accomplished, illustrated in fig 1.1. The beige adipocytes are either transdifferentiated from existing white adipocytes (Cinti, 2009) or recruited by differentiation from white precursor cells (Wang et al., 2013). Both pathways are thought to be initiated upon β3-AR stimulation or cold exposure. As the literature describe the process of browning in detail, less attention is given to the mechanisms causing whitening of BAT, seen in an obese state. Whitening can occur during a positive energy balance with accumulation of white mononuclear cells in the BAT (Bachman et al., 2002).

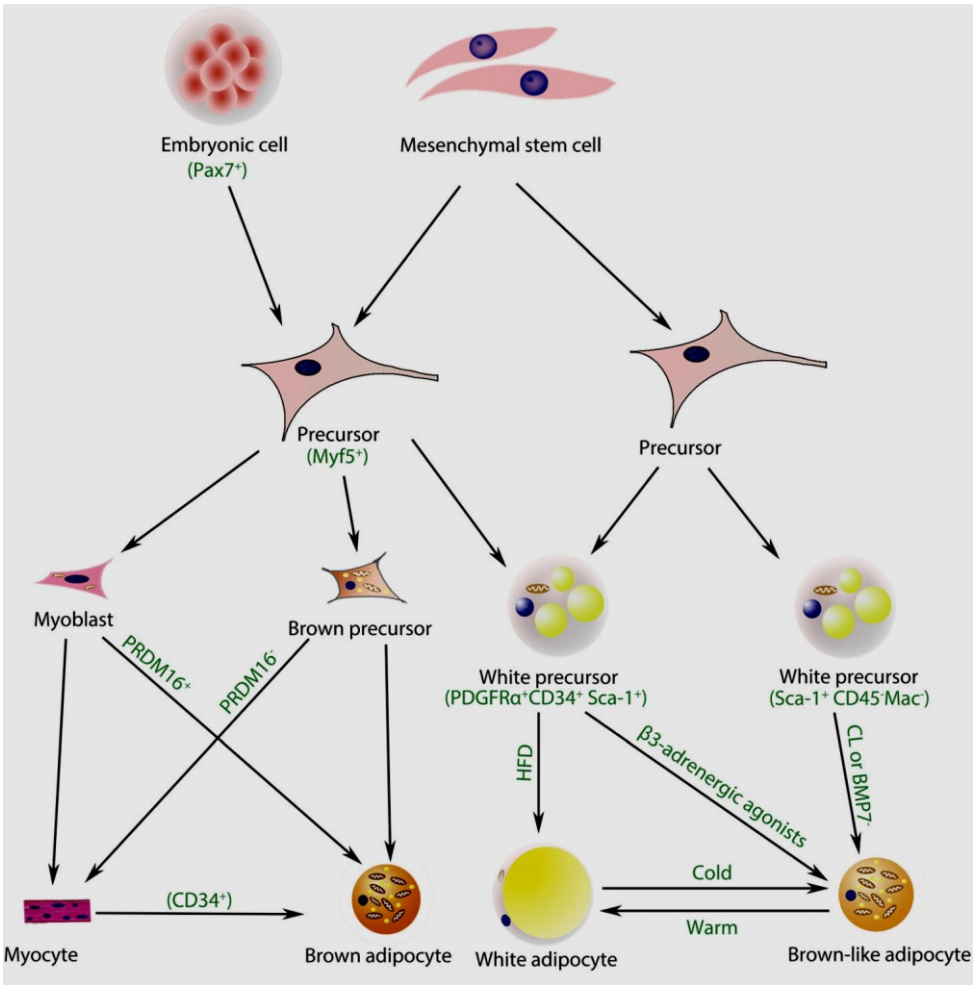

Figure 1.1: *Developmental basis for brown, white and beige adipocytes. Embryonic cells and mesenchymal stem cells can differentiate into adipocyte precursors. The precursor expressing Myf5 give rise to brown and possibly also white precursors. The brown - like adipocyte may be recruited from white precursors upon stimulation of a B-adrenergic agonist, or transdifferentiate from mature white cells. Warm- and cold exposure affect this transformation of white to beige adipocytes, and conversely (Fu et al., 2014).*

1.3 Insulin sensitivity and glucose tolerance

Decreased insulin sensitivity is a common complication to obesity. Insulin is a powerful anabolic hormone, and after a carbohydrate rich meal it allows glucose flow over cell membranes and stimulates energy storage in the adipose tissues (Dimitriadis et al., 2011). After long-term excessive intake of a diet rich in sugar, peripheral tissues such as muscles and adipose tissue develop a reduced response to insulin; a condition is known as peripheral insulin resistance (IR). Insulin loses its effectiveness, and consequently, less glucose is taken up via the glucose transporters (GLUTs) into the muscles and adipose tissues, causing an elevation of blood glucose. As an attempt to decrease blood glucose levels, the pancreas will produce more insulin, causing hyperinsulinemia, further decreasing the sensitivity to insulin

(Kanno et al., 2015). Furthermore, IR in the adipose tissues causes the free fatty acids (FFA) uptake in adipose tissue to fail, resulting in an increase of FFA in plasma. IR in the liver, termed central insulin resistance, responds to the non-accessible circulating glucose with a decreased hepatic glycogen synthesis and an elevated gluconeogenesis, leading to further release of glucose into the bloodstream (de Luca and Olefsky, 2008, Dimitriadis et al., 2011).

Insulin resistance is an important element of the metabolic syndrome, which also encompasses obesity, dyslipidemia and hypertension. All these conditions increase the risk of developing T2DM (Xu et al., 2003, de Luca and Olefsky, 2008). Because of the adipocyte hypertrophy that occurs in obese subjects, inflammatory pathways will be upregulated and pro-inflammatory cytokines circulate in higher levels (de Luca and Olefsky, 2008). In obese mice, detection of elevated levels of pro – inflammatory cytokines, among others the Tumor necrosis factor alpha (TNF-α) and Interleukin – 6 (IL-6) have been hypothesized to take part in the development of insulin resistance through downregulation of the insulin signaling pathway (Olefsky and Glass, 2010). The role of obesity in development of IR is illustrated in Figure 1.2.

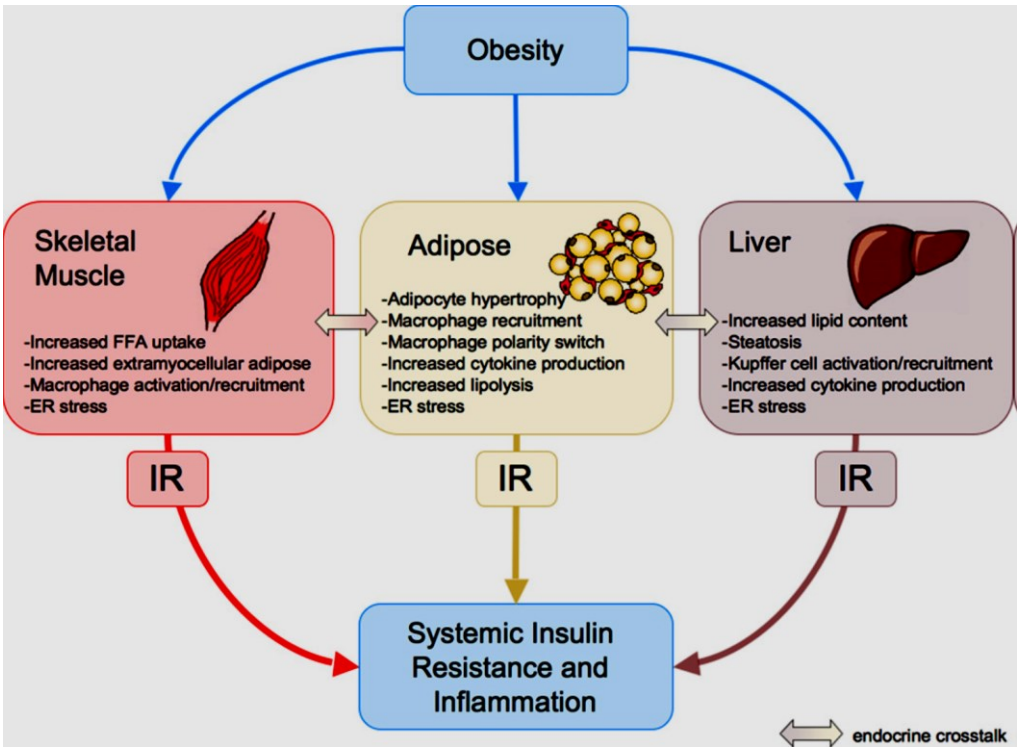

Figure 1.2: *The role of obesity in development of inflammation and insulin resistance (de Luca and Olefsky, 2008). ER: Endoplasmatic reticulum, IR: Insulin resistance*

An impaired glucose tolerance is closely related to insulin resistance, and is described as a decreased insulin-mediated glucose uptake in the tissues (Dimitriadis et al., 2011). Glucose intolerance is defined when blood glucose 2 hours after 75 g glucose load is < 7.8mmol/l or fasting blood glucose is < 6.1-6.9mmol/L (WHO, 2006). Impaired glucose tolerance, along with insulin resistance can be regarded as a pre–diabetic state. Diabetes occurs when pancreatic ß-cells fail to produce sufficient insulin, as a consequence of hyperinsulinemia and IR (Zou et al., 2014). According to WHO, a fasting blood glucose > 7.0 or > 11.0, 2h after a 75 g glucose load is a diagnostic criteria for type 2 diabetes (WHO, 2006). Prevention and alleviation of obesity and the metabolic syndrome is essential to improve this public health issue.

1.4 Weight reduction and macronutrients

In obese and overweight, weight loss have several beneficial effects, amongst them an improved insulin sensitivity and glucose tolerance (Westerterp-Plantenga et al., 2012, Roman et al., 2015). According to Knowler and colleagues, a low fat low calorie-diet has largely reduced the incidence of T2DM in a group of humans predisposed to T2DM (Knowler et al., 2002). A restriction of energy has furthermore reversed the metabolic syndrome in obese adults. A reduced body weight following a concomitant improvement of the lipid metabolism with lower levels of circulating low density lipoprotein (LDL), TAG and elevated high-density lipoproteins (HDL) was seen after 12 weeks (Heilbronn et al., 1999).

Much effort has been put into exploring how the macronutrient composition can influence weight development and weight loss, and it is currently debated what kind of diet composition that is most applicable to achieve or maintain a healthy body weight. There are several suggestions to obtain this, such as low fat, low carbohydrate, high protein or a calorie-restricted variant.

1.4.1 Dietary fat

A high intake of dietary fat has been claimed to cause obesity, although the type of fat plays a vital role. In research, much attention has been given to the polyunsaturated (PUFA) ω-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from fish oil. The intake of these fatty acids is by many believed to decrease the occurrence of coronary heart disease, several types of cancer, along with an improved psychological health (Murru et al., 2013). When given diets enriched with fish oil, effective weight loss

(Thorsdottir et al., 2007) and improvement of lipid profile have been reported among obese humans (Augustine et al., 2014). There is a growing interest in the impact of dietary fatty acid amount and composition, and the increasing ratio between the ω–6 and the ω-3 fatty acids is of importance. In the Paleolithic Stone age, the ω–6:ω–3 was as low as 1:1, compared to the modern American diet with a 10:1 ratio of ω–6:ω–3 (Eaton and Eaton, 2000). This ratio, and in which form the fat is presented in the diet, are thought to have great impact on health. Dietary EPA and DHA ingested incorporated in phospholipids (PL) are believed to have better bioavailability. Research suggests this can be alleviating to disturbances in glucose metabolism, dyslipidemia and inflammation in WAT in high fat (HF) fed mice. Higher levels of DHA were observed in the liver, WAT and muscle PL in mice given EPA and DHA as PL, compared to when fed as TAG (Rossmeisl et al., 2012). A diet high in ω-6, especially linoleic acid (LA) (18:2 ω-6) a common component in a typical western diet, has been linked to the increasing prevalence of obesity through its role in the endocannabinoid system (Alvheim et al., 2014).

The endocannabinoid system

The endocannabinoid system is under dietary influence and plays an essential role in energy metabolism. This system consists of the cannabinoid receptors 1 and 2 (CB1 and CB2), their lipid ligands endocannabinoids, and enzymes involved in the metabolism of these (Piomelli, 2003, Boon et al., 2014). The endocannabinoids are synthetized from the fatty acids on the PL cell membrane, which composition is determined by the dietary intake of ω-3 and ω-6 fatty acids. The best known ω-6–derived endocannabinoids are Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG), synthetized from PL Arachidonic acid (ARA), which precursor is Linoleic acid (LA) (Engeli et al., 2005). A high level of ω-6–derived endocannabinoids is associated with increased energy intake and obesity, and it is shown that mice given a diet with 8% LA, compared to 1%, increased their synthesis of 2-AG and AEA significantly. Adding EPA and DHA to these diets, led to a decrease in 2-AG and AEA synthesis, emphasizing the importance of dietary fatty acid on PL composition (Alvheim et al., 2012).

The endocannabinoid receptor CB1 is present in the brain and other tissues such as liver and adipose tissue, while CB2 is predominantly located in immune cells (Engeli et al., 2005). Obesity increases circulating levels of AEA and 2-AG, and alters the expression of these receptors. Activation of the central cannabinoid receptors promotes an orexigenic stimuli

and consequently obesity (Lafontan et al., 2007). Furthermore, activating peripheral CB1 receptors promote increased *de novo* lipid synthesis and fat storage in liver and adipose tissue (Osei-Hyiaman et al., 2005, Lafontan et al., 2007). On the other hand, blockage of CB1 have shown to reduce obesity in diet-induced obese (DIO) mice and humans and may be important contributor in future therapeutic strategies (Boon et al., 2014).

Low- fat diets

As fat contains more calories per gram than the other macronutrients, the Norwegian Health Authorities recommends limiting dietary fat intake to < 40 E%. Furthermore, as an attempt to reduce the increasing obesity prevalence and to maintain good health, these guidelines include limitations regarding the intake of saturated and trans-fatty acids (Helsedirektoratet, 2014). Studies performed with DIO rodents indicate that a low-fat diet can promote weight loss, improve insulin and glucose metabolism, both with *ad libitum* feeding and energy restriction (ER) (Muurling et al., 2002). In obese and overweight individuals, reducing dietary fat to < 30 E% can induce weight loss and improve the blood lipid profile and visceral fat deposition when consumed *ad libitum* (Skov et al., 1999). The effects of low - fat diets are debated, where many advocate for a large quantity protein at the expense of carbohydrates for effective weight loss and health promotion (Johnston et al., 2004, Blair et al., 2014)

1.4.2 Dietary proteins

According to the Norwegian Health Authority recommendations, energy from dietary protein should range from 10 – 20 E % (Helsedirektoratet, 2014). Numerous studies have demonstrated that a high protein diet (> 25 E % from protein) has promoted weight loss in both animals and humans (Skov et al., 1999, Blair et al., 2014, Noakes et al., 2005, Johnston et al., 2004). Similar results have been presented in unpublished data within our research group, where increasing the protein:carbohydrate ratio led to improvements in weight loss and glucose and insulin metabolism, foremost in mice fed casein. Casein and whey are the constituent proteins in dairy products, possessing a slow and fast absorption rate, respectively (Boirie et al., 1997, Bendtsen et al., 2013). Inclusion of dairy products in the diet provides calcium, associated with increased fat oxidation (Zemel, 2004, Teegarden et al., 2008). Studies that have evaluated differences between dietary protein sources, and observed that protein obtained from scallops (Tastesen et al., 2014a) or whey (Huang et al.,

2008) can prevent obesity development. Improvements in weight development, glucose tolerance and insulin sensitivity have been reported in obese rats and humans when given a diet rich in fish protein (Ouellet et al., 2007, Thorsdottir et al., 2007, Lavigne et al., 2000).

Proteins effect on satiety

Dietary protein seems to yield a more dominant feeling of satiety than carbohydrates or fat (Johnston et al., 2004, Pichon et al., 2006), hence the decrease in energy intake observed in humans given HP diets (Skov et al., 1999, Weigle et al., 2005, Johnston et al., 2004). Observations has been made during animal feeding trials, where feeding low-protein diets, lead to an increased energy intake. This is termed the protein leverage hypothesis, where an increased feed intake aims to provide the animal with indispensable amino acids (Huang et al., 2013). Conversely, ingestion of protein at the expense of other macronutrients reduces energy intake compared to diets with adequate levels of protein (Bensaid et al., 2002, Pichon et al., 2006, Blair et al., 2014, Huang et al., 2013). The amino acid (AA) profile in the protein source can be of great importance, as intake of the AA tryptophan has been associated with increased satiety through serotonin – signaling (Uhe et al., 1992). Moreover, research suggests that high protein diets and the AA leucine (Leu) stimulates mammalian target of rapamycin (mTOR) signaling, subsequently decreasing feed intake through hypothalamic signaling (Cota et al., 2006). The energy sensor enzyme adenosine monophosphate-activated protein kinase (AMPK) in hypothalamus decreases its activity during high protein intake. This is associated with a lower energy intake through decreased neuropeptide Y (NPY) secretion. AMPK in muscle and adipose tissue is elevated when the cell is deprived of ATP, which initiates catabolic processes and is also thought to increase insulin sensitivity (Hardie et al., 2012, Ruderman et al., 2013). Leu is believed to interact with the activity of neural AMPK. In addition, leptin both suppress neural AMPK activity and increase mTOR signaling in a reciprocal fashion (Ropelle et al., 2008), disclosing a complex regulated neural pathway in which dietary proteins are possible modulators. Furthermore, a diet high in protein increase secretion of cholecystokinin (CCK), a gut hormone that increase satiety and decrease energy intake (Potier et al., 2009).

Proteins effect on energy expenditure

In addition to increasing satiety, dietary protein has a greater potential to increase energy consuming processes. Our energy expenditure involves the basal metabolic rate, the cost of

physical activity and the diet-induced thermogenesis (DIT), or thermic effect of foods. DIT is determined by the diet composition and energy requiring processes in the post-prandial period (Westerterp, 2004, Paddon-Jones et al., 2008). To which extent the different nutrients cause DIT, there is a theoretical value. For dietary fat this value is 0-3%, for carbohydrates 5-10% and for protein 20-30% (Westerterp-Plantenga et al., 2012, Tappy, 1996). The body does not store AAs, so they are immediately metabolized in energy-costing processes such as gluconeogenesis, ureagenesis and protein synthesis. (Halton and Hu, 2004). A study demonstrated that isocaloric meals with protein increased energy expenditure more than meals with carbohydrates, along with a greater thermic effect (Acheson et al., 2011). This means a lower energy efficiency from protein, which is favorable if weight loss is desired. The association between satiation and DIT is hypothesized to be caused by an increased oxygen consumption and increase in body temperature. The sensation of oxygen deprivation can be translated into a sensation of satiety by the brain (Westerterp-Plantenga et al., 1999).

1.4.3 Dietary carbohydrates

To an extent, the increased intake of refined carbohydrates and sugars and low intake of fibers may be factors in obesity development (Ma et al., 2011). Therefore, a popular weight loss plan is a diet low in carbohydrates. The idea behind the beneficial effects of this type of diet is that carbohydrates largely controls insulin secretion, and indirectly affects lipid metabolism by increasing lipogenesis. Another theory is the fact that the hunter-gatherer society had a diet consisting of limiting amounts of carbohydrates, and the modern population are evolutionally designed to eat the same way (Volek et al., 2008).

1.5 Introduction to the study

In relation to the increasing rate of obesity worldwide, several measures have been adopted to find an applicable dietary approach to the challenge this presents. Macronutrient composition influence several aspects of metabolism, such as regulation of hormones, insulin signaling and transcription of genes (Madsen et al., 2008a). HP diets are a popular approach to combat obesity, and research support that HP diets can increase energy expenditure, decrease satiety and hepatic lipid synthesis and promote weight loss (Huang et al., 2013, Pichon et al., 2006). In nutritional research, casein, whey or soy are commonly administered as protein sources (Eller and Reimer, 2010, Lillefosse et al., 2013, Tastesen et al., 2014b, Petzke et al., 2005). However, the amount of studies on the weight reducing potential of different protein sources on obese subjects are sparse. Data obtained within our research group indicate that varying the protein source had differential effects, where casein-fed mice were protected from obesity compared to mice fed cod, soy, chicken, beef or pork. Low fat diets have proven effective in animal studies (Vieira et al., 2009, Muurling et al., 2002), and ingestion of marine protein sources have resulted in weight loss in human and animal studies (Thorsdottir et al., 2007, Tastesen et al., 2014b). In the current study, we wanted to further investigate the weight reducing potential of different dietary protein sources. To accomplish this, we initiated a feeding experiment where DIO C57BL/6J mice were given low-fat diets enriched with the protein sources casein, cod or pork.

1.6 Aims of the study

This study primarily aimed to investigate whether different protein sources could exert differential effects on weight reduction and body composition in diet-induced obese mice.

- Hereunder, we wanted to explore the dietary effects on insulin sensitivity and glucose tolerance.
- Moreover, we aimed to determine any differences in energy intake, to evaluate the satiating effects of the different protein sources. In addition, we wants to investigate if the different diets had the capacity to increase energy expenditure.
- Lastly, we aimed to evaluate whether the same protein sources produced different results when given in an energy-restricted diet.

2.0 MATERIALS AND METHODS

2.1 The animal model

Sixty male C57BL/6J Bomtac mice were obtained at 8 weeks of age from Taconic Europe, Ejby, Denmark. Upon arrival, the mice had a mean weight of 25.79 ± 1.71 g. The C57BL/6J (depicted in Figure 2.1) is a preferred strain for obesity research due to its ability to become obese, develop adipocyte hyperplasia, insulin resistance and glucose intolerance when given a diet very high in fat and/or sucrose (Black et al., 1998).

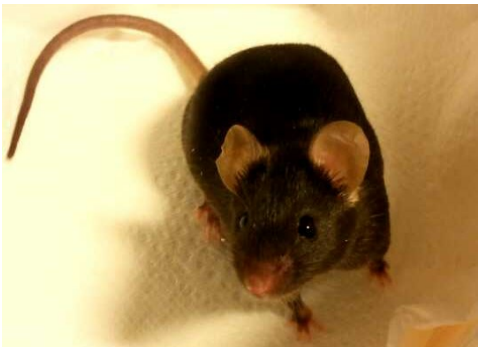

Figure 2.1: *The C57BL/6J mice (private photo).*

2.1.1 Ethical statement

The animal experiments were carried out in accordance with the guidelines of the Norwegian Animal Research Authority. The care and handling were in conformity with the local guidelines and procedures (NIFES, 2013b).

2.2 The animal experiment

2.2.1 Experimental design

Upon arrival, the mice were fed a very high fat (VHF) diet (Ssniff, Soest, Germany) until obesity was induced. Thereafter, the mice were assigned into three experimental groups and one control group (n=10), according to average fat- and lean mass. The experimental groups were given low-fat diets containing either casein, cod or pork as the protein source.

The experiment was carried out in a thermoneutal room (29 ± 0.2 °C) with controlled high humidity (40.2 ± 0.2 % RH) and a 12-hour light/dark cycle. The mice were housed individually in cages enriched with a house, nesting material, bedding and a piece of wood for chewing. Every other week the animals were provided with clean cages and contents, and once a

week the water and bottles were changed. Weights were recorded once a week using a Mettler Toledo weight. The mice were fed three times a week, and all the feed and feed remains were weighed and documented. All the animals had access to *ad libitum* food and water during the entire experiment. Using a magnetic resonance imaging (MRI) technique with a Minispec mq 7.5, NMR analyser (Bruker, Germany), the mice were scanned three times during the experiment, enabling a distinction of fat mass, lean mass and free water.

Samples of feces were collected from a selection of the mice over a course of 7 days, both prior to and during the experimental feeding. Subsequent fat and protein content analyses were carried out, and the apparent fat- and protein digestion of the mice could be calculated based on feed intake during this period. The apparent digestibility (AD) was calculated with the following formula:

$$\mathbf{AD} = \frac{(\text{Amount of nutrient eaten} - \text{Amount of nutrient excreted})}{\text{Amount of nutrient eaten}} \text{x } 100\ \%$$

An additional animal experiment was conducted and was intended for this thesis, but due to unforeseen events, the collected data could not be used. Instead, raw-data collected previously within our research group will be presented in this thesis: A low fat experiment with calorie restriction.

2.2.2 Experimental diets

The low-fat cod and pork feed were prepared at NIFES. The pork sirloin was obtained from H. Brakstad AS and the cod filet from Lerøy Seafood. The casein powder is based on casein sodium salt from bovine milk (Sigma - Aldrich, USA).

Before preparing the diets, the cod and pork fillets were freeze dried, and the following steps were executed to finish this process: The fillets were put in a steamer at 75 °C, until the core temperature reached 70 °C. Thereafter the fillets were minced and stored at – 20 °C overnight, before being inserted into a freeze-drying machine for 72-96 hours. The dried blocks of fillets now produced were homogenized to powder and stored at – 20 °C in vacuum bags to prevent moist. Samples of the powder were selected for analyzes of nutrient composition and level of dryness (aiming for > 90 % dry). Upon preparing the diets, the powder was weighed and mixed with the other components, using a Crypto Peerless EF20 blender (Crypto, UK). The complete feed was kept at – 20 °C before given to the mice.

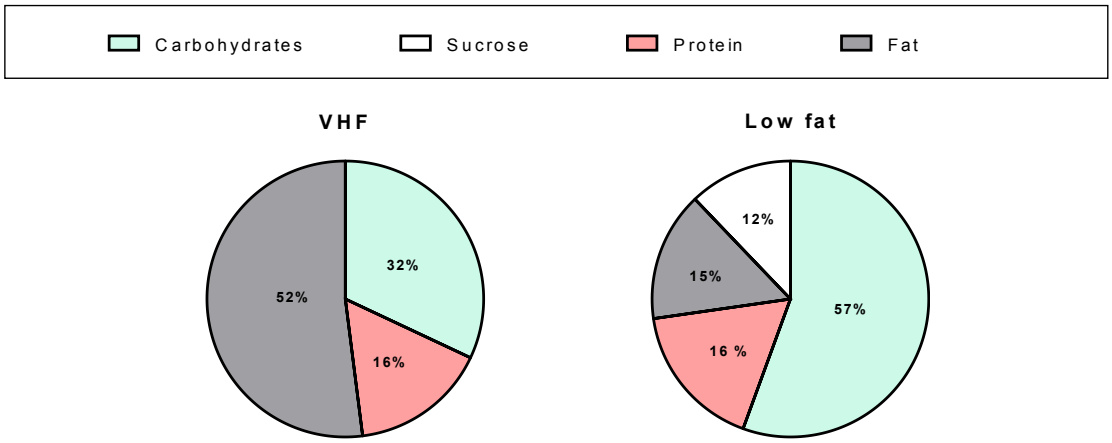

Figure 2.2: *The macronutrient distribution in the experimental diets, presented in Energy percent (E%). For complete diet composition, see Appendix I, Tables A.1 – 4.*

The dietary macronutrient composition is presented in Figure 2.2. The amount of protein in the diets and protein sources was determined by multiplying the nitrogen content with a conversion factor of 6.15 for the casein-diet and 5.6 for the cod and pork diets (Mariotti et al., 2008).

2.3 Insulin tolerance test and glucose tolerance test

To determine whether feeding different protein sources affected glucose tolerance and insulin sensitivity, an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were conducted prior to, and during, the experimental diets. The blood glucose levels were measured with a Contour® next USB glucose meter (Bayer, Canada). Prior to the tests, the mice were put in clean cages, with access to water and a house.

2.3.1 Oral glucose tolerance test

Before commencing the test, the mice were fasted for 6 hours. At baseline, a sample of blood for measuring blood glucose was collected from the tail, determining fasting blood glucose. Thereafter, the mice were subjected to a glucose load orally by gavage. The amount of glucose solution (200 mg/ml) given was calculated on the basis of the mice's lean mass (1 mg/g lean mass). Additional blood glucose measurements were taken after 15, 30, 60 and 120 minutes. Blood samples were collected at 0, 15 and 30 minutes for determination of plasma insulin concentrations. The blood samples were centrifuged at 2500 x g for 5 minutes at 4 °C using a Heraeus™ Fresco 21 Centrifuge (Thermo – Scientific, USA). The plasma obtained was kept at - 80 °C until analysis.

2.3.2 Insulin tolerance test

The mice were at a randomly fed state before the ITT. Blood glucose was measured once before the animals were given an intraperitoneal insulin injection, dose based on their lean mass (1 U/kg lean mass). Blood glucose was measured in blood from the tail at baseline, and after the injection at 15, 30, 45 and 60 minutes. To further explore the tissues' sensitivity for insulin, a homeostasis model assessment - insulin resistance (HOMA – IR) was calculated, using the following formula:

$$\mathbf{HOMA - IR} = \frac{\text{Fasting blood glucose } \left(\frac{\text{mmol}}{\text{L}}\right) \text{ x fasting plasma insulin } \left(\frac{\text{mU}}{\text{L}}\right)}{22.5}$$

2.4 Termination and data collection

2.4.1 Termination

After six weeks of experimental feeding, the mice were anaesthetized with Isoba® vet Isofluran gas (MSD Animal Health, Norway) using a Univentor 2010 Scavenger unit (Agnthos, Sweden) and euthanized by cardiac puncture.

2.4.2 Blood samples

Blood obtained from the heart was collected in a syringe, and put in a tube with Ethylene Diamine-Tetra-acetic Acid (EDTA) to avoid blood coagulation. The samples were then centrifuged at 2500 x g for 5 minutes at 4 °C to separate the plasma from the rest of the blood components. The plasma and the red blood cells (RBC) were stored at - 80 °C until further analysis.

2.4.3 Tissue harvesting

After termination, the liver, musculus tibialis, and several samples of adipose tissue were dissected out; the inguinal -, epididymal - and retroperitoneal white adipose tissue (iWAT, eWAt and rWAT), and interscapular brown adipose tissue (iBAT). All tissues were weighed, and smaller samples were put away for histology. The remaining tissues were separately placed in small plastic bags and snap frozen in liquid nitrogen using a freeze-clamp. The tissue – samples were kept on dry ice until storage at - 80°C.

2.5 Histological methods

2.5.1 Fixation with paraformaldehyde and phosphate buffer

After dissection, the tissues intended for histology were immediately put in cassettes and fixated in a 4 % paraformaldehyde and 0.1 M phosphate buffer (PB) solution. The fixative was stored at 4 °C overnight, before being washed with PB and stored at 4 °C for two days. The PB was prepared with 3.68 g NaH_2PO_4 x H_2O and 16.82 g Na_2HPO_4 x 2 H_2O dissolved in 1000 mL double – distilled water (ddH_2O). Lastly, the pH was adjusted to 7.4.

2.5.2 Dehydration with ethanol and xylene

A series of ethanol washes were performed to remove any water from the tissue samples. See Table 2.1 for time schedule. When the samples were completely dehydrated by 100 % ethanol, the alcohol was replaced with xylene, as xylene is soluble in both ethanol and paraffin (Cinti et al., 2001).

Table 2.1: *Time schedule for dehydration of tissues.*

Dehydration program	
Reagent	**Time (min)**
75% EtOH	45
95% EtOH	45 x 2
100% EtOH	45 x 2
Xylene	45 x 2
Paraffin	15 x 2

2.5.3 Paraffin infiltration and embedding

After dehydration, the cassettes containing the tissue-samples were emerged in baths with liquid paraffin (59 °C) (Histowax, Histolab products AB, Sweden) and stored overnight in a ventilation cabinet. The next morning, the cassettes were relocated and kept in a new paraffin bath for 30 minutes, to remove remaining xylene. The tissues were embedded in paraffin using an EC 350 tissue embedding center (Thermo Scientific, Germany). The tissue samples were then placed in a mold with a very small amount of paraffin in the bottom, before liquid paraffin was poured into the mold, covering the tissue-sample. The moulds were shortly stored at - 20 °C degrees until the samples could be effortlessly removed, and stored at 4 °C awaiting sectioning.

2.5.4 Sectioning and staining

A microtome (Leica RM2165, Germany) was used for the sectioning of the tissue samples. After cutting 3 µm sections of the adipose tissue, the slices were quickly put in a heated bath (60-70 °C) of ddH_2O and 0.5 dL methanol placed on a slide warmer 85 at (ADAMAS instrumenten bv, Netherlands). This made the sections stretch, before they were extracted from the bath onto a microscope slide. The slides were left to dry for a couple of days before commencing the staining. The staff at the Molecular Imaging Center (MIC) at Haukeland University Hospital were most helpful to section some of the samples.

To be able to visualize the tissues in a microscope, the samples required staining. The staining was carried out with use of Hematoxylin and Eosin, one of the preeminent staining procedures used in histology. Hematoxylin provides the nucleus with a blue color, and the cytoplasma is stained pink from the eosin (Fischer et al., 2008). Before commencing, the slides were heated to 57 °C for 1 hour, before being rehydrated, stained and dehydrated according to the schedule in Appendix II, Table A.6. When having completed the staining and dehydration, a xylene-based glue and a glass cover was placed onto the tissues slides, and they were left to dry in a ventilation cabinet. For a complete list of chemicals and reagents used in the histological methods, see Appendix II, Table A.5

2.5.5 Immunohistochemistry

The principle behind immunohistochemistry is to visually detect antigens present in tissues, in this case UCP1 in iBAT. Firstly, the tissues were rehydrated, by xylene and a series of ethanol baths, following the schedule in Appendix II, Table A.8. Furthermore, the proteins that were to be determined were made reactive again by lowering the tissues into a citrate buffer and heated for 30 minutes, using a Heto OBN 18 heated bath (Heto-Holden, Denmark) holding 95 °C. After this, endogen peroxidase activity had to be inactivated to minimize background staining. This was accomplished by lowering the tissues in H_2O_2 in MetOH for 10 minutes. The samples were then washed in phosphate buffered saline (PBS) and T(w)een, before incubating in goat serum (10 %) to further reduce non–specific background staining. Then the primary antibody was added to the samples prior to overnight incubation at 4 °C. It was crucial that the tissues did not dry, so to ensure that the liquid stayed on the desired tissue samples, a liquid blocker super pap-pen was used to form a hydrophobic film around the samples. The following day, the secondary antibody was added, following another PBS

wash. Then a Vectastain® Elite ABC kit was used to prepare and add the Avidin–Biotin Complex (ABC) mix, a marker system to amplify the signal. The next step was to wash and add diaminobenzidin tetrahydrochloride (DAB) to the samples, using Vector® DAB substrate kit. DAB will be oxidized by ABC's peroxidase, and the antigen in the samples will acquire a brown color when DAB precipitates. The last step was to add hematoxylin to ensure a slight color to the tissue samples. The tissues were dehydrated according to the schedule in Appendix II, Table A.8, before mounting a glass cover on the slides to enable examination in a microscope. Time schedule, reagents and equipment used in the procedure are listed in Appendix II, Tables A.7 and A.8. ImageJ (SciJava) was used to quantify UCP1 in photos taken after the staining. During the procedure, sections from a cold – induction experiment was stained, providing a negative and positive control for our experiment.

2.5.6 Microscopy

The cells were examined by using an Olympus BX 51 binocular microscope. Captions of the cells were taken with a Nikon digital sight DS-Fi1 camera.

2.6 Analytical methods

2.6.1 ELISA insulin kit

The Mouse ELISA method is based on the sandwich technique where two antibodies located in the wells are directed against antigenic determinants on the insulin molecules. The commercially available Mouse Insulin ELISA kit (DRG Instruments GmBH, Germany) was used for determination of insulin in mouse plasma. The enclosed reagents and the equipment used are listed in Appendix III, Table A.9. Before commencing the procedure, all reagents were allowed to reach room temperature while the plasma samples were thawed on ice. The enzyme conjugate 1X solution and wash buffer 1X solution were prepared according to the DRG Insulin (Mouse) ELISA protocol. Firstly, 10 µl of calibrators and 10 µl of each sample was transferred to designated wells on a 96-well microplate, along with 100 µl Enzyme conjugate 1X solution. The plate was then incubated for 2 hours on a Delfia® plate shaker (PerkinElmer, USA) at 700-900 rpm, to ensure a reaction between the insulin in the plasma samples and the peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the wells in the microplate. Then each well was washed 6 times with 700 µl wash buffer 1X solution using a Delfia ® platewash (PerkinElmer, USA) to ensure removal of unbound enzyme labeled antibodies. To detect the bound conjugate, 200 µl Substrate TMB

were added into each well, and the plate was incubated for 15 minutes. To stop the reaction, 50 µl of Stop Solution was added to each well, and the plate was put on the shaker for 5 seconds to properly mix the Stop solution with the samples. A VICTOR ™ X5 multilabel spectrophotometric plate reader (PerkinElmer, USA) was used to read optical density at 450 and 620 nm. The results presented in absorbance could be calculated into insulin concentrations in the plasma samples. For more information regarding the method and kit, see DRG Insulin (Mouse) ELISA protocol.

2.6.2 RNA purification with QIAzol

The first step to be able to determine gene expression in the tissues, was to extract the ribo nucleic acid (RNA) from the tissue samples. All work surfaces and instruments were washed with RNase Zap (Sigma-Aldrich, USA) to prevent RNA degradation in the working environment. The frozen iBAT tissue samples were placed in a RNase free tube, and 1 ml of QIAzol (Qiagen, Norway) was quickly added to each sample. QUIazol is a monophase solution that contains phenol and guanidinium salts (NIFES, 2014). Four zirconium beads were added for efficient homogenization of the tissues in a Precellys 24 – machine (Bertin Technologies) at 6000 rpm for 3x10 seconds, with 10 seconds between the intervals. The samples were then incubated at room temperature (RT) for 5 minutes. Furthermore, the samples were centrifuged (Eppendorf centrifuge 5415 R) for 10 minutes (4 °C, 12000 x g), enabling the fat layer to be removed using a pipette. The homogenate was transferred to a clean tube and 200 µl of Chloroform was added to separate RNA from deoxyribonucleic acid (DNA) and proteins. The tubes were shaken forcefully for 15 seconds and incubated at RT for 10 minutes, before 15 min. centrifugation. A clear aqueous phase containing RNA now developed, and was transferred to a clean tube and mixed with 500 µl Isopropanol. The mixture was stored at RT for 10 minutes, and at 4 °C for 10-30 minutes, during which the isopropanol will ensure that the RNA is separated from the water–phase. Thereafter, the samples were centrifuged again for 30 minutes, to separate the RNA pellet from the supernatant. The supernatant was carefully removed with vacuum suction (IBS Integra Biosciences, Vacuboy, Switzerland), and 1 ml cold 75% EtOH was added to the remaining pellet in order to wash it. The tubes were vortexed (Lab dancer S40, IKA) and centrifuged again before the supernatant was removed, and the pellet dissolved in 50 µl ddH_2O.

The RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Savneen-Werner, Sweden). The NanoDrop accurately measures concentrations between 260 and 280 nm, and provide A260/A280 and A260/230 ratios, indicating the degree of purity of the RNA samples. A desired value for the A260/280 ratio is ≥ 1.8 – 2.1. Deviations from these values can indicate the presence of phenol, DNA or protein in the sample. The A260/A230 ratio is preferably > 1.8, where a lower number may indicate salt residues or inhibitors in the sample (NIFES, 2014). The samples were stored at - 80 °C. For a complete list of chemicals and reagents used in RNA purification, see Appendix IV table A.10.

2.6.3 RNA quality - BioAnalyzer

Because RNA is easily degraded in the presence of RNase enzymes, the integrity is determined prior to measuring gene expression (Schroeder et al., 2006). At least 2 µl of 12 selected purified RNA samples were pipetted into new Eppendorf tubes (Agilent Technologies, USA) for integrity testing. The RNA samples exceeding a concentration of 500 ng/µl were diluted using RNase free water and placed on a heating plate holding 70°C (Grant, UK), causing denaturation.

Using the RNA Nano 6000 kit, we were provided with a gel, dye, marker, ladder and a LabChip (See Appendix V, Table A.11 for complete list of reagents and equipment). The LabChip consisted of 16 wells, 12 for RNA samples, 1 for the ladder and 3 for a gel – dye mixture. The reagents were allowed to reach room temperature before the gel and the dye were mixed, vortexed and centrifuged for 10 minutes (RT, 13000 x g) (Eppendorf centrifuge 5415 D). The gel-dye-mix was added to its designated wells on the LabChip, and was distributed into the other wells using a Chip Priming station (Agilent Technologies, USA). Furthermore, the marker was added to each well, along with 1 µl of RNA sample. Before the chip was analyzed, 1 µl of ladder was added to its designated well, and the chip was vortexed for 1 minute using a Bioanalyzer Chip vortexer (IKA).

A BioAnalyzer (2100 Agilent Technologies, USA) detects the RNA samples with laser – induced fluorescence, and the results can be visualized as an electropherogram, where the amount of fluorescence correlates with the amount of RNA. These results are quantified by the software, which yields a RIN value (RNA integrity number) that range from 1 to 10, where 10 is an indicator of an intact RNA (Schroeder et al., 2006). In this experiment, a RIN >7 was recommended for running qPCR.

2.6.4 Reverse Transcription reaction

Principle

During a reverse transcription reaction (RT-reaction) complimentary DNA (cDNA) is synthetized from RNA (Figure 2.3). In this experiment we used Multiscribe Reverse Transctiptase (50U/µl) and a random primer mix in a 30 µl reaction. The RNA is reversely transcribed to cDNA, which is more stable then RNA (Valasek and Repa, 2005). For a complete list of reagents, see Appendix VI, Table A.11

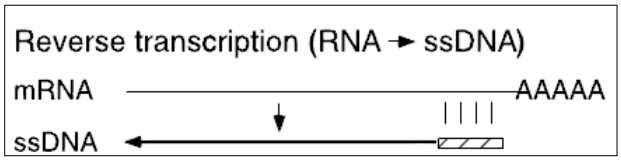

Figure 2.3: *Reverse transcriptase creates a single stranded cDNA. DNA polymerase converts the single stranded cDNA into double stranded cDNA (Valasek and Repa, 2005).*

Procedure

The RNA samples were thawed on ice, and samples of each RNA – specimen was diluted with ddH_2O to achieve a concentration of 50 ng/µl. A mixture for the standard curve was also prepared by mixing 2 µl of all the diluted RNA – samples, and prepare concentrations of 100 ng/µl, 50ng/µl, 25 ng/µl, 12.5 ng/µl and 6.25 ng/µl in triplicates. The diluted RNA – samples, in duplicates, and samples for the standard curve were added to a 96-well RT plate (Thermo – Scientific, USA), along with RT-reaction mix (appendix VI, Table A.12). Two negative controls: non-amplication control (nac) and non template control (ntc) were also run. The well with nac did not have the enzyme multiscribe transcriptase, and the ntc had no RNA. The plate was covered with a lid, centrifuged for 1 minute (Eppendorf Centrifuge 5810 R), and run on GeneAmp® PCR System 9700 (Applied Biosystems, USA) for 75 minutes, with the program represented in Table. 2.2. The RT plate was stored at -20 °C.

Table 2.2: *Reverse Transriptase reaction instrument setup. From 279 RT reaction method description (NIFES, 2013a)*

Step	**Incubation**	**RT**	**RT inactivation**	**End**
Temperature (°C)	25	48	95	4
Time (min)	10	60	5	∞

2.6.5 Quantitative polymerase chain reaction

Principle

A quantitative polymerase chain reaction (qPCR) was applied to enable measurement of the cellular gene expression in iBAT. During a qPCR, the cDNA samples are copied exponentially and specific nucleic acid sequences are amplified with the help of sequence specific primers and DNA polymerase (Figure 2.4). TATA box binding protein (TBP) was used as housekeeping gene, to correct for variations and errors between the different cDNA samples (Valasek and Repa, 2005, Arya et al., 2005). For a complete list of target genes, see appendix VII, Table A.14.

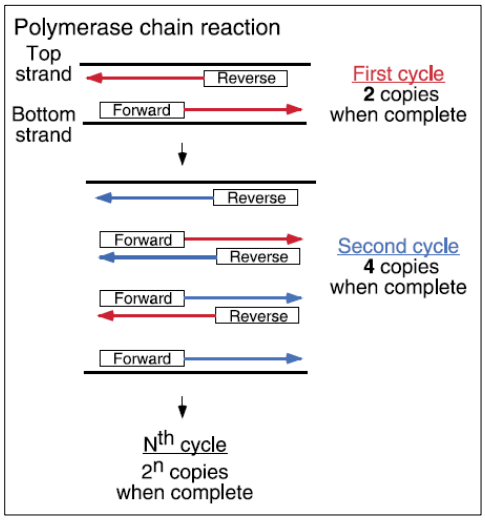

Figure 2.4: *The amplifications of the DNA samples in a qPCR (Valasek and Repa, 2005).*

During amplification, three steps are completed in 45 cycles. The first is denaturation of the DNA double strands (95 °C), melting it into single strands. Thereafter, the primers attach to their specific sequences (60 °C). The third step is allowing elongation of complementary DNA with DNA polymerase (72 °C) (Valasek and Repa, 2005). The program for the PCR machine is presented in Table 2.3. While the DNA is amplified, the process makes it possible to use a fluorescent dye, commonly SYBRGreen, to accumulate proportionally to the amount of PCR products generated in each cycle. SYBRGreen is a DNA – binding dye, which has high affinity for the minor groove of double stranded DNA (dsDNA), and upon binding, it fluoresces strongly. When in presence of much dsDNA, it will emit a strong fluorescent signal and reach threshold early (Arya et al., 2005, Valasek and Repa, 2005).

Table 2.3: *Time and temperature cycle program for LightCycler 480 Real Time PCR System.*

Step	Pre - incubation	Amplification			Melting curve analysis			Cooling
Temperature (°C)	95	95	60	72	95	65	97	40
Time	5 min	10s	10s	10s	5s	1 min		10s
Number of cycles	1	45			1			

Procedure

The cDNA plate was thawed on ice while the reaction mix was prepared (see Table 2.4 for details), and then vortexed for 3 minutes (MixMate® Eppendorf). The SYBRGreen Master reagent was mixed with RNase free ddH_2O and a primer pair forward and reverse. The volumes listed in Table 2.4 were multiplied by 115 (the number of wells + dead volume). The mix was aliquoted into a set of eight Eppendorf – tubes, one set per primer pair. Once the mix was ready, it was placed on a Biomek 3000 Laboratory Automation Workstation pipetting robot (Beckman Coulter, USA) along with the cDNA plate, and 2 µl of cDNA sample and 8 µl of master mix were transferred into each of the 384 designated wells on a LightCycler 480 mulitiwellplate (Roche, Norway). The new plate was covered with a LighCycler 480 sealing foil (Roche, Norway) and centrifuged (Eppendorf centrifuge 5810 R) for three minutes at 1500 x g. Lastly, it was placed in the LightCycler® 480 Real Time PCR System (Roche, Norway) and qPCR was performed.

Table 2.4: *SYBRGreen reaction mix for 10 µl reaction.*

Reagent	Volume (µl) per sample
ddH_2O	2.9
Primer forward	0.05
Primer reverse	0.05
SYBRGreen Master	5

To determine gene expression, the LightCycler® 480 software calculates a cycle threshold value (C_T), which is when the fluorescence reaches threshold. This value is inversely proportional to the number of DNA sequences in the original template (Valasek and Repa, 2005) and is further used in calculations to determine the gene expression. For a complete list of chemicals, reagents and equipment used in qPCR see Appendix VII, Table A.13.

2.7 Statistical analyses

Microsoft Excel 2013 (Microsoft) was used to assemble the data into tables, and to calculate mean and standard error of the mean (SEM) of the raw data. Graph Pad Prism 6 was used to identify outliers using ROUT test ($p < 0.05$) and to calculate the differences between the experimental groups, using a one-way analysis of variance (ANOVA) with a post hocFisher Least significant difference (LSD) test. The normality of the data was tested with a D'Agostino – Pearson normality test. P values < 0.05 were considered statistically significant.

3.0 RESULTS

3.1 Body mass development

3.1.1 Body weight changes

After being fed a VHF diet for 18 weeks the C57BL/6J mice were assigned to three experimental groups and one control group. The experimental diets (dietary compositions are presented in appendix I, Table A.1) were consumed over a course of six weeks. The mice in the previously completed experiment with energy restriction were fed VHF for 13 weeks.

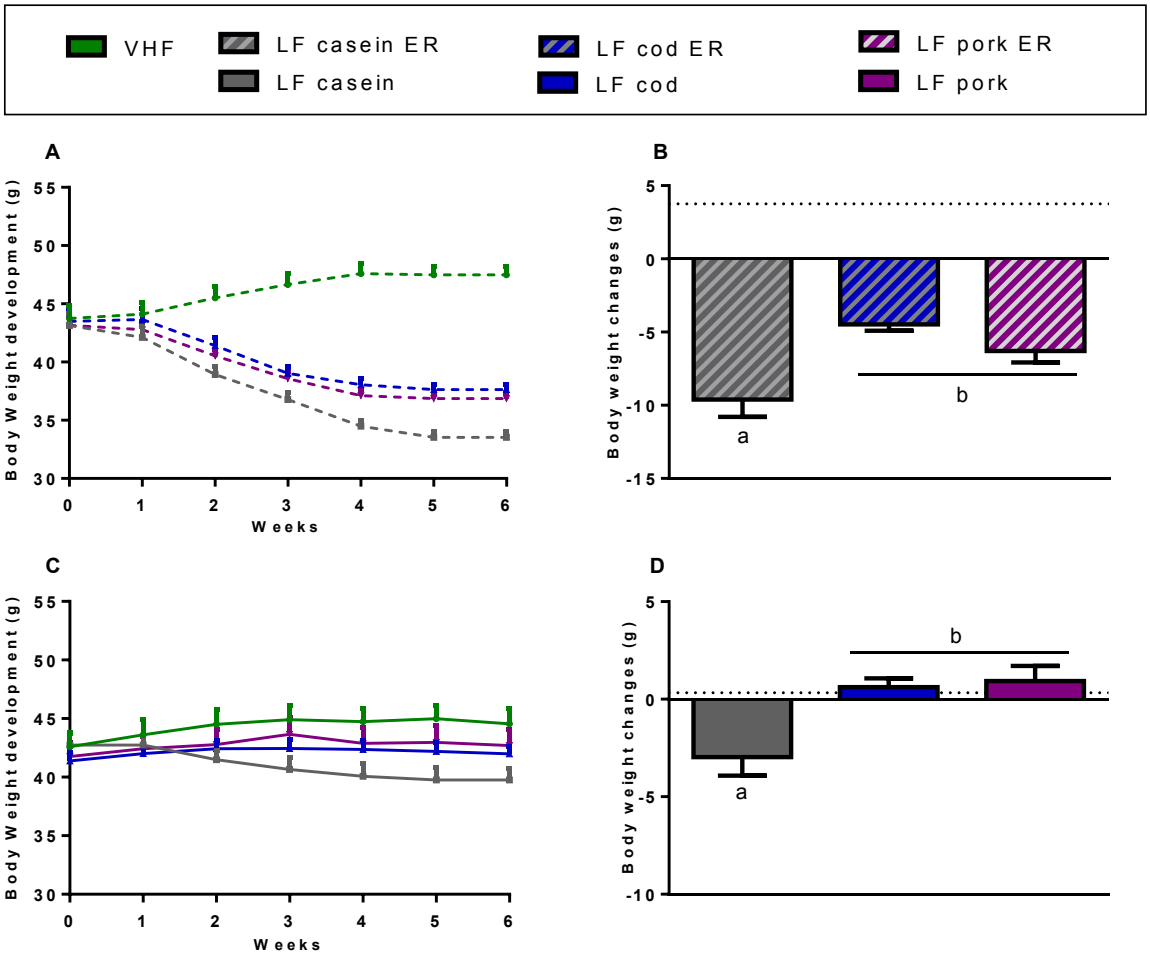

Figure 3.1: *Body weight development on experimental diets.* ***A,C:*** *Body weight development presented per week of feeding.* ***B,D:*** *Body weight changes during the entire experiment. The dotted lines represents the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ($p<0.05$), according to a one-way ANOVA with multiple comparisons of the mean of each group, with post hoc Fisher's LSD test.*

Feeding the experimental diets resulted in differences in weight-development. In the experiment with calorie-restriction, the casein–fed mice lost significantly more weight than the cod – and pork-fed mice during 5 weeks of feeding (Fig 3.1 B). This group lost a mean of 9.6 g body weight. In the experiment where the mice had *ad libitum* access to feed (Fig 3.1 D), the casein-fed mice's weight loss was also of statistical significance compared to the cod– and pork-fed mice. Here, the mean weight loss was 2.9 g.

3.1.2 Body mass composition

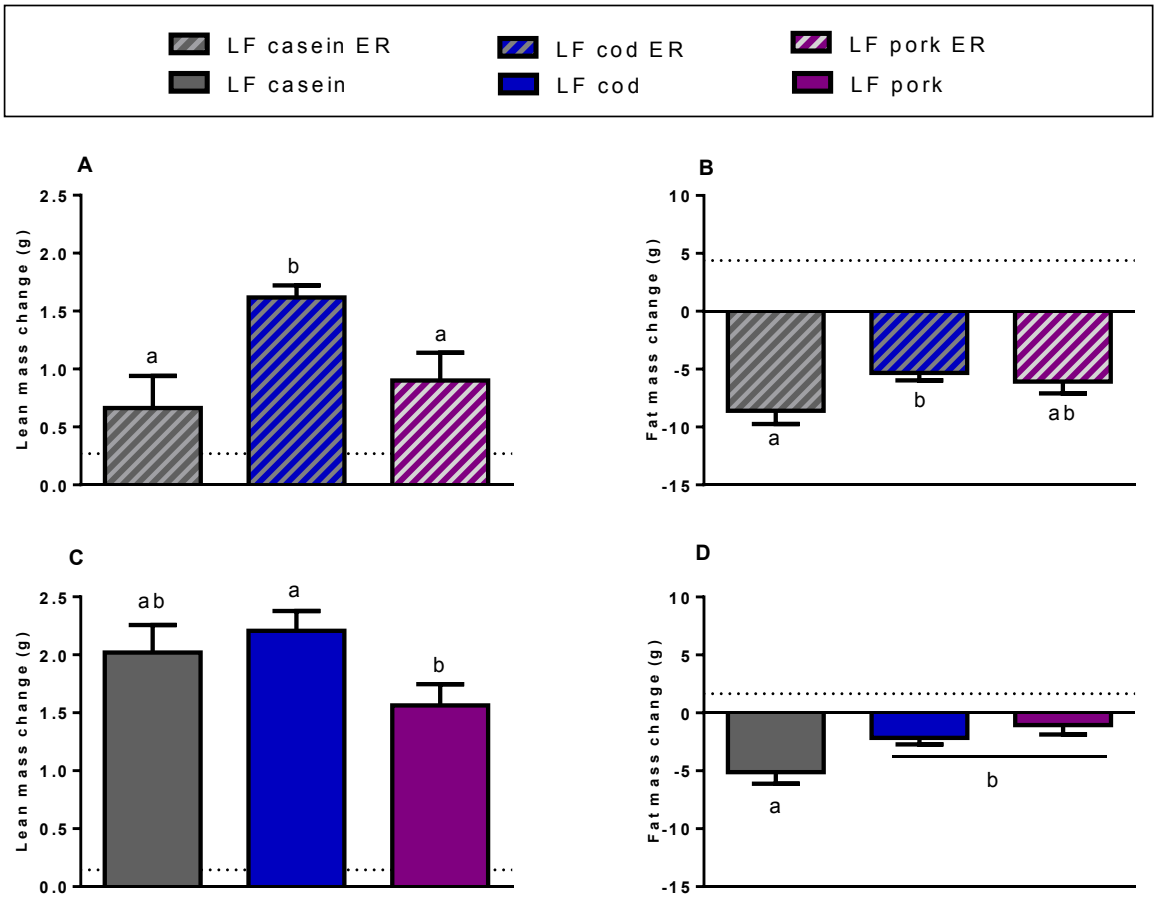

Figure 3.2: *MRI-scan of fat and lean mass changes after consuming the experimental diets for three weeks.* ***A,C:*** *Lean mass change during experimental feeding* ***B,D:*** *Fat mass change during experimental feeding. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ($p<0.05$), according to a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

During the experimental feeding, the mice were MRI scanned to determine any changes in body mass composition, thus the fat– and lean mass content. The scan took place at week 0, three weeks into the experimental period, and at week 6, the same week as termination. In the experiment with ER, figure 3.2 A illustrates that the cod-fed mice gained significantly more lean mass than the other experimental groups. The casein – fed mice lost significantly more fat mass compared to the cod – fed mice (Fig 3.2 B). Figure 3.2 C illustrates a significant increase in lean mass in the *ad libitum* cod - fed mice, compared to the pork-fed mice. As presented in Figure 3.2 D, there was a significant decrease in the fat mass in the *ad libitum* casein-fed mice, compared to the other two experimental groups.

3.1.2 Tissue masses

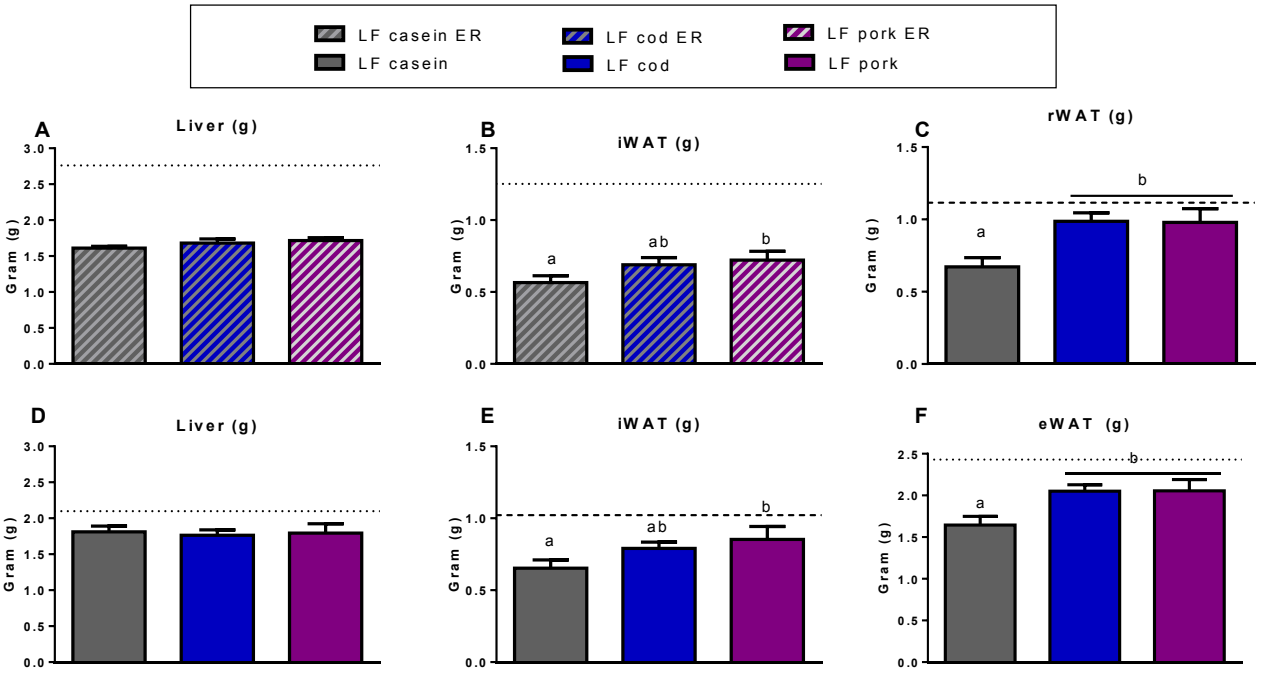

Figure 3.3: *Weights of tissue-masses presented in grams. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ($p<0.05$), according to a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

The different dietary protein sources led to changes in fat – deposition, but not in liver weights (Fig 3.3 A and D). In the ER–experimental groups, the casein–fed mice had a significantly lower iWAT mass than the pork – fed mice (Fig. 3.3 B). In the *ad libitum* - experiment, giving different protein sources resulted in significant differences in weights of eWAT and rWAT, where the casein-fed mice had reduced fat depot weights (Fig. 3.3 C and

F). The iWAT weight of the casein-fed mice were significantly lower compared to the pork – fed mice (Fig. 3.3 E).

3.1.3 Inguinal white adipose tissue

To assess whether there had been any dietary effect on the adipocyte sizes, photos of histological sections of iBAT, iWAT and eWAT were taken.

The captions of iWAT (Fig. 3.4) illustrates small differences between the experimental groups. The *ad libitum* casein - and cod – fed mice appear to have smaller adipocytes than the pork – fed mice, which also reflects on differences in the iWAT weights between casein and pork (Fig. 3.4 E). In the ER–experiment, there were no obvious differences in adipocyte size between the experimental groups, but a slightly larger phenotype in mice fed VHF control was observed. There is little noticeable difference between mice fed ER and *ad libitum*, except a modest reduced adipocyte size in iWAT from mice fed the ER pork diet compared to mice fed the *ad libitum* diet with pork.

The captions of eWAT (Appendix IX, Figure A.1) show small differences between the experimental groups. A slightly greater degree of adipocyte hypertrophy can be seen in the pork – fed mice, especially compared to the the casein –fed mice, which is consistent with the adipose tissue weights of eWAT (Fig. 3.3 F).

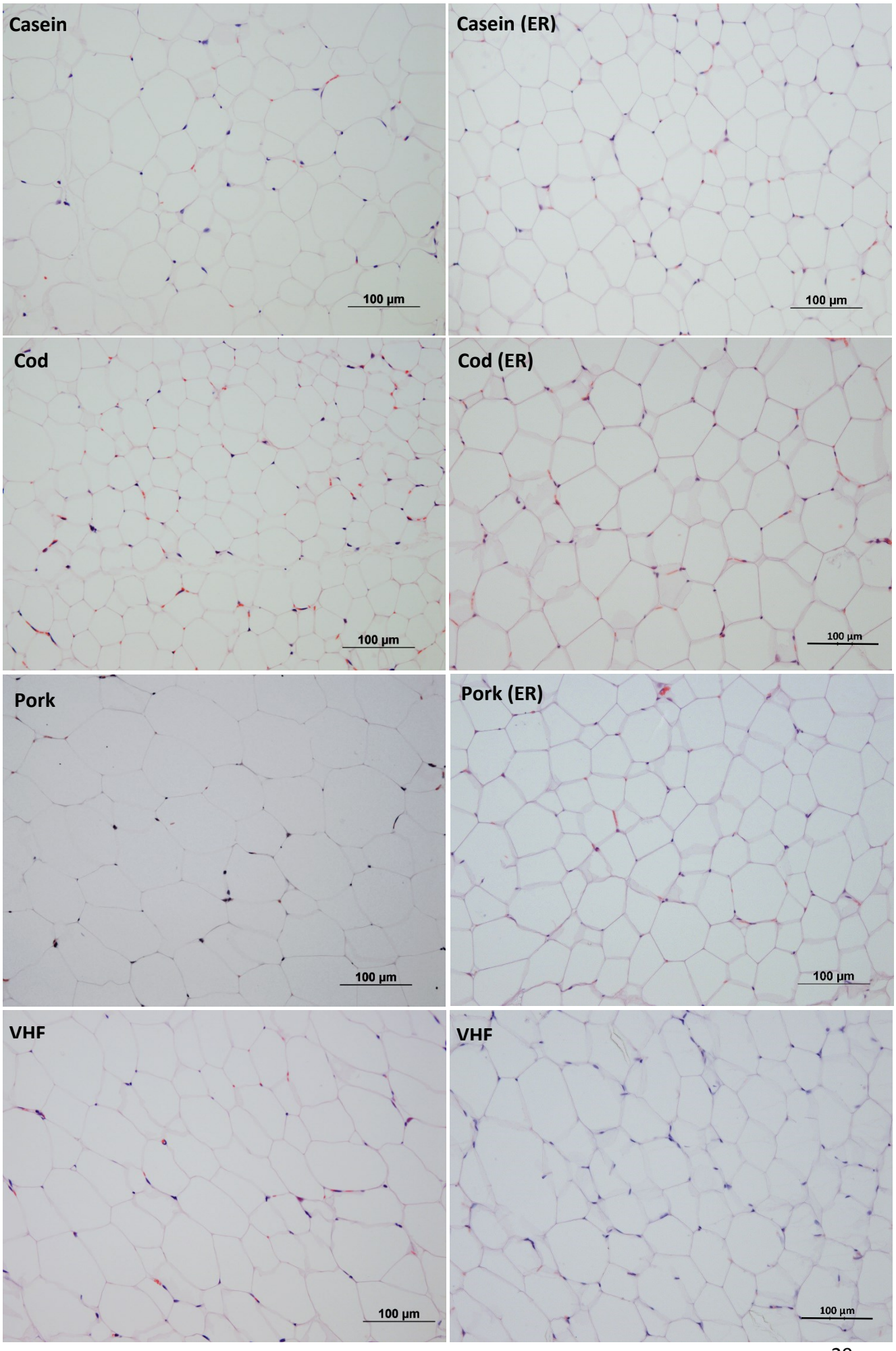

Figure 3.4: *Microscopy photos of the inguinal white adipose tissues. The photos presented are from a representative mouse in each experimental group. All photos are magnified 20x.*

3.3 Glucose tolerance and insulin sensitivity

3.3.1 Oral glucose tolerance test

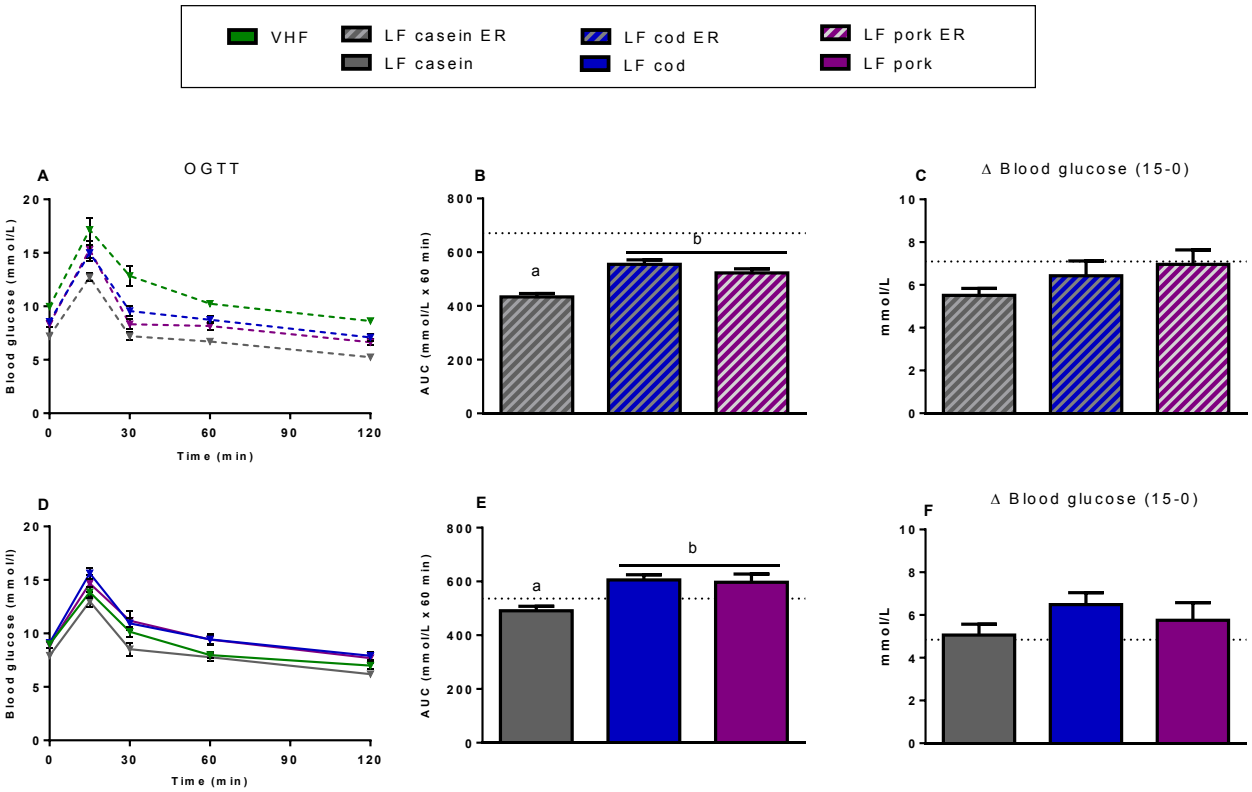

Figure 3.5: *Results from the 120 min oral glucose tolerance test.* ***A,D:*** *Blood glucose values plotted against time (min).* ***B,E:*** *Calculated 1 hour area under the curve (AUC).* ***C,F:*** Δ blood glucose (15-0)*. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), according to a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

An OGTT was carried out in week 5 of both the experiment with ER and with *ad libitum* access to feed. Figure 3.5 A implies an improvement of glucose tolerance in the mice on an ER diet, compared their respective control group. Results from the group fed *ad libitum* show a less clear difference in values between the groups (Fig 3.5 D). As presented in Figures 3.5 B and E, the calculated area under the curve (AUC) for both experiments reached statistical significance between the casein–fed group and the groups fed cod and pork, indicating a more effective glucose clearance in the group fed casein. The Δ blood glucose levels (0-15 min) among the experimental groups did not reach statistical significance in either experiments (Fig 3.5 C and F).

3.3.2 Plasma insulin concentrations

During the OGTT, samples of blood were collected at baseline and at 15 and 30 minutes for plasma insulin analysis, and the results are presented in Figure 3.6 A and B. The same analysis was performed on plasma collected at termination, at a randomly fed state (Fig. 3.6 C and D). During the OGTT, significant differences in plasma insulin concentrations only occurred in the *ad libitum* experiment. Here, the casein–fed group had a lower insulin concentration in plasma compared to the other experimental groups at baseline and after 15 minutes (Fig 3.6 B). At termination, the ER casein–fed mice had significantly lower insulin levels compared to cod (Fig 3.6 C). In the *ad libitum* experiment, the mice fed casein had a significantly lower plasma insulin concentration at termination compared to mice fed pork (Fig 3.6 D).

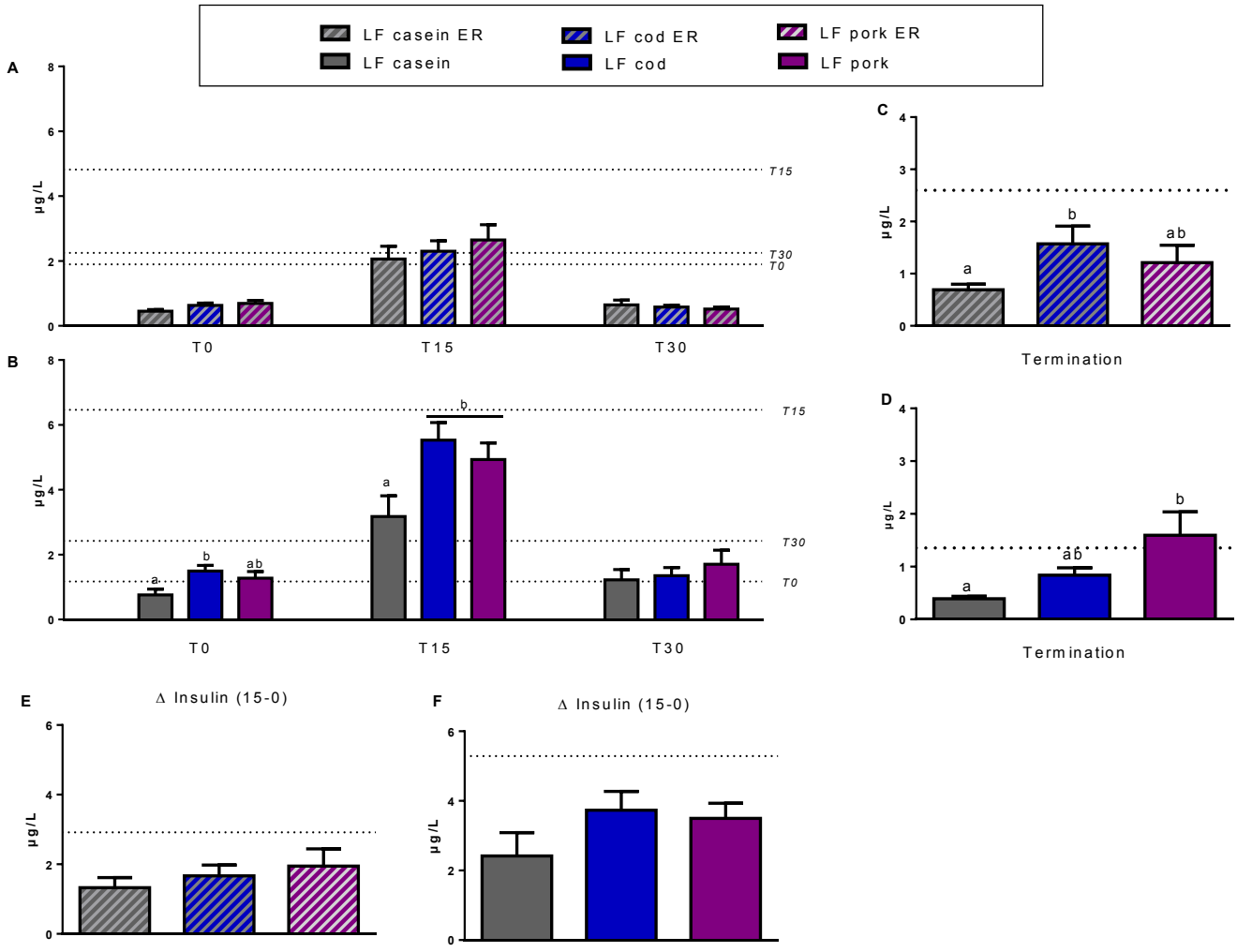

Figure 3.6: *Plasma insulin concentrations (µg/L)* ***A,C:*** *during the OGTT and* ***B,D:*** *at termination.* ***E,F:*** *Δ insulin (15-0). The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), according to a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

3.3.3 Insulin tolerance test

An ITT was performed after 4 weeks on the experimental diets. As presented in Figure 3.7 B, the group fed casein reached a significantly lower AUC compared to the other groups in the ER - experiment. The same is observed in the *ad libitum* experiment (Fig 3.7 E). In addition, the *ad libitum* casein–fed mice had significantly higher decremental area under the curve (DAUC) compared to mice fed cod (Fig 3.7 F). A homeostatic model assessment–insulin resistance (HOMA – IR) was also calculated. In both experiments, the group fed casein had the lowest HOMA – IR, associated with improved insulin sensitivity (Fig 3.7 G and H).

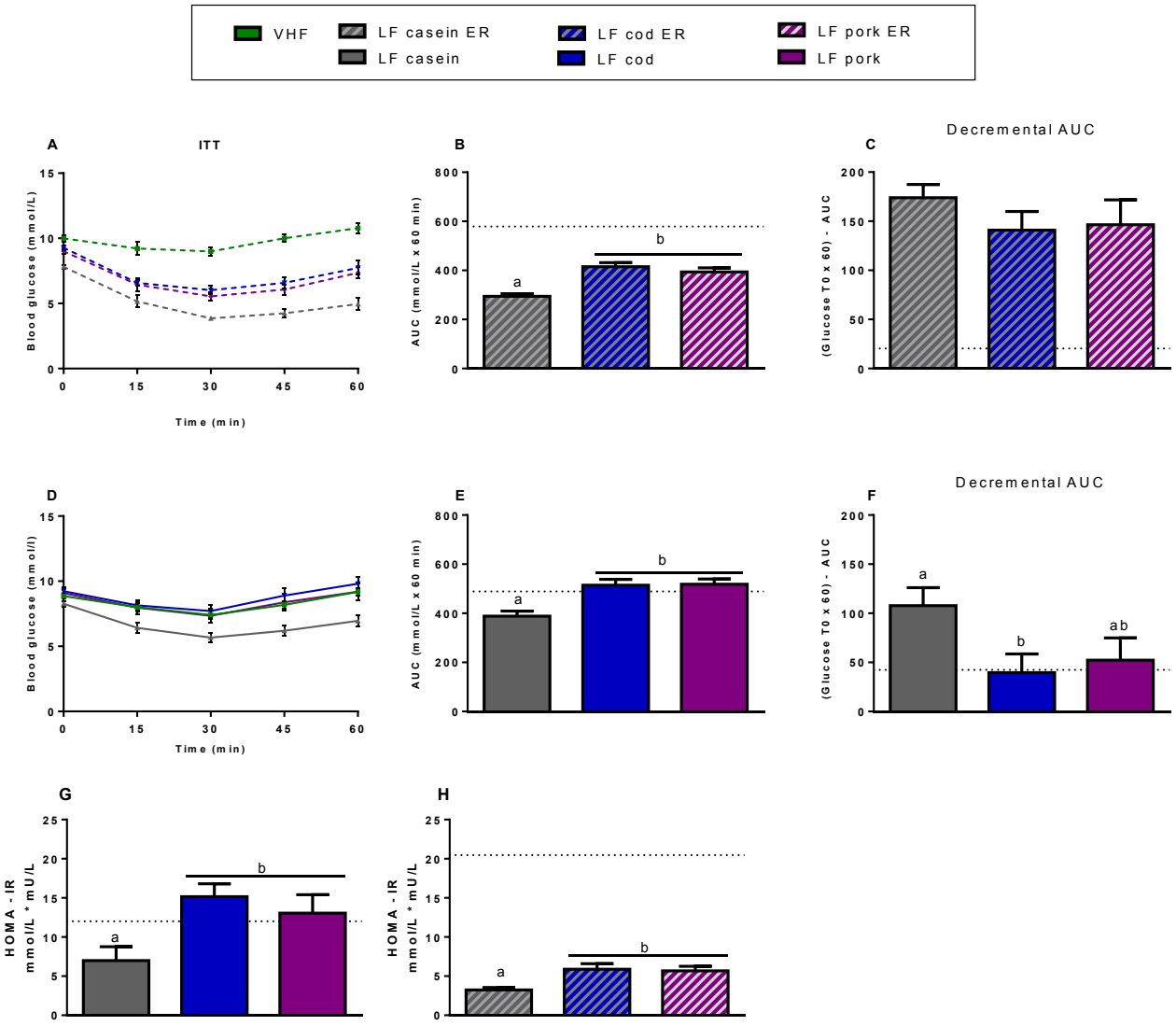

Figure 3.7: *Results from the 60 min insulin tolerance test.* ***A,D:*** *Blood glucose during the test plotted against time.* ***B,C:*** *Calculated area under the curve.* ***C,F:*** *Calculated decremental AUC (Glucose T0 x 60) – AUC.* ***G,H:*** *Calculated HOMA – IR (mmol/L * mU/L). The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), according to a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

3.4 Energy intake and metabolism

3.4.1 Energy intake and feed efficiency

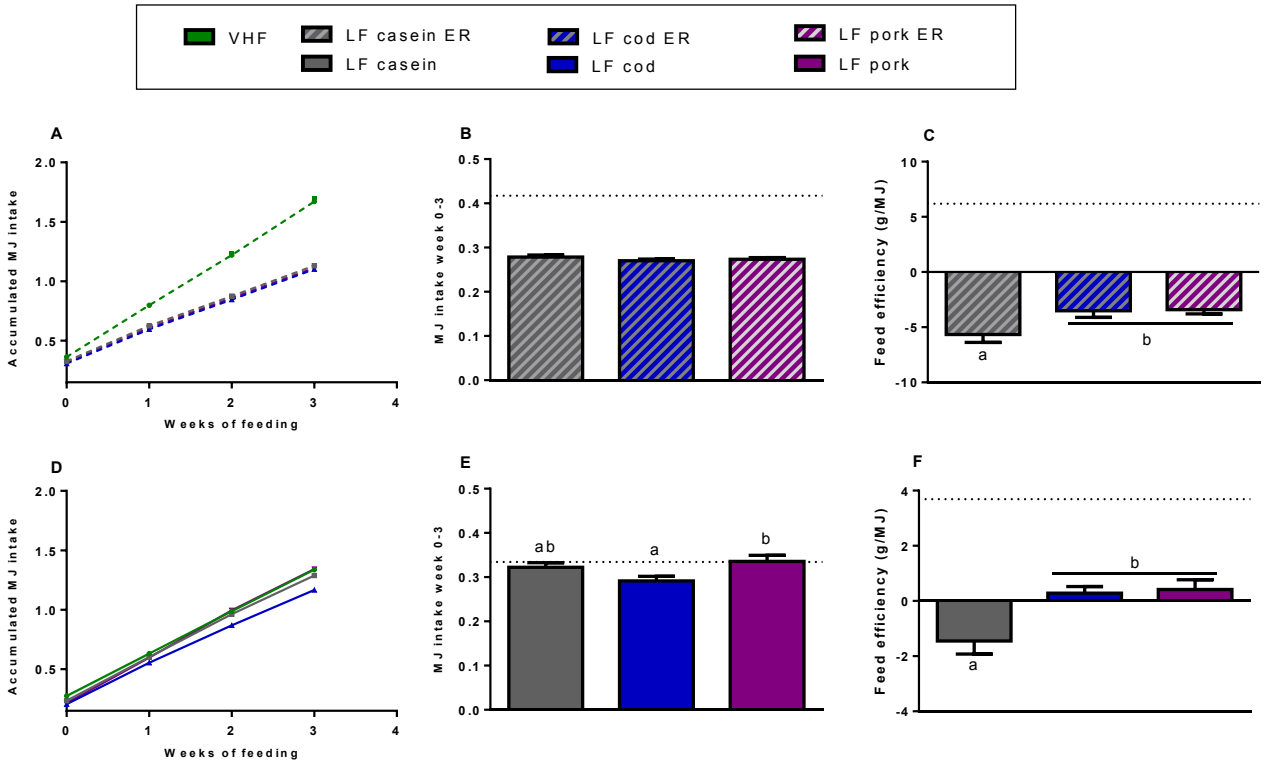

Figure 3.8: *The energy intake calculated during 3 weeks of the experiments.* ***A,D:*** *Accumulated MJ intake.* ***B,D:*** *Total MJ intake during 3 weeks.* ***C,F:*** *Feed efficiency (g/MJ). The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ($p<0.05$), according to a one-way ANOVA with multiple comparisons of the mean of each group, with post hoc Fisher's LSD test.*

The energy intake, presented in megajoules (MJ) was calculated during the first three weeks of the feeding experiments. Because feed intake was influenced by testing, energy intake documented during this period was not included in the calculations presented above (Fig. 3.8). We observed that mice fed *ad libitum* cod, consumed less energy than the pork – fed group (Fig 3.8 E). In the ER experiment, the control group had no calorie restriction, so these mice naturally had a higher feed intake than the other groups (Fig. 3.8 B). As presented in Figure 3.8 C and F, the mice fed casein had a lower feed efficiency compared to the other experimental groups, in both experiments.

3.4.2 Expression of genes involved in regulation of energy metabolism

Genes coding for leptin and proteins associated with energy metabolism through the endocannabinoid system were measured in iBAT by qPCR. As presented in Figure 3.9, expression of the genes coding for the cannabinoid receptors 1 and 2 (*Cnr1* and *Cnr2*) was lower in the casein–fed mice than in the pork–fed mice (Fig. 3.9 A and B). The casein–fed mice also presented a lower expression of N – acyl phosphatidylethanolamine phospholipase (*Napepld*) (Fig. 3.9 C). The expression of *Leptin* reached a higher value in the pork–fed mice compared to the casein–fed mice (Fig. 3.9 E).

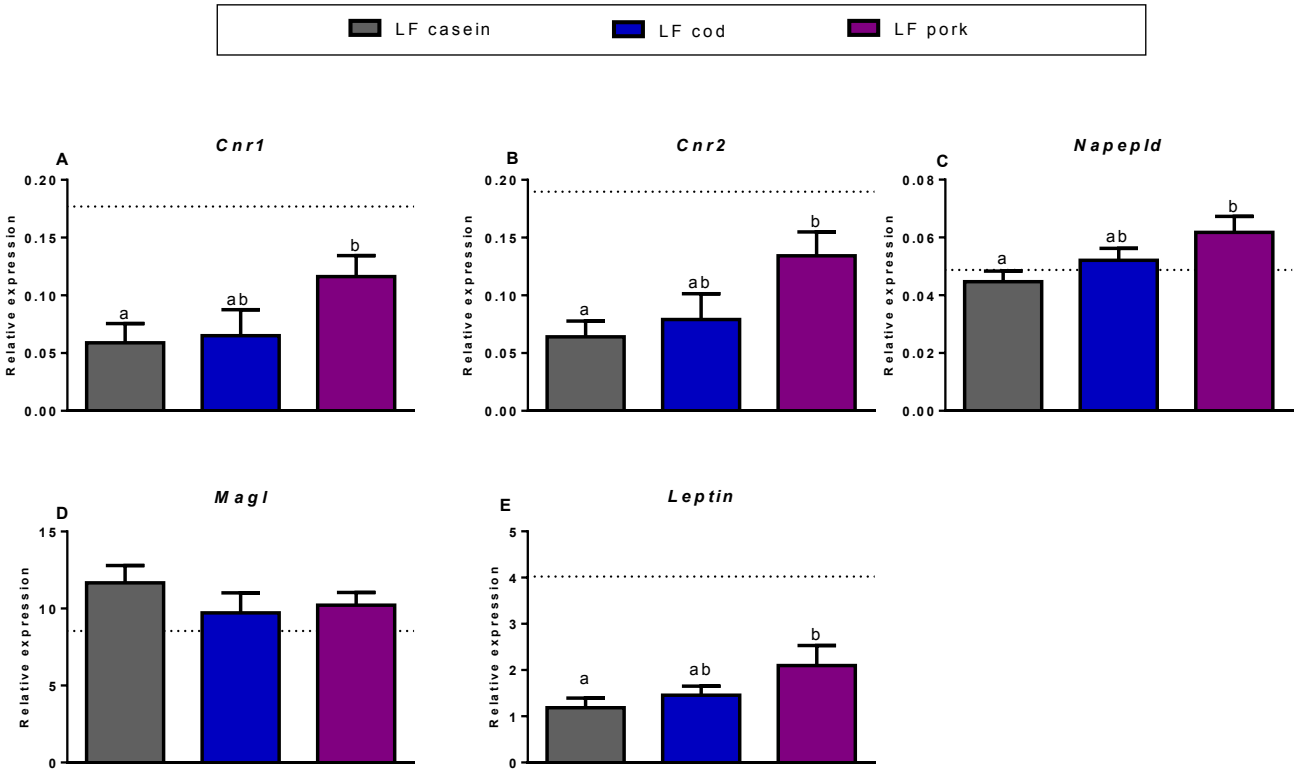

Figure 3.9: *Relative expression of Leptin and four genes acting as markers for the endocannabinoid system: Cnr1, Cnr2, Napepld, and Magl. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), determined by a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

3.4.3 Apparent digestibility

Samples of feces were collected and calculations were made to determine apparent digestibility for fat and nitrogen for the mice in each experimental group. No differences in digestibility for neither protein nor fat could be observed (Fig. 3.10).

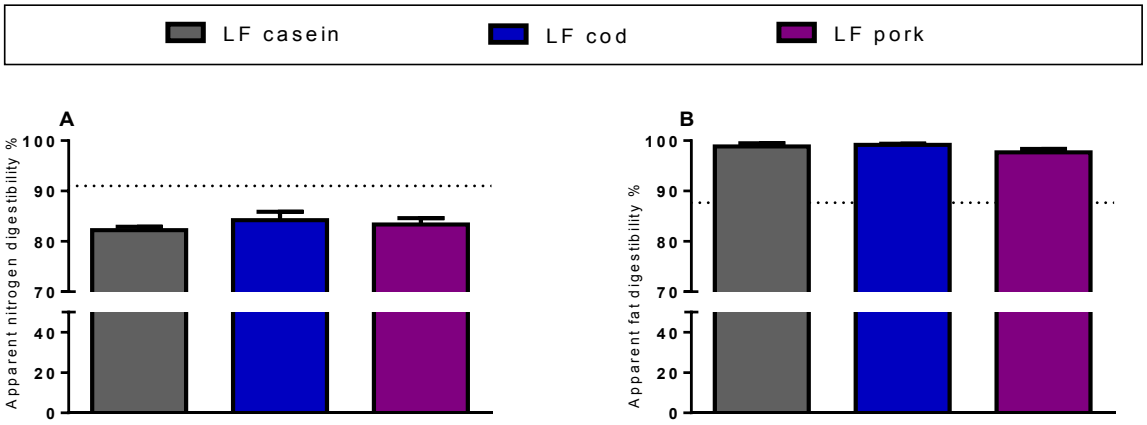

Figure 3.10: *Calculated apparent digestibility for protein and fat, presented in %. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups. Differences in digestibility ($p<0.05$) were determined by a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

3.4.4 Red blood cell analysis

The fatty acid composition of the *ad libitum*-fed mice's RBC was analyzed. The RBC of the cod–fed mice contained most ω-3 fatty acids, whereas the lowest ω-3 levels were found in the casein–fed mice's red blood cells (Fig. 3.11 A). The ω-3:ω-6 ratio was also higher in the cod–fed mice's blood cells compared to the other groups (Fig. 3.11 C). Furthermore, the cod–fed mice had a lower ω-6 content in their RBC than the other two experimental groups (Fig. 3.11 B). The content of ARA was lowest in the cod–fed mice, but this group had the highest level of LA (Fig. 3.11 E and F) in their RBC.

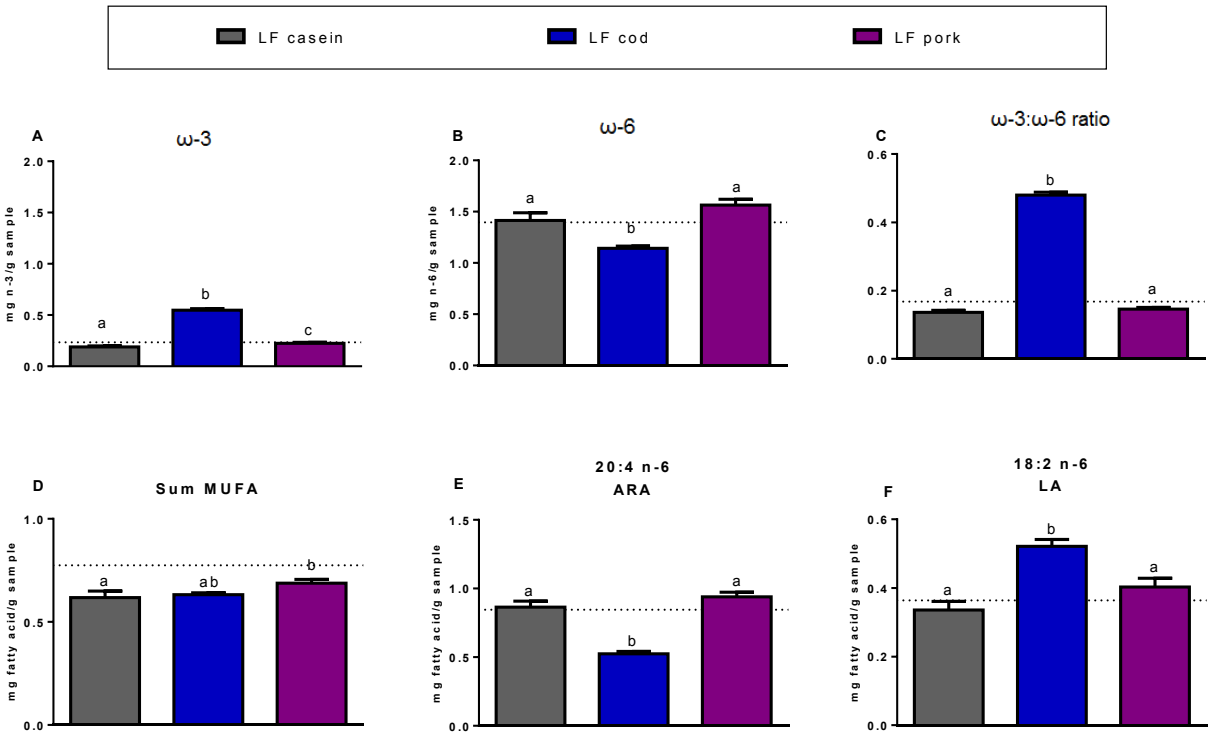

Figure 3.11: *The fatty acid content of the red blood cells, presents in mg/g sample, and* the ω-6/ω-3 – ratio. *The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ($p<0.05$), determined by a one-way ANOVA with multiple comparisons of the mean of each group, with post a hoc Fisher's LSD test.*

3.5 Interscapular brown adipose tissue

3.5.1 Adipose tissue weights

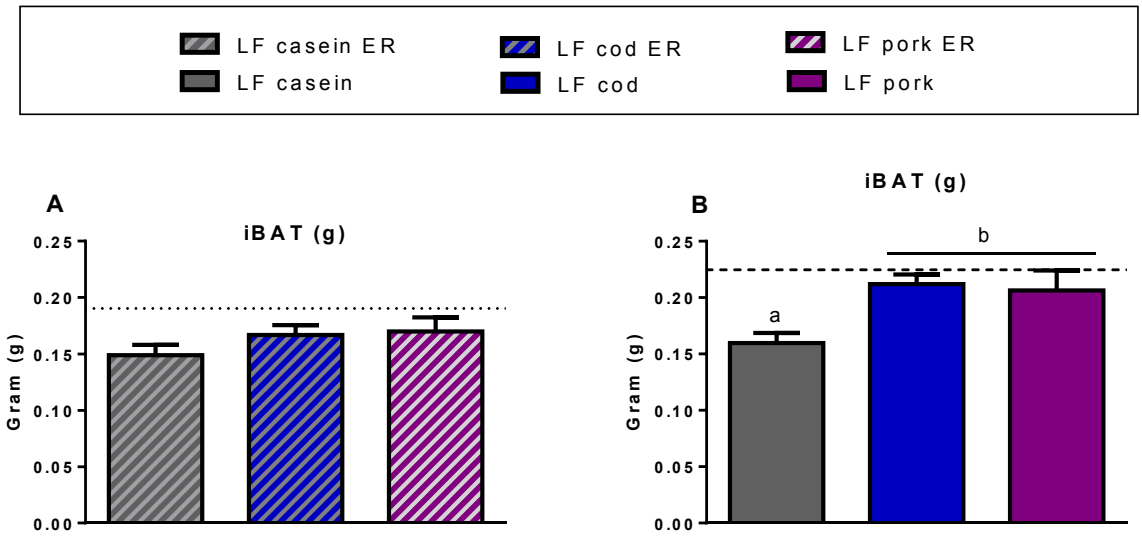

Figure 3.12: *Weights of iBAT presented in grams. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), according to a one-way ANOVA with multiple comparisons of the mean of each group, with post hoc Fisher's LSD test.*

The iBAT weights in the ER experiment did not differ among the experimental groups (Fig 3.12 A). In the *ad libitum* experiment, the casein group had a significantly lower iBAT weight compared to the two other experimental groups (Fig 3.12 B).

3.5.2 Adipocyte morphometry

The captions of the iBAT from the different groups (Fig. 3.13) illustrate that the casein–fed mice in both experiments have smaller adipocytes and a greater amount of multilocular cells in their BAT compared to the other groups. The pictures of iBAT from mice fed cod and pork illustrate larger unilocular adipocytes and the tissue seem to have a greater amount of white components, more similar to the control groups.

There is a clear resemblance in iBAT morphometry in mice fed ER and *ad libitum*. The only exception is a slightly higher presence of multilocular cells in the mice fed ER cod, compared to mice fed *ad libitum* cod.

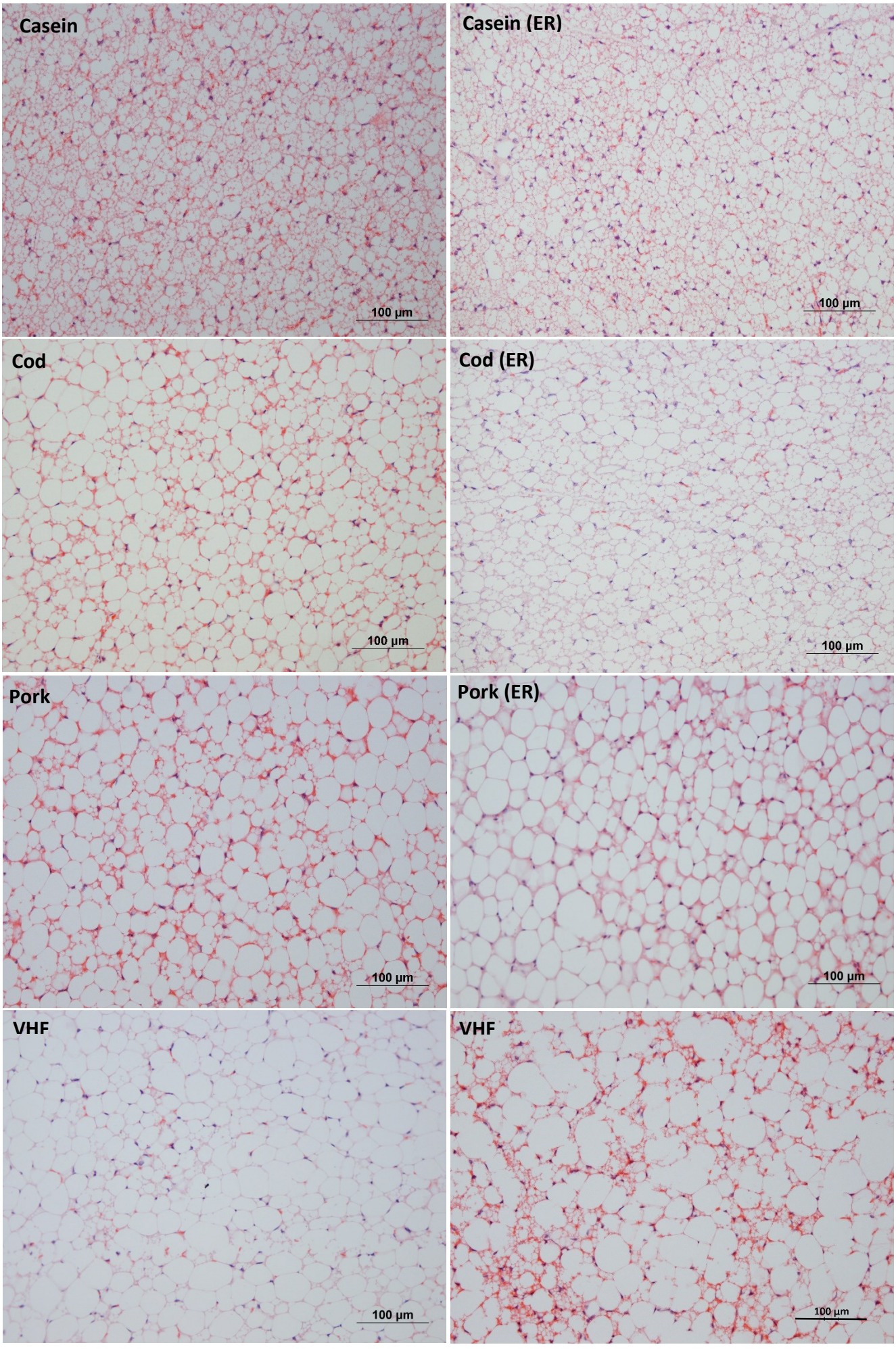

Figure 3.13: *Microscopy photos of the interscapular brown adipose tissue. The photos presented are from a representative mouse in each experimental group. All photos are magnified 20x.*

3.5.3 Immunohistochemistry

The UCP1–staining of iBAT resulted in non-significant differences between the experimental groups (Fig 3.14 B). A positive and negative control is presented in the same figure (Fig. 3.14 C), where the positive control was exposed to a cold environment and have an expected high expression of UCP1, whereas the negative was not exposed to cold. The negative control present a similar result of the staining as our experimental groups.

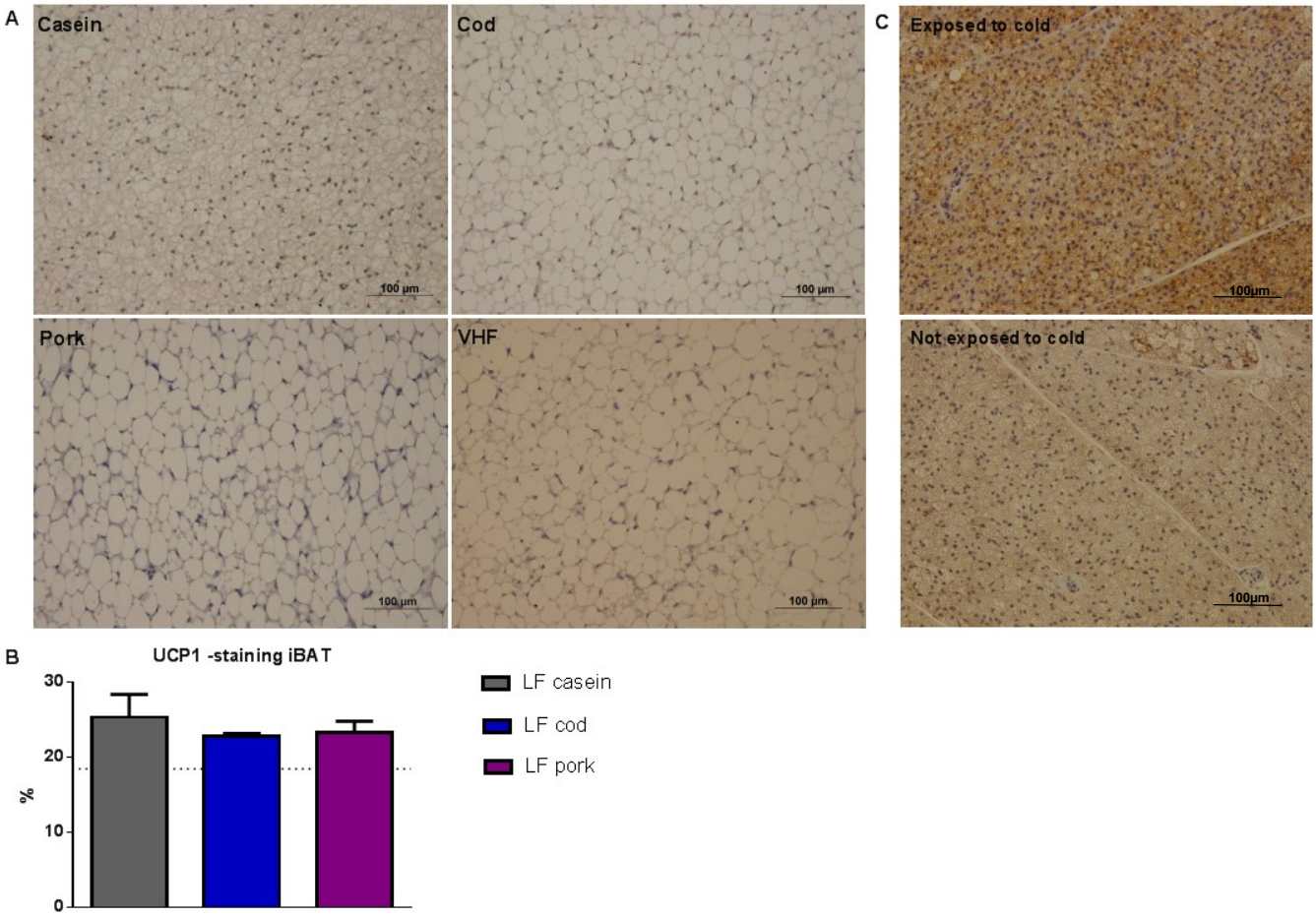

Figure 3.14: A: *Pictures of the UCP1 – stained iBAT.* ***B:*** *The results of the immunohistochemistry presented as percentage (%) presence of stained UCP1.* ***C:*** *A positive and negative control. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups ((p<0.05), determined by a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test. All photos are magnified 20x.*

3.5.4 Expression of genes involved in brown adipose tissue function

Figure 3.15 presents the expression of genes coding for proteins that relate to the BAT's thermogenesis function. Feeding different protein sources affected gene expression of the *Ucp1* gene, which was expressed highest in the casein–fed mice, compared to the other two experimental groups (Fig. 3.15 A). Furthermore, the C*d36* gene expression was higher in iBAT of the pork–fed mice compared to the cod – fed mice (Fig. 3.15 F).

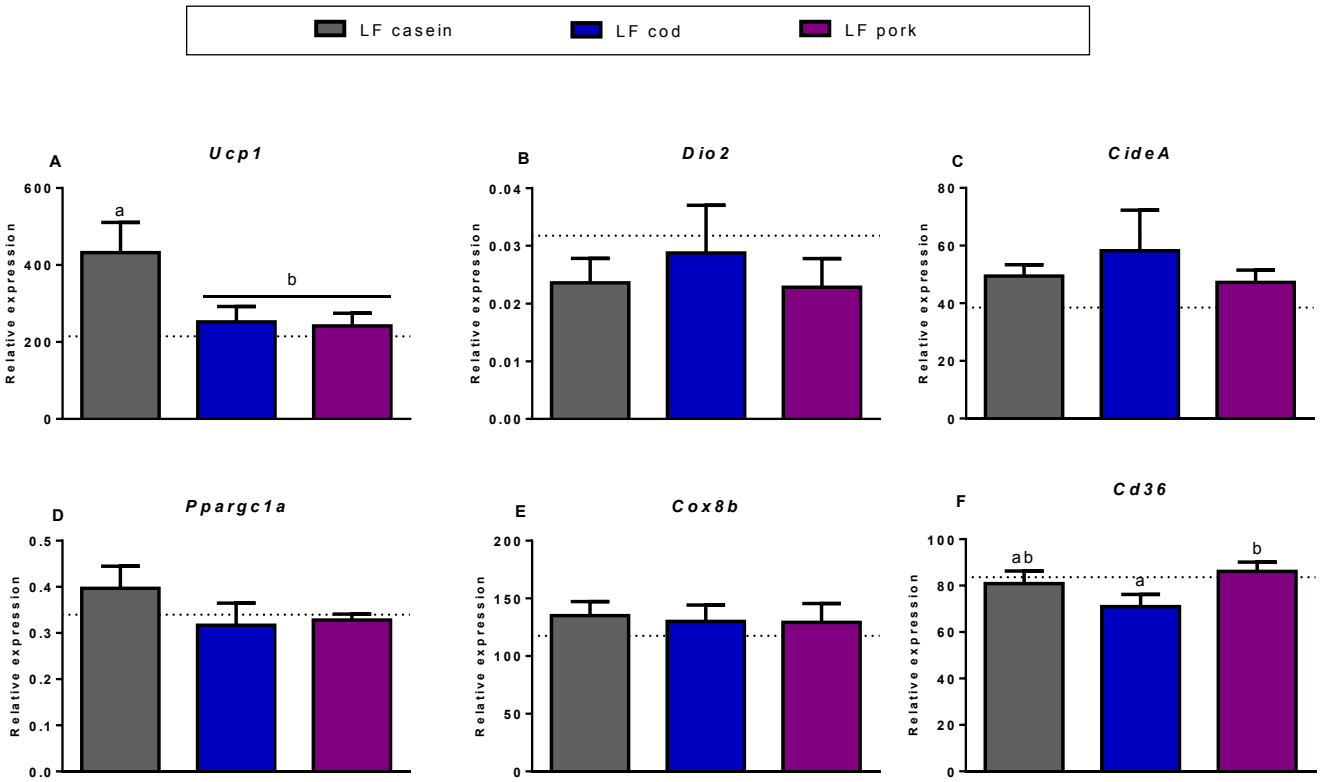

Figure 3.15: *Relative expression of markers of brown adipose tissue activity: Ucp1, Dio2, CideA, Ppargc1a, Cox8b and Cd36. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), determined by a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

3.4.4 Expression of genes involved in redox pathways

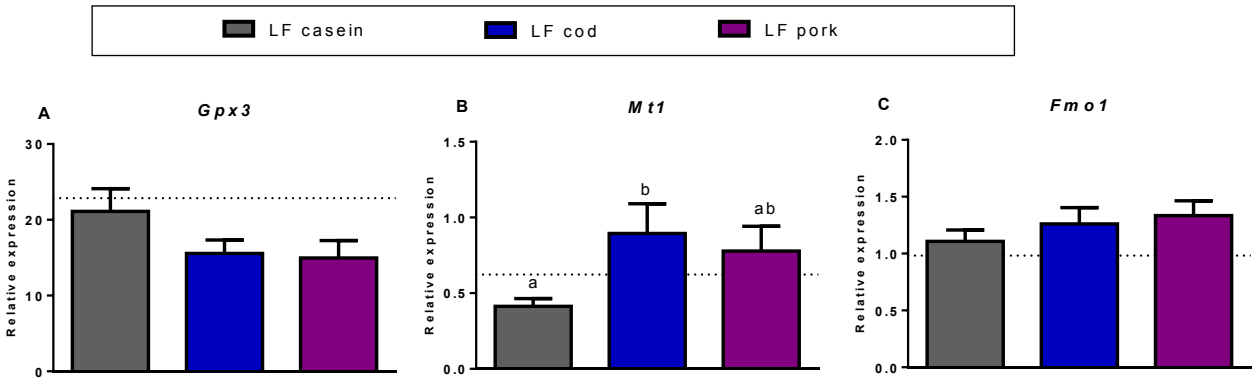

Figure 3.16: *Relative expression of three genes in redox pathways: Gpx3, Mt1 and Fmo1. The dotted lines represent the control group. The results are presented as mean ± SEM. Different letters designates statistical significance between the groups (p<0.05), determined by a one-way ANOVA with multiple comparisons of the mean of each group, with a post hoc Fisher's LSD test.*

Measuring genes involved in redox pathways in iBAT resulted in a lower expression of Metallothionein 1 (*Mt1*) in iBAT from mice fed casein compared to the cod–fed mice, with the highest expression of this gene (Fig. 3.16 B).

4.0 DISCUSSION

Although extensive research on substituting dietary carbohydrates with protein have been performed, both within our group and elsewhere (Ma et al., 2011, Madsen et al., 2008b, Skov et al., 1999), there is limited information regarding the weight reducing effects of different protein sources. Unpublished data from our research group revealed that mice fed casein was protected against obesity development, whereas mice fed diets with protein derived from soy, pork, cod, beef and chicken was not. Casein has previously exhibited these advantageous effects, and is believed to be able to mimic the effect of a HP diet in HF fed mice and thus ameliorate adiposity (Lillefosse et al., 2013, Tastesen et al., 2014b). In addition, an increased ingestion of fish has resulted in weight loss and improved insulin sensitivity on several occasions (Lavigne et al., 2000, Lavigne et al., 2001, Thorsdottir et al., 2007). Based on these previous findings, we induced obesity with a very high fat diet, and attempted to reduce the weight with LF, normal protein diets. Hereunder, we evaluated whether different protein sources could modulate the effects of the LF-diet. We demonstrate that the type of protein ingested is of importance, and that dietary casein exhibits a striking weight reducing effect compared to cod and pork, especially when ER is enforced. To elucidate the mechanisms in which casein induces weight loss, we investigated possible contributing factors in dietary fatty acid- and AA composition and gene expression.

4.1 The dietary protein source alters body composition

Nutritional research frequently revolve around the capability of different diets to induce weight loss and to correct metabolic disturbances linked to obesity. A popular dietary approach is to reduce the amount of dietary fat (Muurling et al., 2002, Skov et al., 1999). In addition, both LF and HF energy restricted diets have proven effective to induce weight loss (Johnston et al., 2004, Heilbronn et al., 1999, Noakes et al., 2005). This is in concordance with our study, where all the mice with ER reduced their weight compared to baseline. When given *ad libitum* feed access, weight loss was only induced in mice fed casein.

Observations regarding the dietary composition of macronutrients are numerous. Research suggests that a HP diet promotes weight loss (Pichon et al., 2006, Blair et al., 2014), and that mice given a diet with a low protein:carbohydrate ratio (P:C ratio) gain heavier fat depots compared to a diet with a high P:C ratio. Lowering the P:C ratio have also caused a

simultaneous increase in energy intake to compensate for an unsatisfying dietary protein content (Huang et al., 2013). The dietary protein amount seem to influence weight reduction considerably, because compared to ours, a diet with somewhat less carbohydrates and more protein successfully induced weight loss in mice (Muurling et al., 2002). We can argue whether the dietary P:C ratio could account for the modest weight reduction in the mice fed *ad libitum* cod and pork, and if small adjustments in dietary composition might have given different results. However, a successful decrease in body weight was obtained in all mice fed ER, suggesting that the low amount of dietary fat has less impact than restriction of energy.

Further observations of the development of body composition revealed a decreased fat mass in all the LF-fed groups. Mice fed casein had the greatest decrease, consistent with the differences observed in adipose tissue weights and to some extent histologic appearance of the adipose tissue. Furthermore, all the LF-fed groups increased their lean mass during experimental feeding. The *ad libitum*-fed mice had a lean mass increase exceeding total grams of body weight lost, suggesting a conversion of fat mass to lean mass. The ER mice had a sparse lean mass increase compared to the reduction in body weight and fat mass. Despite equal percentage of energy from protein in both diets, the absolute protein amount ingested was lower in the ER experiment, so a decreased muscle protein synthesis could be expected. Interestingly, the ER experiment's control group had *ad libitum* access to feed, but still gained less lean mass and more fat mass than the experimental groups. This could indicate that lowering the dietary fat content has a favorable effect on lean mass development and fat mass loss.

Within the experimental groups, we made an interesting discovery; the mice fed *ad libitum* cod gained significantly more lean mass than mice fed *ad libitum* pork. Furthermore, the ER cod-fed mice gained more lean mass compared to both experimental ER groups. Giving attention to this phenomenon, we see that administrating fish protein supplementation to obese humans, resulted in a small increase in muscle mass after four weeks (Vikoren et al., 2013), and heavier muscle weights in rats (Kawabata et al., 2015). Furthermore, giving fish oil supplements have resulted in increased muscle mass and prevented further weight loss in cancer patients (Murphy et al., 2011). Although we could not establish that the ingestion of fish protein induced weight loss compared to ingestion of casein, fish protein seems to benefit the preservation or gain of lean mass.

The AA composition of the diet may provide more insight to why casein was the only protein source to induce weight–and fat mass loss in the mice fed *ad libitum*. Previous studies from our research group indicate that dietary AAs reflect plasma AA profile, and assuming the same can be applied here, we can explore the possible effects of dietary AA composition. The casein diet contains more of the branched chain amino acid (BCAA) Leu than the cod and pork diets (Appendix I, Table A.3 and A.4). The impact of Leu has been vigorously debated, and Leu is believed to alter several metabolic pathways that can influence or be influenced by weight development (Layman and Walker, 2006). Mice given a HF diet with Leu-supplemented drinking water lost body weight and fat mass compared to the control group (Zhang et al., 2007). Supporting studies confirm these findings, and indicate that Leu can mimic the effects of a HP diet, yet not entirely replace it (Freudenberg et al., 2012).

We have demonstrated that inducing weight loss with a low-fat normal protein diet is best achieved with ER, and that feeding casein as the protein source have greater impact on weight loss than feeding protein from cod or pork.

4.2 The dietary protein source affects glucose and insulin metabolism

As a reduced glucose tolerance is related to the degree of adiposity, we observed that the development of body composition could readily be associated with results from the OGTT. According to calculations of glucose AUC, the casein–fed mice had a significantly better glucose clearance after sustaining a glucose load orally, compared to mice fed cod or pork. We saw this in direct relation to insulin concentrations in plasma, where mice fed *ad libitum* casein had lower fasting insulin concentrations compared to the mice fed cod. Moreover, at glucose peak, the casein–fed mice had lower insulin concentrations compared to both pork– and cod–fed mice, indicating a better tissue response to insulin. A study in humans predisposed to T2DM show that a lifestyle change effectively decreases the incidence of T2DM compared to using an anti–diabetic drug (Knowler et al., 2002). Our data confirm that improvements in glucose homeostasis relates to the weight reduction. However, we can speculate whether the casein–fed mice's more efficient blood glucose clearance is indirectly due to lower adiposity, or directly due to dietary composition. In relation to this, we have considered that various metabolic systems respond differently to BCAAs, especially Leu. Leu supplements are believed to efficiently improve glucose tolerance by stimulating insulin secretion and consequently glucose uptake via the mTOR signaling pathway (Devkota and

Layman, 2010, Yang et al., 2010, Macotela et al., 2011). Studies show that to give humans a mix of glucose and Leu resulted in increased insulin secretion and glucose clearance (Kalogeropoulo et al., 2008), suggesting that Leu is a potent stimulator of insulin secretion, especially in the presence of glucose.

Furthermore, along with a lower AUC from the ITT, the casein–fed mice in our experiment presented with a lower HOMA–IR index, indicating an improved insulin sensitivity compared to mice fed cod and pork. This is in agreement with a publication where casein is compared to chicken (Tastesen et al., 2014b). With regards to this, studies associate Leu intake with improved insulin sensitivity through the mTOR signaling pathway (Macotela et al., 2011, Zhang et al., 2007), and that Leu augments the insulin–mediated glucose uptake (Eller et al., 2013). The mTOR pathway's role in development of insulin sensitivity is still unresolved, and some claim that BCAA supplements can induce insulin resistance, possibly through excess insulin secretion (Newgard et al., 2009). Moreover, several studies with rodents associate ingestion of protein from cod with an improved insulin sensitivity and glucose tolerance compared to dietary casein (Lavigne et al., 2000, Lavigne et al., 2001, Tremblay et al., 2003, Tastesen et al., 2014b) and in humans, compared to ingestion of a mix of different proteins (Ouellet et al., 2007). Nonetheless, we did not observe these effects using LF-fed mice as our experimental model.

Being two separate studies, the two experiments described in this thesis are not statistically comparable. We observe that both ER and *ad libitum* casein-fed mice had an improved insulin sensitivity and glucose tolerance. However, the group fed *ad libitum* casein presented with an increased DAUC compared to mice fed cod, while no significant differences in DAUC occurred between the ER groups. All the ER mice lost weight during LF-feeding, but casein was the only dietary protein source to induce weight loss when given *ad libitum*. These findings emphasize the powerful effect weight reduction exerts on glucose homeostasis.

The results herein indicate that feeding a LF-diet with casein as protein source influences blood glucose regulations in a positive manner, compared to diets with cod and pork. This is possibly influenced by the amount of dietary Leu, although research in this area presents conflicting results. Further research is necessary to elucidate the mechanisms regarding dietary protein source and AA composition.

4.3 The dietary protein source affects energy intake

Both animal and human studies present results promoting a HP diet as a satiating agent, and thus a strategy to reduce energy intake (Pichon et al., 2006, Huang et al., 2013). In our study, the mice fed cod had an inferior energy intake compared to the mice fed pork. We have seen the same tendency in unpublished data within our research group, with a lower energy intake amongst cod–fed mice compared to mice fed casein. Borzoei and colleagues observed that humans given fish for lunch, reported a higher feeling of satiety and consumed less calories during their next meal, compared to a group given beef for lunch (Borzoei et al., 2006). The satiating effect of fish is further supported by Uhe and colleagues, who argue whether the elevated plasma Trp:LNAA (Tryptophan:large neutral amino acids) ratio observed after fish ingestion, could be connected to satiety signaling by serotonin via the hypothalamus (Uhe et al., 1992). The brain requires Trp to synthesize serotonin, and an increased Trp:LNAA ratio can promote this synthesis (Geeraerts et al., 2011). Since the amount of Trp in the cod diet exceeded the diet with casein, but not the pork diet, feed intake in the cod–fed group could be related to these signaling pathways. However, plasma analyses of Trp:LNAA or serotonin would further elucidate these possible effect of Trp.

The variations we observed in energy intake could also relate to the dietary fatty acid composition and the influence of the endocannabinoid system. Alvheim and colleagues confirmed that a high amount of dietary LA increases PL–ARA and circulating AEA and 2-AG (Alvheim et al., 2012, Alvheim et al., 2014). However, fish intake, especially ω-3 PUFA in the form of PL seem to reduce formation of AEA and 2–AG (Rossmeisl et al., 2012), potentially suppressing energy intake. The cod diet in our experiment contained more LA than the other diets, which reflects the amount of LA in the cod–fed mice's RBC. However, the amount of ARA in RBC was significantly lower in the cod – fed mice, compared to the other experimental groups. This could be associated with a decreased endocannabinoid production and energy intake. Nevertheless, relative expression of the cannabinoid receptors *Cnr1* and *Cnr2* was decreased, but did not reach statistical significance in iBAT from the mice fed cod compared to the mice fed pork. The expression of *Napepld*, one of the enzymes that participates in the formation of AEA (Okamoto et al., 2007), was also intermediate in cod-fed mice, compared to mice fed casein and pork. We can discuss whether the differences in RBC-composition and gene expression in the cod–fed mice would

have been more pronounced with a higher dietary fat content. As the measurements of endocannabinoids in plasma were not finalized before this thesis was completed, we can only speculate whether the cod–fed mice had a lower level of circulating endocannabinoids in plasma.

Furthermore, the expression of *Cnr1* and *Cnr2* was significantly elevated in the pork–fed mice compared to the mice fed casein. This can be seen in relation to the pork–fed mice's heavier body weight, as elevated circulating endocannabinoids are seen in obese, along with elevation of CB1 expression and promotion of fatty acid synthesis and storage (Osei-Hyiaman et al., 2005, Lafontan et al., 2007). Several studies show that giving obese mice a CB1 antagonist promotes weight reduction, and this may be an important future contributor in the treatment of obesity (Trillou et al., 2003, Bajzer et al., 2011, Jbilo et al., 2005). In addition, compared to the casein–fed mice, the pork–fed mice had a higher expression of *Napepld*, which could help explain their increased feed intake compared to mice fed cod.

Although a decreased endocannabinoid action can cause a decline in energy intake, this did not occur in the mice fed casein, and does not explain why this diet provoked the greatest weight loss. However, the casein–fed mice's low expression of *Cnr1* and *Cnr2* could be associated with their lower body weight compared to mice fed cod or pork (Engeli et al., 2005). Furthermore, we know that obesity can cause leptin secretion to exceed physiological ranges, decreasing leptin sensitivity and causing an impaired satiety signaling (Ren, 2004). Although circulating leptin was not measured, explaining the increased *Leptin* expression in pork-fed mice with their higher adiposity remains a possible factor. However, it is reasonable to think that the increased *Leptin* expression in the iBAT of pork-fed mice compared to iBAT of mice fed casein, is caused by the amount of white adipocytes present in the iBAT, as leptin is predominantly secreted from WAT (Cinti et al., 1997).

4.4 The dietary protein source affects energy expenditure

We observed that giving mice different protein sources has an impact on the appearance of the iBAT. These findings are in concordance with unpublished studies from our research group are in, in which it is clearly visible that the iBAT morphometry in mice fed casein differ from the iBAT from mice fed cod or pork. We observe smaller adipocytes and a higher degree of multilocular cells in representative pictures of iBAT from the mice fed casein. The sections of iBAT from cod-and pork-fed mice show a closer resemblance to WAT, along with

heavier fat depots and body weight. In addition, a significantly lower iBAT weight was observed in the casein–fed mice, compared to the cod and pork–fed mice. The differences in tissue weights could be related to the total reduction in body- and fat mass weight of the casein-fed mice, but also indicate a higher conservancy of the brown component in their iBAT.

In addition to visible changes in iBAT from mice fed casein, we observed significant elevations in the relative expression of *Ucp1*, compared to cod– and pork–fed mice. When feeding a HP diet, an increased gene expression of genes involved in thermogenesis regulation of BAT, *Ucp1*, *Dio2* and *Ppargc1a* have been reported (Huang et al., 2013, Petzke et al., 2005). We observe that still with an adequate protein intake, iBAT from the mice fed casein had an elevated *Ucp1* expression accompanied by a reduced body weight. Thus, it is possible that casein hold properties that could increase the thermogenesis in the BAT in a similar fashion to HP diets.

To further evaluate the thermogenic effects of different protein sources on the iBAT, we performed an immunohistochemical staining for UCP1. According to the quantification of these results, the dietary protein source did not cause significant differences in the occurrence of UCP1. Comparing these images to the histological appearance of a cold–exposed positive control, clearly confirms lower amounts of UCP1 in iBAT from the mice in our experiment, indicating a less metabolically active iBAT. Unlike qPCR, which only supply information about gene expression at mRNA level, UCP1 – staining identifies the presence of the protein, thus the thermogenic capacity of iBAT in mice fed different protein sources. On the occasion that the presence of UCP1 in iBAT is not exceeded in the casein–fed mice, we can speculate whether an increased energy expenditure could originate from other tissues. Although the mechanisms in which this is accomplished is still debated, WAT can undergo a "browning" and take on similar properties to BAT (Fu et al., 2014). An increased glucagon:insulin ratio can increase intracellular cAMP activity, which has an ability to induce UCP1 action in WAT (Madsen et al., 2010). Since the mice fed casein had lower insulin levels in both a fed and fasting state, we can speculate whether this contributes to a higher energy expenditure.

On the other hand, obesity can lead to a "whitening" of the BAT with accumulation of lipid droplets. This is possibly due to a decreased vascular innervation and hypoxia, and causes

dysfunctionality of important BAT functions (Shimizu et al., 2014). This may have occurred in the iBAT from mice fed pork or cod, as we can see a characteristic change in the histological images of their iBAT compared to iBAT from the mice fed casein. In relation to this, we observed unexpected levels of expression of two genes that normally are indicators of an increased thermogenesis. We found a decreased *Cd36* expression in iBAT from mice fed cod compared to mice fed pork. Elevated levels of CD36 has been associated with an increased thermogenesis in BAT, as it promotes FA uptake into the adipose tissues (Boon et al., 2014, Goldberg et al., 2009). A study demonstrates that BAT lacking CD36 have increased whitening (Anderson et al., 2015), a possible explanation to the *Cd36* levels found in iBAT from mice fed cod. Furthermore, an increased *Mt1* expression in BAT has been associated with an increased thermogenesis (Jbilo et al., 2005) and it has a preventative effect on oxidative stress during increased FA oxidation (Beattie et al., 2000). Interestingly, we observed an elevated expression of *Mt1* in iBAT from cod-fed mice compared to mice fed casein, despite any clear indication of an increased thermogenesis. Since *Mt1* has been detected in WAT, we could speculate whether also these findings could be associated with an increased whitening of the brown adipose tissue in mice fed cod (Trayhurn et al., 2000).

In concordance with a study comparing casein to chicken (Tastesen et al., 2014b), we observed a lowered feed efficiency in mice fed casein, compared to cod- and pork-fed mice, illustrating a possible reason for the weight reduction. Although the mechanisms in this field of research is still not fully understood, we can discuss whether this may be induced by Leu's ability to decrease AMPK in hypothalamus and increase energy expenditure via UCP1 (Lopez et al., 2010, Beiroa et al., 2014). In addition, antagonism of CB1 has been associated with an increased thermogenesis (Boon et al., 2014), allowing us to question whether despite non–significant difference in UCP1 presence, low expression of CB1 may indicate increased UCP1 function. Furthermore, a look into the dietary AA composition provides information that the casein–diet contains higher amounts of tyrosine (see appendix I, Tables A.2 and A.3). Tyrosine has been given attention regarding its potential role in energy metabolism, as it is a precursor to the catecholamines dopamine and norepinephrine (NE) (Daubner et al., 2011). A decreased NE turnover could cause a reduction thermogenesis caused by reduced β–adrenergic stimulation of BAT. In light of these findings, mice fed casein seem to have an increased energy expenditure, possibly through thermogenesis via UCP1 in iBAT.

4.5 Experimental considerations

The two experiments presented in this thesis were performed at separate times, and that precludes statistical comparison. We aimed for the two experiments to have similar start–weights, causing a prolonged duration of VHF feeding in the *ad libitum* experiment. Taking into consideration that the ER mice were younger at termination, we can argue whether this could affect the results. For instance, a reduction of *Ucp1* expression is seen with increasing age in rats (Iritani et al., 2002). In addition, there were noticeable variations between the control groups in both experiments during the OGTT and ITT (see appendix IX, Figure A.1), altering the impression of improvement in glucose homeostasis in the respective LF groups.

Also worth noticing is the use of casein as protein source. Casein is naturally found in dairy protein such as cheese. However, in this study, the casein diet is prepared with casein powder, whereas the cod–and pork diets are produced from fillets. Comparing protein from an intact source to a protein extracted from its source may exert different effects.

4.6 The animal model and relevance to humans

Due to its genetic and metabolic similarities to humans, the C57BL/6J strain is a widely used animal model in medical and nutritional research. This strain's ability to develop DIO, hyperglycemia and hyperinsulinemia makes it an ideal model in which to study the weight reducing effects of different diets (Black et al., 1998).

An important aspect of animal research is the controlled environment, with feed intake, activity and temperatures being closely monitored. In addition, the C57BL/6J is an inbred strain with less genetic variations than a human population. These factors prevent direct transferability to a human life and consumption. Furthermore, a diverse human diet will never solely consist of one food group. Nonetheless, these factors also contribute to very accurate results. Monitored feed intake allows the effects observed when feeding different diets to be regarded as diet-induced. The mice in our experiments are kept in a thermoneutral environment, because when exposed to lower temperatures (18 – 22 °C) the mice will increase their metabolism to keep the body temperature at a satisfactory level, and as a consequence, obesity is not as easily induced (Feldmann et al., 2009). The thermoneutral surroundings help increase the relevance to humans, as a modern society provides with clothes and a tempered indoor environment.

Studies with a rat model have provided results indicating improvements in weight development and glucose homeostasis with an increased fish intake (Pilon et al., 2011, Lavigne et al., 2000, Lavigne et al., 2001), which is also confirmed in human studies (Thorsdottir et al., 2007, Vikoren et al., 2013). We did not obtain supporting results with feeding cod fillet to mice, which indicates that general observations do not apply to all species. It is imperative that similar research is carried out in a human model, to conclude or verify the results' impact on human health.

4.7 Future perspectives

Observations made throughout our experiment pave the way for many interesting future approaches. To further investigate the effects of different protein sources on energy expenditure, it would be interesting to perform qPCR or an immunohistochemical staining of WAT. In addition, considering the considerable weight - loss in mice fed ER it would be interesting to measure *Ucp1* levels in iBAT from this experiment.

We can speculate whether the differences in iBAT phenotype in mice fed casein are caused by weight reduction, or whether the dietary protein sources differentially affects the BAT function, and consequently the mice are leaner. Performing an indirect calorimetry have shown that NE induced O_2–consumption in *Ucp1* $^{(+/+)}$ mice increased compared to *Ucp1* $^{(-/-)}$ mice and that UCP1 is the only source of adaptive adrenergic induced thermogenesis (Feldmann et al., 2009, Golozoubova et al., 2006). It would be interesting to initiate such a test after feeding casein, cod and pork with caloric adjustment to clamp weight gain. Testing the physiological response to a diet instead of e.g. determining gene expression on mRNA levels could provide information whether the dietary protein source induces a difference in UCP1–function.

Furthermore, it would be of importance to consider the results from ongoing analyses of endocannabinoids AEA and 2-AG in plasma. Lastly, considering that Leu seems to be able to mimic the favorable effects of HP diets, it would be interesting to compare a LF normal protein diet with casein to a HP diet with other protein sources.

Lastly, it would be interesting to do a human intervention and compare the effects of giving diets with emphasis on different protein sources, such as dairy protein and fish protein.

5.0 CONCLUSION

The current study presents several findings. Primarily, we observe that feeding diet-induced obese mice a low-fat diet with casein as the protein source attenuates obesity largely compared to diets prepared with cod–and pork fillets. The weight reducing effect is possibly associated with increased energy expenditure, which further can be discussed to involve several metabolic pathways.

Secondly, glucose tolerance and insulin sensitivity was improved in the casein-fed mice compared to mice fed cod or pork.

Moreover, there seem to be a greater satiating effect from ingestion of cod compared to pork. In addition, feeding cod seem to lead to an increase in lean mass, especially compared to feeding pork.

A clear body weight and fat mass reduction is seen in mice fed an ER diet compared mice fed *ad libitum*. However, it is not easy to distinguish differences in adipocyte morphometry between the mice fed ER and *ad libitum*.

Collectively, our findings together with earlier publications suggest a growing body of information to support that feeding casein as the dietary protein source can induce weight loss and prevent obesity. Further research is necessary to establish whether these findings have an impact on human nutrition.

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APPENDIX

Appendix I – Diets

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

Ingredients		VHF	Casein	Cod	Pork
Protein					
Casein	g/kg		207.18	-	-
Cod	g/kg		-	215.15	-
Pork	g/kg		-	-	216.89
L-cysteine	g/kg		3	3	3
Carbohydrates					
Sucrose	g/kg		91.8	91.8	91.8
Cellulose	g/kg		50	50	50
Potato starch (Dextrin)	g/kg		531.4	523.86	530.02
Dextrin from melis	g/kg		1.84	1.84	1.84
Fat					
Corn oil	g/kg		69.11	68.68	60.78
Fat from protein	g/kg		0.89	1.32	9.22
Vitamin/mineral mix					
t-Butylhydroquinone	g/kg		0.01	0.01	0.01
Min.mix: SDS.AIN93Gminmix	g/kg		35	35	35
Vit.mix: SDS. AIN93VX NCR95compliant	g/kg		10	10	10
Choline Bitartrate	g/kg		2.5	2.5	2.5
Energy percent (E%)					
Protein	%	16.3	17.1	15.8	16.5
Carbohydrates	%	31.9	67.5	69.3	68.9
Fat	%	51.8	15.4	14.8	14.6
Analyzed nutrients					
Crude fat	g/kg	332.7	78.8	71.79	70.5
Fat in protein source	g/kg		4.3	6.11	42.5
Crude protein	g/kg	235.5	182	171.9	178.6
Protein in protein source	g/kg		86.87	83.66	82.99
Cholesterol	mg/kg	158.4	30.16	841.32	474.602
Vitamin D_3	mg/kg	0.02	0.03	0.037	
Energy					
	kJ/g	24.2	17.79	18.2	18.15
	kcal/g	5.7	4.2	4.3	4.3

Table A.2: Amino acid composition of the diets (mg/g)

Amino acid	VHF	Casein	Cod	Pork
Histidine	6.5	4.9	3.7	6.3
Taurine	0	0	0.8	0.1
Serine	13.9	10.3	8.4	7.5
Arginine	8.8	6.7	11.4	11.4
Glycine	4.4	3.5	8.7	7.8
Aspartic acid	17.9	13.7	20.4	19.3
Glutamine	53.8	41.8	30.2	30.7
Threonine	10.4	7.8	8.5	8.8
Alanine	7.25	5.5	11.1	10.8
Proline	26.4	20.5	6.8	7.1
Lysine	20.7	15.3	18.3	18.5
Tyrosine	12.2	8.6	5.5	5.3
Methionine	7.6	6.2	6.8	6.5
Valine	16.5	12.6	9.8	10.1
Isoleucine	12.4	4.7	8.5	9.2
Leucine	22.7	17.5	15.4	15.7
Phenylalanine	12.1	9.8	7.5	7.6
Tryptophan	2.7	2.2	2.7	4.8
Hydroxyproline	0	0.5	0.5	0.4
Essential aa	88.5	52.8	64.1	88.5
Non – essential aa	165.3	121.8	118.4	165.3
BCAA	51.6	34.8	33.7	35
Total amount aa	253.8	174.6	182.6	253.8

Table A.3: Amino acid composition of the protein sources (mg/g)

Amino acid	Casein	Cod	Pork
Histidine	27.46	16.86	30.1
Taurine	0	3.91	0.52
Serine	54.2	40.2	36.48
Arginine	35.02	53.51	55.8
Glycine	18.38	39.93	37.3
Aspartic acid	61.22	99.3	90.65
Glutamine	204.4	146	142.9
Threonine	40.19	39.91	42.02
Alanine	27.13	53.4	50.71
Proline	101.4	31.06	32.69
Lysine	69.40	91.72	85.59
Tyrosine	53.96	29.21	30.44
Methionine	27.49	28.07	25.92
Valine	64.13	45.79	45.03
Isoleucine	47.64	39.31	41.06
Leucine	87.59	72.07	73.60
Phenylalanine	52.85	33.33	36.43
Tryptophan	11.4	10.6	11.9
Hydroxyproline	0	2.09	1.93
Essential aa	389.3	367.2	379.2
Non – essential aa	583.3	496.5	471.6
BCAA	199.4	157.2	159.7
Total amount aa	972.5	863.6	850.8

Table A.4: Content of lipids in neutral and phospholipids in the diets (mg/g)

Lipid	Casein		Cod		Pork	
	Neutral	Phospholipid	Neutral	Phospholipid	Neutral	Phospholipid
16:0	6.6	0.1	6.9	0.3	7.5	0.6
18:0	1.3	0	1.4	0.1	1.9	0.3
Sum SFA	8.5	0.1	8.9	0.4	10.1	1
Sum MUFA	19.2	0.2	19.8	0.4	20.7	0.8
Sum PUFA	36.2	0.3	38.4	0.9	33.7	1.8
18:2 ω-6 (LA)	35.6	0.3	37	0.4	32.9	1.4
18:3 ω-6 (GLA)	0	0	0	0	0	0
20:4 ω-6 (ARA)	0	0	0.03	0.02	0.05	0.23
Sum ω-6	35.6	0.3	37	0.4	33	1.6
18:3 ω-3	0.5	0	0.5	0	0.5	0
20:5 ω-3 (EPA)	0	0	0.2	0.1	0	0
22:6 ω-3 (DHA)	0	0	0.4	0.4	0	0
Sum EPA + DHA	0	0	0.6	0.6	0	0
Sum ω-3	0.6	0	0.3	0.6	0.6	0.1
ω-3:ω-6 ratio	0.02	0.02	0.04	1.5	0.02	0.1
Sum identified	63.9	0.5	67.1	1.8	64.5	3.6
Sum unidentified	0.1	0	0.1	0	0.1	0.3
Sum fatty acids	64.1	0.53	67.2	1.8	64.6	3.9

SFA: Saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, LA: Linoleic acid, GLA: Gamma-linoleic acid, ARA: Arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaeonic acid

Appendix II – Histological methods

Table A.5: Chemicals and reagents used for fixation, dehydration, embedding and sectioning, staining of the tissues

Product name	Supplier/catalog no.
Formaldehyde 37 %	Sigma-Aldrich/F1635
NaH_2PO_4 x H_2O	Sigma-Aldrich
Na_2HPO_4 x H_2O	Sigma-Aldrich
Phosphate buffered saline	Sigma-Aldrich/107K8217
Ethanol (100%)	Kemetyl Norge/200-578-6
ddH_2O	MilliQ Biocel apparatus, Lab-tec, Norway
Methanol	Sigma-Aldrich/32213
Xylene	VWR Chemicals/28975.291
Hematolxylin Gill no. 2	Sigma-Aldrich/GHS116
Eosin Y	Sigma-Aldrich/E4382
DPX mountant	Sigma-Aldrich/06522

Table A.6: Time schedule for rehydration, staining and dehydration of the tissues

Reagents	Time (min)
Xylene	2 x 10
100 % EtOH	2 x 10
95 % EtOH	2 x 5
75 % EtOH	5
50 % EtOH	5
ddH_2O	5
Hematoxylin	2
H_2O	wash
Eosin	0.5
H_2O	wash
ddH_2O	1
50 % EtOH	2
75 % EtOH	2
95% EtOH	2 x 5
100 % EtOH	2 x 5
Xylene	2 x 5

Table A.7: Chemicals and reagents used for immunohistochemistry

Product name	Supplier/catalog no.
100 % EtOH	Kemetyl Norge/200-578-6
Xylene	VWR Chemicals/28975.291
Phosphate buffered Saline	Sigma-Aldrich/107K8217
Methanol	Sigma-Aldrich/32213
Hydrogen peroxide	Sigma – Aldrich/216763
Phosphate buffer saline	Sigma-Aldrich
Tri-Sodium citrate dihydrate	Merck/1.06448.1000
Goat serum	Vector laboratories, USA
Liquid blocker super pap pen	Ted Pella, inc., USA
Primary antibody – anti UCP1 (C4/98)	Prof. Jan Kopecky and Dr. Pavel Flachs
Secondary antibody (anti rabbit IgG)	Vector laboratories, USA
T(w)een	VWR/28829.296
VECTASTAIN® Elite ABC universal kit	Vector laboratories, USA
Vector® DAB Substrate kit	Vector laboratories, USA
Hematoxylin	Sigma-Aldrich/GHS116
Mounting solution	Entellan® New Merck , USA

Table A.8: Time schedule for immunohistochemistry

Reagent	Time (min)
Xylene	15
100 % EtOH	5 x 2
95 % EtOH	5
75 % EtOH	5
50 % EtOH	5
ddH_2O	5 x 2
Citrate buffer (92°C)	30
Cool down	20
ddH_2O	5 x 2
3 % H_2O_2 in MetOH	10
ddH_2O	10
PBS buffer + 0.1 % T(w)een	20
PBS buffer	15
Goat serum incubation	30
Primary antibody incubation	over night
PBS buffer	15 x 2
Secondary antibody incubation	60
PBS buffer	15 x 2
ABC	60
PBS buffer	15 x 2
DAB	5
H_2O wash	2
Hematoxylin	1
H_2O wash	2
95 % EtOH	20 s
100 % EtOH	20 s
Xylene	30 s

Appendix III – ELISA insulin kit

Table A.9: Reagents in and equipment used when performing Insulin Mouse ELISA kit

Product name	Supplier
Insulin Mouse ELISA kit	DRG Instruments GmbH, Germany
Coated Plate	
Calibrators 1, 2, 3, 4 and 5 (5 vials)	
Calibrator 0 (1 vial)	
Enzyme Conjugate 11X (1 vial)	
Enzyme Conjugate Buffer (1 vial)	
Wash buffer 21X (1 bottle)	
Substrate TMB (1 bottle)	
Stop Solution (1 vial)	
Adhesive PCR film	Thermo-Scientific, USA/ AB-0558

Appendix IV – RNA purification

Table A.10: Chemicals and reagents used in RNA extraction

Product name	Supplier/catalog no.
RNase Zap	Sigma-Aldrich/R2020
Deconex 11	
QIAzol Lysis Reagent	Qiagen/5.346.994
Chloroform	VWR Chemicals/1.02444.1000
Isopropanol	Kemetyl Norge AS/200-661-7
Ethanol	Kemetyl Norge AS/200-578-6
Zirconium beads	

Appendix V – RNA quality - BioAnalyzer

Table A.11: Chemicals and reagents used in determination of RNA quality

Product name	Supplier/catalog no.
BioAnalyzer 2100	Agilent Technologies, USA
RNase Zap™	Sigma/R2020
RNA 6000 Nano LabChip kit	Agilent Technologies/5067-1511
RNA 6000 ladder	Agilent Technologies/5065-4476
Chip Priming Station	Agilent Technologies/5056-4401
Bioanalyzer Chip Vortexer	IKA/3617036

Appendix VI – Reverse Transcription reaction

Table A.12: Chemicals and reagents used in a RT-reaction mix for a 30 µl reaction

Product name	Supplier
RNase free ddH_2O	MilliQ Biocel apparatus
TaqMan® RT Reagents	**Applied Biosystems/N8080234**
10X TaqMan® RT buffer	
25 mM $MgCl_2$	
10mM deoxyNTPs Mixture	
50 µl random hexamers	
RNase Inhibitor (20 U/µl)	
Multiscribe Reverse Transcriptase (50 U/µl)	

Appendix VII – Real time qPCR

Table A.13: Chemicals and reagents used in real time qPCR

Product name	Supplier/catalog no.
LightCycler® 480 SYBRGreen Master	Roche, Norway
Primers (see table A.10)	Invitrogen Ltd, UK
RNase free ddH_2O	MilliQ Biocel apparatus

Table A.14: List of primers used in real time qPCR

Housekeeping gene	Sequence 5' → 3'
β-*actin*	Forward: ATG GGT CAG AAG GAC TCC TAC G
	Reverse: AGT GGT ACG ACC AGA GGC ATA C
Calnexin	Forward: GCA GCG ACC TAT GAT TGA CAA CC
	Reverse: GCT CCA AAC CAA TAG CAC TGA AAG G
TBP	Forward: ACC CTT CAC CAA TGA CTC CTA TG
	Reverse: ATG ATG ACT GCA GCA AAT CGC
Primer	**Sequence 5' → 3'**
Cd36	Forward: AAT TAG AAC CGG GCC ACG TA
	Reverse: CGC CAA CTC CCA GGT ACA A
CideA	Forward: TGC TCT TCT GTA TCG CCC AGT
	Reverse: GCC GTG TTA AGG AAT CTG CTG
Cnr1	Forward: AAGTCGATCTTAGACGGCCTT
	Reverse: TCC TAA TTT GGA TGC CAT GTC TC
Cnr2	Forward: ATG GCC GTG CTC TAT ATT ATC CT
	Reverse: ATG GTC ACA CTG CCG ATC TTC
Cox8b	Forward: GAA CCA TGA AGC CAA CGA CT
	Reverse: GCG AAG TTC ACA GTG GTT CC
Dio2	Forward: GCC CAG CAA ATG TAG AC
	Reverse: TGG CAA TAA GGA GCT AGA A
Fmo1	Forward: GCC AGT CTT TAC AAG TCT GTG G
	Reverse: TCC AGG AAT AGA GAA TTT GGC AC
Gpx3	Forward: CCT TTT AAG CAG TAT GCA GGC A
	Reverse: CAA GCC AAA TGG CCC AAG TT
Leptin	Forward: ATT TCA CAC ACG CAG TCG GTA T
	Reverse: AAG CCC AGG AAT GAA GTC CA
Magl	Forward: AGG CGA ACT CCA CAG AAT GTT
	Reverse: ACA AAA GAG GTA CTG TCC GTC T
Mt1	Forward: AAG AGT GAG TTG GGA CAC CTT
	Reverse: CGA GAC AAT ACA ATG GCC TCC
Napepld	Forward: AGC GCC AAG CTA TCA GTA TCC
	Reverse: TCA GCC ATC TGA GCA CAT TCG
*Ppargc1*α	Forward: CAT TTG ATG CAC TGA CAG ATG GA
	Reverse: CCG TCA GGC ATG GAG GAA
Ucp1	Forward: AGC CGG CTT AAT GAC TGG AG
	Reverse: TCT GTA GGC TGC CCA ATG AAC

Appendix XIII – Red blood cells

Table A.15: The fatty acid composition of the red blood cells

Fatty acid	VHF	LF casein	LF cod	LF pork
16:00	0.94 ± 0.03^{a}	0.97 ± 0.04^{ab}	1.03 ± 0.02^{b}	1.03 ± 0.03^{ab}
18:00	0.45 ± 0.02^{a}	0.33 ± 0.02^{bc}	0.3 ± 0.01^{c}	0.35 ± 0.01^{db}
Sum SFA	1.43 ± 0.05	1.33 ± 0.05	1.36 ± 0.02	0.35 ± 0.01
Sum MUFA	0.77 ± 0.04^{a}	0.62 ± 0.03^{b}	0.63 ± 0.01^{b}	0.69 ± 0.02^{b}
Sum PUFA	1.64 ± 0.05^{ab}	1.61 ± 0.08^{a}	1.69 ± 0.03^{ab}	1.79 ± 0.06^{b}
18:2 ω-6 LA	0.36 ± 0.03^{a}	0.34 ± 0.02^{ac}	0.52 ± 0.02^{b}	0.40 ± 0.03^{ac}
18:3 ω-6 GLA	0	0	0	0
20:4 ω-6 AA	0.85 ± 0.03^{ab}	0.86 ± 0.04^{b}	0.51 ± 0.02^{c}	0.94 ± 0.03^{ab}
Sum ω-6	1.40 ± 0.05^{a}	1.42 ± 0.08^{ac}	1.14 ± 0.02^{b}	1.56 ± 0.06^{c}
18:3 ω-3 ALA	0	0	0	0
20:5 ω-3 EPA	0	0	0.08 ± 0	0
22:6 ω-3 DHA	0.22 ± 0.01^{a}	0.19 ± 0.01^{b}	0.44 ± 0.01^{c}	0.22 ± 0.01^{a}
Sum EPA + DHA	0.22 ± 0.01^{a}	0.19 ± 0.01^{b}	0.52 ± 0.01^{c}	0.22 ± 0.01^{a}
Sum ω-3	0.23 ± 0.01^{a}	0.19 ± 0.01^{b}	0.55 ± 0.01^{c}	0.22 ± 0.01^{a}
ω-3:ω-6 ratio	0.17 ± 0.01^{a}	0.14 ± 0.01^{bd}	0.45 ± 0.01^{c}	0.14 ± 0^{d}
Sum identified	3.85 ± 0.13	3.55 ± 0.16	3.68 ± 0.05	3.88 ± 0.12
Sum unidentified	0.01 ± 0	0.01 ± 0.01	0.01 ± 0	0.02 ± 0.01
Sum fatty acids	3.86 ± 0.13	3.56 ± 0.16	3.69 ± 0.05	3.9 ± 0.12

Appendix IX – ITT and OGTT before diets and control groups

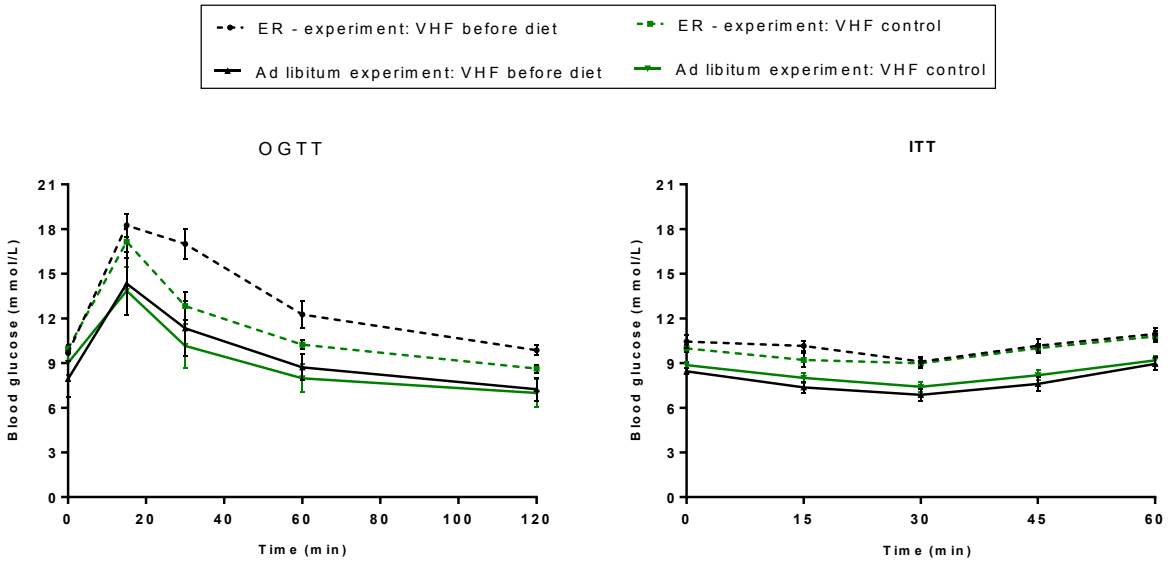

Figure A.1: Results from ITT and OGTT before diets and control groups from both experiment.

Appendix X – Adipocyte size

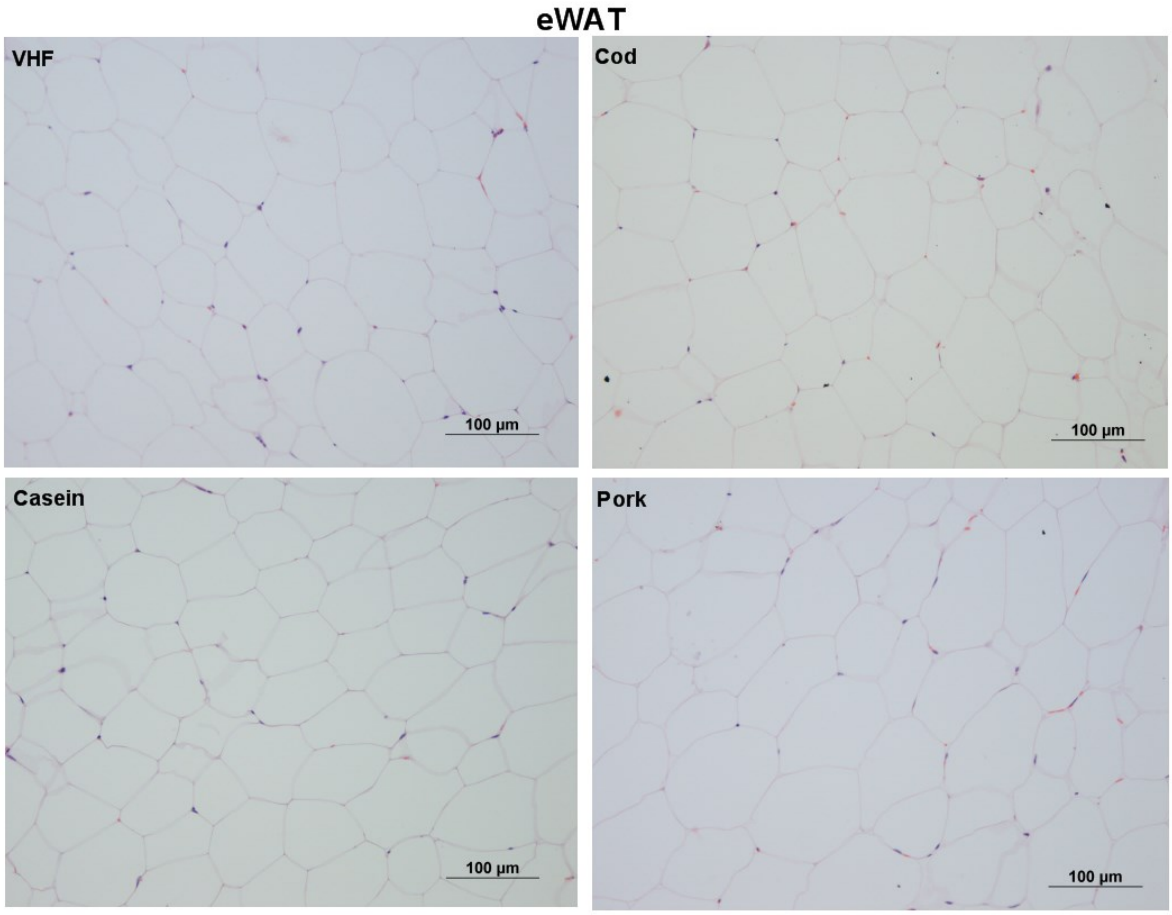

Figure A.2: Microscopy photos of the epididymal white adipose tissues. The photos presented are from a representative mouse in each experimental group. Magnified 20x

Appendix XI – Musculus tibialis weights

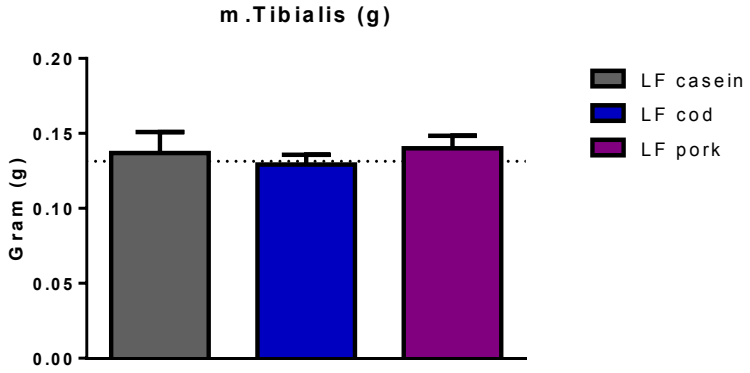

Figure A.3: Weights of m. tibialis presented in grams. The dotted line represents the control group. No significant differences were observed between the experimental groups.