COMBINING A LOW FAT DIET WITH EXERCISE REVERSES DIET-INDUCED OBESITY AND IMRPROVES GLUCOSE HOMEOSTASIS IN C57BL/6J MICE

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MASTER THESIS IN HUMAN NUTRITION

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N I F E S

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Hanne Andersen

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LIST OF ABBREVIATIONS

AA	Amino acid
ABC	Avidin- biotin – peroxidase- complex
AFD	Apparent fat digestibility
ANOVA	Analysis of variance
ATP	Adenosin- 5'-trifosfat
AOC	Area over the curve
AUC	Area under the curve
BAT	Brown Adipose Tissue
BMI	Body mass index
BW	Body weight
cAMP	Cyclic-adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
DAB	Diaminobenzidin tetrahydroklorid
DIO	Diet-induced obesity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme- linked immunsorbent assay
eWAT	Epididymal white adipose tissue
EX	Exercise
FA	Fatty acids
FAO	Food and Agriculture Organization
FFA	Free fatty acids
GSIS	Glucose stimulated insulin secretion
G6P	Glucose-6-phospat
HF	High fat
HF/HP	High fat and high protein
HF/HS	High fat and high sucrose
HP	High protein
HSL	Hormone sensitive lipase
iBAT	Interscapular brown adipose tissue
IL-6	Interleukin 6
iNOS	Inducible nitric oxide
IPGTT	Intraperitoneal glucose tolerance test

ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue
LF	Low fat
MCP	Monocyte chemotactic protein 1
MRI	Magnetic resonance imaging
NAFLD	Non- alcoholic fatty liver disease
PB	Phosphate buffer
PBS	Phosphate buffer Solution
PKA	Protein kinase A
PUFA	Polyunsaturated fatty acid
RD	Reference diet
RM	Repeated measurements
RNA	Ribonucleic acid
SED	Sedentary
SEM	Standard error of the mean
TAG	Triacylglycerol
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor Alfa
UCP1	Uncoupling protein-1
VHF	Very high fat
WAT	White adipose tissue
WHO	World health organization
qPCR	Quantative polymerase chain reaction

ABSTRACT

Background: The prevalence of obesity and obesity- related disorders, including type 2 diabetes and non-alcohol fatty liver disease is alarmingly high. These conditions can mostly be attributed to increased intake of energy dense food and decreased physical activity. It is also proposed that the obesogenic effect of a unfortunate diet is influenced by protein source. There is disagreement in defining the best way to deflect the obesity trend; however, most people agree that dietary changes and/or exercise are crucial in the treatment of obesity and its related disorders. Whether it is the effect of exercise or diet that is essential, is highly debated. Furthermore, little is known about the impact of various protein sources in terms of weight loss.

Methods: Male C57BL/6J BomTac mice were fed a high fat – high sucrose diet with casein, salmon and entrecôte for seven weeks to promote obesity**.** Weight loss was induced by changing to either a low fat diet with salmon or entrecôte, exercise with continuous high fat – high sucrose feeding or with a combination of a low fat diet and exercise. We evaluated the effect of exercise and a low fat diet with different protein sources on weight loss, body composition, glucose homeostasis and hepatic gene expression. Body composition was determined by MRI scan prior to, in the middle and at the end of the experiment. Glucose tolerance test and insulin tolerance test were performed to evaluate glucose homeostasis and insulin sensitivity. Real time qPCR was conducted to analyze relative gene expression in terms of fat accumulation and fatty acid oxidation in the liver.

Results: Obesity was induced and insulin sensitivity was impaired when 2/3 of casein was replaced with salmon and entrecôte in a high fat – high sucrose diet. Exercise was not of significance in terms of weight loss in mice fed high fat – high sucrose diet, but a low fat diet reduced body weight and exercise decreased it further. A low fat diet and exercise intervention for 5 weeks decreased fat mass and increased lean mass. Glucose tolerance and insulin sensitivity were improved by low fat diet and the combined treatment of low fat diet and exercise. Mice fed low fat diet with salmon had generally a higher expression of genes involved in fatty acid oxidation and lower expression of genes involved in fat accumulation compared to the mice fed a low fat diet with entrecôte.

Conclusion: A mixture of meat, fish and casein proteins promote obesity compared to proteins solely from casein. A low fat diet combined with exercise has beneficial effects in terms of weight loss, body composition and insulin sensitivity. Low fat diet alone improve plasma glucose levels and expression of genes involved in fat accumulation and fatty acid oxidation. The importance of protein source in a low fat diet seem insignificant with the exception of relative gene expression in the liver, where mice fed salmon exerts beneficial effects.

1.0 INTRODUCTION

1.1 Overweight and obesity

Overweight and obesity are defined as an unusual or excessive fat accumulation that represents a major risk factor for a number of chronic diseases (WHO, 2012). Body mass index (BMI) is a widely used index to classify overweight and obesity in adults. It is defined as weight in kilograms divided by the square of height in meters (kg/m^2). World health organization (WHO) classify overweight as a BMI equal to or greater than 25, whereas a BMI greater than or equal to 30 is considered obesity. As BMI is the same for both sexes and for all ages of adults, it provides a valuable population based information about overweight and obesity. However, BMI does not consider a person's lean mass and neither does it consider fat distribution (Doherty et al., 2014). To obtain information about the distribution of body fat, methods such as waist circumference or waist – hip ratio are more appropriate measures for obesity (Observatory, 2009). A number of studies have indicated that measurements of visceral fat or central obesity may be a better predictor of obesity- related disorders than BMI (Wang et al., 2005, Schneider et al., 2007, Lee et al., 2008). This may be due to the fact that visceral fat is more likely to cause health complications compared to subcutaneous fat. Nevertheless, these methods also have limitations and in order to better identify individuals with increased risk of obesity-related disorders, an assessment of both general and central obesity should be applied (Observatory, 2009).

1.1.1 Prevalence of overweight and obesity

The prevalence of obesity has nearly doubled worldwide since 1980, and in many European countries the prevalence have tripled (WHO, 2012). In 2013, the number of overweight and obese individuals worldwide had reached 2.1 billion (Ng et al., 2014). The proportion of overweight and obese men has increased to 36.9%, while the proportion of women has increased to 38%. To define overweight and obesity as a problem concerning only developed countries is no longer valid, as the prevalence is increasing more rapidly in middle- and low income countries (WHO, 2012). Today, overweight and obesity are linked to more deaths worldwide than underweight, where 65% of the world's population live in countries where overweight and obesity is causing more deaths than underweight. Simultaneously, the prevalence of childhood obesity is rising, in both developed and developing countries (Ng et al., 2014). In 2013, the prevalence of overweight and obesity in developed countries was 23.8%

for boys and 22.6% for girls. Furthermore, in developing countries, 12.9% of the boys and 13.4% of the girls were overweight or obese. In Norway, the average body weight has increased by 6 kg from 1985-2008 (Folkehelseinstituttet, 2012), where the greatest increase was among young men. Results from the child growth study in Norway indicates that 16% of 8 year olds were overweight or obese in 2012 (Folkehelseinstituttet, 2013).

1.1.2 Factors contributing to overweight and obesity

The main cause of obesity and overweight is an energy imbalance between calories consumed and calories expended (WHO, 2012). During the last decades, the food production has evolved from self-sufficient farming to processed industrialized food, containing high amounts of fat, sugar and salt (FAO, 2013). A possible environmental factor contributing to overconsumption of calories is the wide variety and availability of these energy dense foods (Hill et al., 2003). Additionally, other environmental factors seem to reduce total energy expenditure due to decreased physical activity. Factors contributing to reduced physical activity are among others the reductions in jobs requiring physical labor, decreased energy expenditure at school and in daily living, as well as an increase in time spend on sedentary activities. However, in addition to energy imbalance, genetic factors can affect the development of overweight and obesity on many levels, including appetite regulation and energy metabolism as well as hereditary traits (Walley et al., 2006).

1.1.3 The pathophysiology of overweight and obesity

Overweight and obesity is a major cause for a variety of human diseases, including cardiovascular disease, type 2 diabetes and cancer (Eyre et al., 2004). Increased body fat, and possibly specific depots of body fat are clearly associated with an increased risk of type 2 diabetes and cardiovascular disease (Goran et al., 2003). Insulin resistance is a major characteristic of type 2 diabetes and is causally related to obesity (Kahn, 1994). There are evidence that obesity is a low grade chronic inflammatory state, where especially adipose tissue inflammation is closely linked to obesity-induced insulin resistance (Weisberg et al., 2003, Wellen and Hotamisligil, 2005). Furthermore, non-alcoholic liver disease (NAFLD) often co-occur with insulin resistance (Farese et al., 2012), and both of these diseases are strongly associated with inflammation (Glass and Olefsky, 2012). This indicates that inflammation and lipid accumulation in other areas than adipose tissue may be of importance in the development of insulin resistance.

1.2 The adipose tissue

The adipose organ consists of both white (WAT) and brown (BAT) adipose tissue. While WAT mainly functions as a lipid storage used for fuel between meals, brown adipocytes consume lipids to produce heat.

1.2.1 White adipose tissue

White adipocytes are spherical, unilocular adipocyte cells that vary in size, where the size of the adipocyte is mainly determined by the size of the lipid droplet (Cinti, 2009). The lipid droplet is mainly comprised of triacylglycerol (TAG) which accounts for most of the cell's volume. In addition to function as an energy storage, WAT has also been recognized as a major endocrine and signaling organ (Trayhurn et al., 2008). White adipocytes secrete a large number of adipokines that enables extensive crosstalk within adipose tissue and with other tissues, including adiponectin, leptin, interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor alfa (TNF-α). Obesity alters the metabolic and endocrine function of adipose tissue, which implies an increased production of proinflammatory molecules and acute phase proteins from macrophages (Weisberg et al., 2003). The elevated production of proinflammatory adipokines induces chronic inflammation, which seems to be highly associated with, if not causative of, insulin resistance (Xu et al., 2003). The macrophage infiltration is also linked to increased BMI and adipocyte size (Weisberg et al., 2003, Cancello et al., 2005). Furthermore, proinflammatory molecules such as inducible nitric oxide (iNOS) and TNF-α have been implicated in the development of insulin resistance (Perreault and Marette, 2001, Sartipy and Loskutoff, 2003).

1.2.2 Brown adipose tissue

In brown adipocytes, TAG's are stored in the form of multiple small vacuoles, making them multilocular adipocytes (Cinti, 2009). The most characteristic organelles of these cells are the large, spherical mitochondria, which are highly represented in the cytoplasm. Brown adipocytes use energizing substrates to produce heat through non-shivering thermogenesis. This process can increase energy expenditure and is activated through the uncoupling of oxidative metabolism from Adenosin-5'-triphospat (ATP) production, accompanied by mitochondrial uncoupling protein-1 (UCP1). UCP1 is located in the inner mitochondrial membrane of brown adipocytes and catalyzes a leak of protons across the inner membrane, dissipating the electrochemical gradient that is necessary for ATP- production (Krauss et al., 2005). The

metabolic activity of BAT s is regulated through the release of catecholamines by sympathetic nerve terminals, acting on β-adrenic receptors located on the surface of brown adipocytes (Wu et al., 2013). This binding initiates signaling cascades including cyclic-adenosine monophosphate (cAMP) and protein kinase A (PKA), which further phosphorylates and activates hormone sensitive lipase (HSL) which cleaves TAG into free fatty acids (FFAs) (Townsend and Tseng, 2012). FFAs are transported into the mitochondria and used for β-oxidation or UCP1 thermogenesis. Numerous of studies demonstrates that UCP1 is found exclusively in brown adipocytes (Cannon et al., 1982, Cinti et al., 1989, Ricquier et al., 1991, Cannon and Nedergaard, 2004, Frontini et al., 2007), however, Wu et al. (2013), mentions that UCP1 is highly selective for both brown *and beige* adipose cells (Wu et al., 2013). In the condition of a positive energy balance and obesity, BAT undergoes changes, in which the brown adipocytes gradually morph into cells similar to WAT (Cinti, 2009). These changes include the transformation of the lipid depot from multilocular to unilocular.

1.2.3 Occurrence of brown adipose tissue

In mammals and infants, brown adipocytes are most numerous in the anterior subcutaneous, mesenteric and retroperitoneal adipose depots (Cinti, 2009). Primarily, BAT is known for its ability to maintain body temperature in newborns without shivering. Although the need for BAT is higher in newborns, the application of positron emission tomography (PET) has revealed metabolically active BAT in human adults (Hany et al., 2002, Gelfand et al., 2005). However, the detection of UCP-1 positive brown adipocytes in human BAT is quite recent (Cypess et al., 2009, van Marken Lichtenbelt et al., 2009, Virtanen et al., 2009, Zingaretti et al., 2009), and have rekindled a interest in human adipose tissue biology for its potential role in obesity prevention and treatment.

1.2.4 Transcriptional development of brown and beige adipocytes

In adipose tissue, the relative amount of white, brown and mixed areas are genetically programmed and depends on several factors, including age, environmental temperature and nutritional status (Cinti, 2009). Until the identification of a cold induced PPARγ – binding partner, peroxisome proliferator-activated receptor gamma coactivator 1 (now PGC-1α), the molecular basis for the BAT program has remained unknown (Puigserver et al., 1998). PGC-1α is activated by a pathway downstream from the β-adrenic receptor (Herzig et al., 2001) and is mostly expressed in tissues with a high oxidative metabolism, including liver, kidney and muscle (Lin et al., 2005). PGC-1α regulates thermogenesis by inducing the expression of UCP1

and is identified as a regulator of brown fat development and function (Wu et al., 2013). Another transcriptional factor more present in classical BAT relative to WAT is PRDM16, which seems to be necessary for the brown fat phenotype and is effective in browning of visceral fat under the stimulation of adrenoreceptors (Seale et al., 2007). Although the majority of brown and white adipose depots have different anatomical locations, multilocular UCP1 positive cells exist within certain areas of WAT. These cells become more prominent upon prolonged cold exposure (Young et al., 1984, Loncar et al., 1986) and when treated with β3- adrenergic receptor agonists (Cousin et al., 1992). These so called beige or brite adipocytes can develop from transformation of differentiated white adipocytes, *de novo* differentiation or a combination of these phenomena (Cinti, 2009). Cinti and colleagues concluded that the cold- induced browning of WAT was most likely caused by transdifferentiation of mature white adipocytes. Brown adipocytes in the intrascapular and perirenal depots arise from similar precursors as skeletal muscle, which implies a $Myf5^+$ - derived cell lineage (Seale et al., 2008, Sanchez-Gurmaches et al., 2012). In contrast, the beige $UCP1^+$ adipocyte cells found in WAT under prolonged β-adrenic stimulation originates from a non – Myf5- derived cell lineage. These findings suggest that the brown fat cells detected in WAT are from a different cell linage than classical brown adipocytes and a hypothesis for the origin of the different fat cells is illustrated in Figure 1.1.

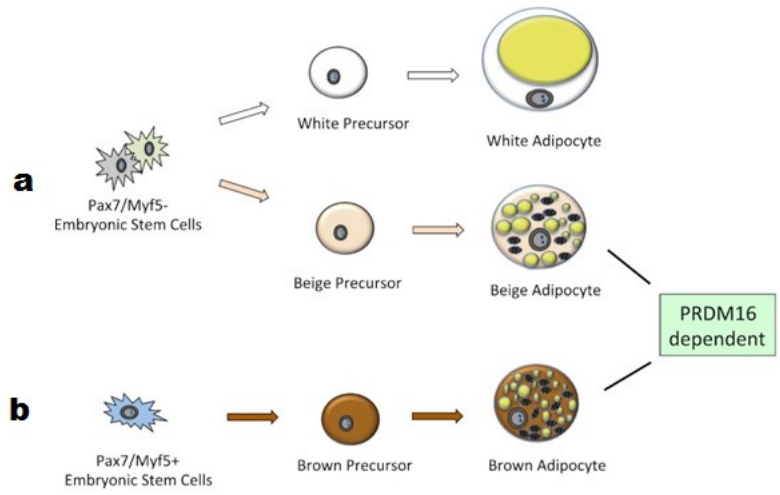

Figure 1.1: Origins of fat cells. At least three types of precursors give rise to white, beige and brown adipose cells separately. Precursors for brown adipocytes developmentally originate from dermomyotome and express Pax7 and Myf5. White and beige arise from two distinct populations of precursors of Pax\ and Myf5-negative lineages. While PPARγ is essential for adipogenesis of all fat cells, various transcriptional components play different roles in the development, commitment and differentiation of white, beige and brown fat, and PRDM16 has been shown to play an important role in regulating both brown and beige fat. Figure is adapted from (Wu et al., 2013).

1.3 Possible pathogenic effects of obesity on the liver

The liver plays a major role in regulating metabolism, and is therefore a target for numerous metabolic disorders (Burra, 2013). NAFLD increases worldwide and is recognized by a spectrum of chronic liver diseases, ranging from hepatic fat accumulation to more serious conditions such as cirrhosis and cancer (Maheshwari and Thuluvath, 2011). The metabolic syndrome, obesity, hyperlipidemia and type 2 diabetes are closely linked to NAFLD, and insulin resistance seems to play a relevant role in the pathogenesis of this disease. The hepatic accumulation of lipids is associated with the buildup of neutral lipids as TAGs and cholesterol esters in lipid droplets of hepatocytes (Farese et al., 2012). Besides neutral lipids, others lipids and their metabolites, including fatty acids (FAs) ceramides and diacylglycerols (DAG) often accumulate in the liver. These lipids are known to be potentially bioactive and could therefore interfere with hepatocyte function and subsequently reduce the ability to respond to changes in insulin levels. In insulin resistant hepatocytes, the insulin stimulated signal transduction pathway that suppresses hepatic glucose production is impaired (Haas and Biddinger, 2009). However, whether insulin resistance is a cause or a consequence of NAFLD is a debated question that needs further investigation. Figure 1.2 illustrates development of fatty liver in the setting of insulin resistance.

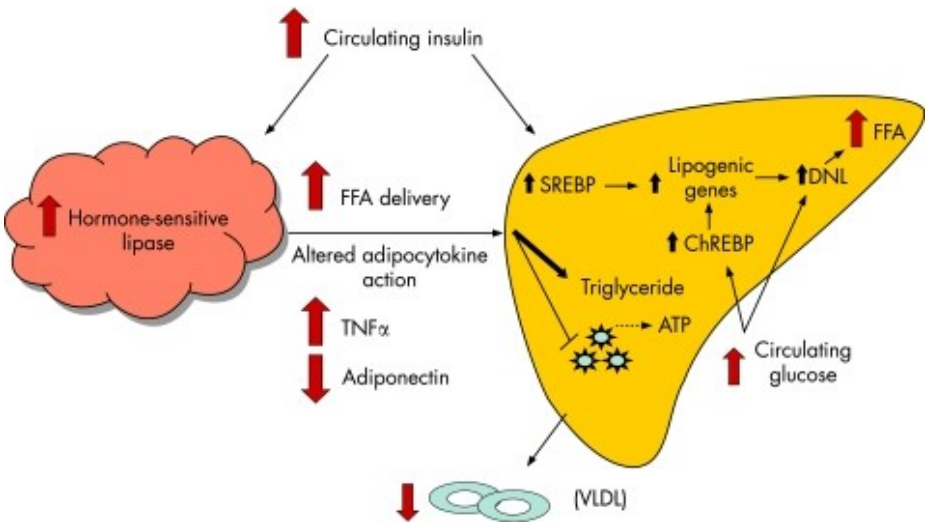

Figure 1.2: Mechanisms for development of fatty liver in the setting of insulin resistance. In the setting of insulin resistance, there is increased adipose tissue hormone-sensitive lipase activity that results in enhanced lipolysis and increased non – esterified fatty acid (NEFA) delivery to the liver. NEFAs are preferentially esterified to triglycerides. Additionally, hyperinsulinemia leads to increased sterol regulatory element binding protein (SREBP) expression, resulting in de novo lipogenesis (DNL) and decreased FA oxidation. Carbohydrate response element-binding protein (ChREBP) is induced by hyperglycaemia and leads to further increases in DNL. Decreased hepatic lipid transport may also occur, in part via altered synthesis of apolipoprotein B, leading to decreased very low-density (VLDL) production. Figure adapted from (Harrison and Day, 2007).

1.4 Weight reduction and prevention of obesity

1.4.1 Macronutrients

Although many underlying factors may contribute to obesity, the main cause is an imbalance between energy intake and energy expenditure over a prolonged period. The unfavorable diet consumed in large parts of the world is energy dense and consists of high amounts of saturated FAs (SFAs) and refined carbohydrates, including sucrose. An elevated amount of sucrose in the diet is associated with an increased feed efficiency and accumulation of WAT (Hao et al., 2012). By introducing more carbohydrates in the diet, the intake of dietary protein has been reduced, especially compared with typical stone age and hunter- gatherer diets (Cordain et al., 2000, Cordain et al., 2005). Furthermore, the elevated level of SFAs and n-6 polyunsaturated fatty acids (PUFAs) in the diet, compromise the intake of n-3 PUFA, which also represent an unfortunate turn in the macronutrient composition. In addition to a more sedentary lifestyle, these changes in macronutrient composition may contribute to the high prevalence of obesity and type 2 diabetes. The optimal distribution of macronutrients is highly debated, with each composition representing its pros and cons. Nevertheless, there is a certain consensus in the fact that the amount of protein should not be reduced, while the optimal amount of fat and carbohydrates in the diet are still debated. The Norwegian health authorities recommend a diet consisting of 45 – 60 E% from carbohydrates, 25 – 40 E% from fat and 10 – 20% from protein (Helsedirektoratet, 2014), which can be referred to as a low fat (LF) diet.

1.4.2 Low fat diets

LF diets are based on the restriction of dietary fat, and Norwegian health authorities especially recommend to reduce intake of SFAs and trans FAs (Helsedirektoratet, 2014). Limiting the amount of dietary fat is an efficient way to reduce calorie intake, as fat is the most energy dense macronutrient (9 kcal/g). In obese individuals, a LF diet has the ability to reduce weight and fat mass, as well as improving plasma cholesterol levels (Wood et al., 1991, Stefanick et al., 1998). Furthermore, a LF diet has a higher potential than a 70% energy restricted high fat (HF) diet to improve insulin sensitivity in obese mice (Muurling et al., 2002). However, compared to diets high in protein, LF diets have been observed to promote a higher feed intake (Sorensen et al., 2008). Nonetheless, a LF diet does not have to compromise the recommended intake, 10 – 20 E% protein.

1.4.3 Exercise

In addition to specific dietary – advices, Norwegian health authorities recommend children and youth to be physically active at least one hour per day and adults is recommended to have either a moderate activity level of 150 minutes or a level of high intensity 75 minutes per week (Helsedirektoratet, 2014). These suggestions are based on the well-established fact that physical activity promotes health, gives surplus and is an important factor in the prevention and treatment of several different diagnoses and conditions. Physical activity is an overarching term, which, among others include exercise. Whether we discuss physical activity or exercise, it is the most variable and thus most determinative component of total energy expenditure (Hunter et al., 1998). For this reason, exercise is an important tool during weight loss, and is proposed as an effective intervention for both treatment and prevention of type 2 diabetes and obesity (Knowler et al., 2002). Furthermore, exercise may have a beneficial impact on the liver, as it enhances the expression of a metabolite β- aminoisobutyric acid (BAIBA) derived from PGC-1α, which results in increased hepatic β-oxidation trough a PPAR-α mediated mechanism (Roberts et al., 2014). In addition, BAIBA increases the expression of brown adipocyte-specific genes in WAT, which may increase energy expenditure. One study demonstrates that PGC-1α stimulates energy expenditure trough increased expression of Fndc5, a muscle- gene product that is secreted as a hormone named irisin (Bostrom et al., 2012). Results from the latter study hypothesize that irisin activates changes in the subcutaneous adipose tissue by induce browning and increase the expression of UCP1.

1.4.4 The effect of protein on satiety, energy expenditure and glucose metabolism

The amount of dietary protein seems to make a difference in relation to weight loss (Skov et al., 1999). There is evidence that protein is more satiating than carbohydrate and fat, and that the satiating effect can differ between protein sources (Westerterp-Plantenga, 2008). The satiating effect of protein could possibly be due to protein metabolism, higher diet-induced thermogenesis or an elevated concentration of the satiety hormones ghrelin and glucagon-like peptide 1 (Hall et al., 2003, Lejeune et al., 2006). The elevated diet- induced thermogenesis from protein (20–30 %) compared to carbohydrates (5-10 %) and fat (0-3 %) (Tappy, 1996) may be mediated by a high consumption of ATP during postprandial protein synthesis (Tessari et al., 2003). Additionally, amino acid (AA) oxidation may be of importance, although this depends on the protein source (Mikkelsen et al., 2000). The protein source, hence, the AA composition, may be an important determinant of the metabolic efficiency as the oxidative properties of amino acids vary (Tessari et al., 2003). Furthermore, an increased protein intake

has been associated with higher resting metabolic rate and elevated sleeping metabolic rate, indicating that protein affects energy expenditure (Whitehead et al., 1996, Mikkelsen et al., 2000).

High protein (HP) diets have the ability to increase *de novo* synthesis of glucose from gluconeogenic precursors, mainly in the liver (Azzout et al., 1984). An elevated protein intake increases the activity of hepatic phosphoenolpyruvate carboxykinase (PEPCK) in both a fasted and fed state, and the activity of glucose- 6 phosphatase (G6Pase) is up regulated in fasted state and down regulated in fed state (Azzout-Marniche et al., 2005). This suggests that liver gluconeogenesis is stimulated by a HP diet; however, in a fed state the newly synthesized glucose -6 phosphate (G6P) is directed towards glycogen storage, whereas in fasted state G6P is converted to glucose and released from the liver. The activity control of PEPCK and G6Pase have a beneficial effect on hepatic metabolism and glucose homeostasis (Trinh et al., 1998) and the satiating effect of HP diets could be attributable to improvement of glucose homeostasis through enhanced hepatic gluconeogenesis. Protein sources vary in their AA composition as well as their distribution of fat following the protein source. Based on these variations, protein sources may affect the liver differently, as the distribution between n-3 and n-6 FAs following the protein source may differ, and a low n-3/n-6 ratio is associated with NAFLD (Zelber-Sagi et al., 2007).

In fat following protein sources, the n-3/n-6 FA ratio differ, where marine protein sources commonly have higher levels of n-3 FAs than vegetable and animal protein sources. However, as the amount of vegetable oil in fish-feed seems to increase at the expense of fish oil; this alters the distribution of the n-3/n-6 ratio in fish (Liland et al., 2013, Midtbo et al., 2015). In Norway, this issue is particularly relevant for farmed salmon. Within ten years (1997 – 2007), the amount of fish oil in farmed salmon has been halved (Tacon and Metian, 2008), which further reflects a reduction in n-3 FAs. Thus, an altered n-3/n-6 FA ratio makes the commercial salmon fillets more similar to other protein sources, including meat, when evaluating the FA composition.

1.5 Introduction to the study

In today's society, weight reduction and maintenance of lean mass is a highly relevant topic. Whether it is the effect from exercise or from different dietary approaches that is crucial for weight regulation, is a continuously addressed question with contradicting answers. Diets high in protein and/or low in carbohydrate have lately gained much attention as a possible method for weight reduction. A review from Westerterp-Plantenga suggests that an elevated protein intake plays a key role in relation to weight loss, body composition and increased energy expenditure (Westerterp-Plantenga, 2008). LF diets have also demonstrated vigorous effects regarding weight loss (Stefanick et al., 1998, Vieira et al., 2009). Nevertheless, energy expenditure related to physical activity is the most variable factor in energy expenditure and has the greatest potential for increasing total energy expenditure (Hunter et al., 1998). Furthermore, a study done on obese women, demonstrates that exercise minimizes the insulin resistance that may occur with a sedentary lifestyle (Houmard et al., 2004). Thus, the benefits of a healthy diet combined with the advantages of exercise seem useful in the prevention and treatment of obesity and its related disorders.

It is known from both literature and studies done in our research group, that different protein sources have different obesogenic potential. Some studies demonstrate that marine protein sources can attenuate high fat diet-induced obesity (Liaset et al., 2009, Tastesen et al., 2014). Despite a similar energy intake, mice fed cod/scallop had lower feed efficiency than mice fed chicken, which was subsequently reflected by a reduced adipose tissue mass after seven weeks of feeding (Tastesen et al., 2014). These studies were conducted to address the effect of various protein sources in diets that promoted obesity; however, less is known about different protein sources and their effect to reverse obesity.

1.6 Aims of the study

The present study was conducted to evaluate the importance of dietary macronutrient composition, protein source and exercise on obesity development and glucose homeostasis in obesity prone C57BL/6J mice. In light of the discovery that casein based diets attenuate obesity development, we wanted to evaluate if casein proteins had the same protective effects when 2/3 of the casein proteins were replaced with a mixture of salmon and entrecôte in the first part of the experiment. In the second and main part of the experiment we wanted to compare proteins from salmon and entrecôte in a low fat diet and evaluate their effect on reversing diet-induced obesity. Additionally, we wanted to investigate the importance of exercise in reversing diet-induced obesity, and compare it with the impact of a low fat diet with different protein sources.

In this study, we aimed to investigate following:

- Initially, how a mixture of proteins from casein, salmon and entrecôte affects diet-induced obesity and glucose homeostasis compared with a 100% casein based HF/HS diet.
- Secondly, the effect of exercise and LF diet with different protein sources on weight loss and changes in body composition
- Thirdly, the importance of diet, exercise and dietary protein source in relation to glucose tolerance and insulin sensitivity.
- Lastly, the effect of exercise and LF diets with different protein sources on expression of genes involved in fat accumulation and fat oxidation in the liver.

2.0 MATERIALS AND METHODS

2. 1 The animal experiment

The mouse model (C57BL/6J BomTac) used in this experiment was selected based on its ability to develop obesity, hyperglycemia and hyperinsulinemia when fed a high fat (HF) diet (Black et al., 1998). Furthermore, when C57BL/6J mice are fed a low fat (LF) diet, they maintain a low fat mass (Petro et al., 2004).

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

Seventy male mice from the inbred strain C57BL/6J were purchased from Taconic Europe (Ejby, Denmark) at eight weeks of age. Upon arrival, all mice were acclimatized for five days (29.3 ± 0.4 °C) and fed a LF-diet (Appendix I, Table A.1). The experiment encompassed 60 mice (n = 10/group) who were assigned to different experimental groups based on body weight (BW), lean mass and fat mass, followed by seven weeks fed a HF/HS diet (Appendix I, Table A.2) to induce obesity. The mice received three different experimental diets (Appendix I, Table A.2), of which two groups received a LF salmon diet, two groups a LF entrecôte diet and two groups remained on a HF/HS diet. In each diet, one group received a running wheel. Several tests were performed during the experiment, including collection of feces, intraperitoneal glucose tolerance test (IPGTT), glucose stimulated insulin secretion (GSIS) and insulin tolerance test (ITT). The mice were fed experimental diets for seven weeks prior to termination.

2.1.1. Ethical statement

The animal handling and experiments were performed in accordance with local institutional recommendations. The animal experiment was approved by The Norwegian Animal Health Authorities (ID number FOTS: 5358).

2.1.2 Preparation of diets

Casein used in the diets was bought from SIGMA (lot number BCBF8389V). Entrecôte was obtained from H. Brakstad Eftf. AS meat processing and salmon was a gift from Marine Harvest. The protein sources (entrecôte and salmon) were warm - treated, freeze-dried and homogenized at NIFES. Diets were made by weighing the different ingredients on a Mettler Toledo PG42002-S/PH weight, mixed together in a Crypto Peerless EF20 blender and further stored at -20°C. Distribution of macronutrients in the diets are illustrated in Figure 2.2 as energy percent (E%). For a detailed diet composition, see appendix I Table A.2.

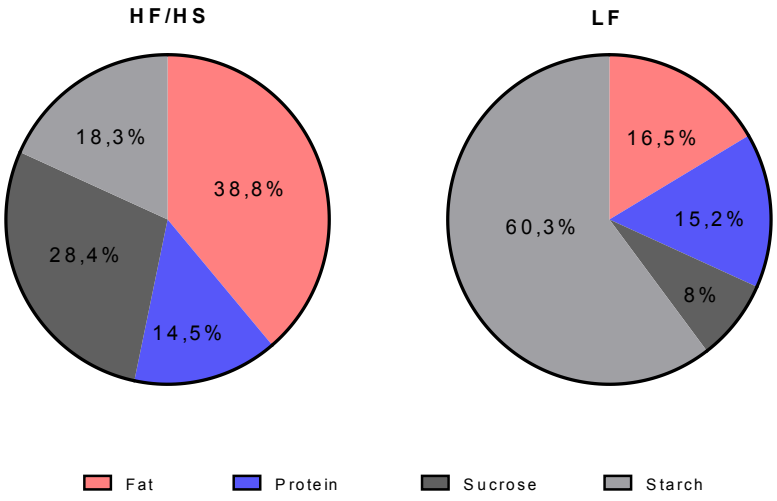

Figure 2.2: Distribution of macronutrients in the HF/HS and LF diet (E%).

2.1.3 Diets

The six groups (n = 10) were given three different diets, presented in Figure 2.3. Three of the groups received a running wheel and where therefore assigned to voluntary exercise (Ex). The groups with no running wheel are termed sedentary (Sed). Two groups received a LF diet which consisted of salmon + casein as protein sources, and two groups were given entrecôte + casein. The two remaining groups continued on a HF/HS diet with casein, salmon and entrecôte as protein sources.

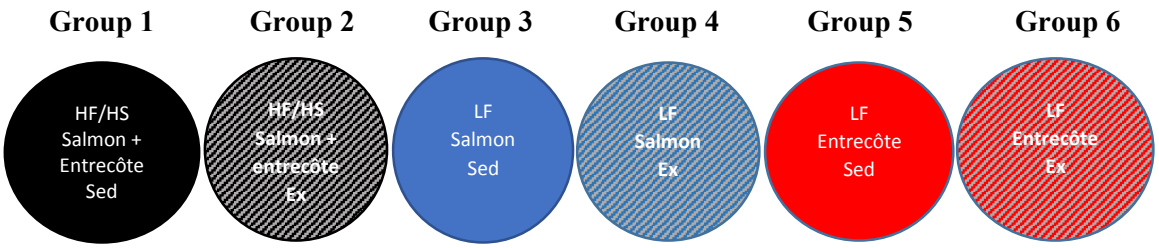

Figure 2.3: Grouping, physical activity, diets and protein sources in the experiment.

2.1.4 Housing and feeding

All mice were individually caged (Techniplast 1291) in a controlled environment throughout the experiment. The cages (20 x 36 x 18 cm) were equipped with a house, nesting material, wooden bedding and a chewing stick. In addition, the Ex- groups had a running wheel (Fast Trac, Bio Serv *or* ENV – 044, Low Profile Wireless Running Wheel for Mice, Med Associates Inc.). The animal room had a 12 hour light/dark cycle and had an average temperature of 29.1 ± 1.7 °C (thermoneutrality) and a humidity of 40% throughout the experiment. The mice were fed *ad libitum* three times a week and received a clean water bottle with fresh water every week.

2.1.5 Measurements

Once a week the mice were weighed on a Mettler Toledo (PG42002-S/PH) weight. The mice were scanned with magnetic resonance imaging (MRI), using a Bruker Minispec LF50mq 7.5 apparatus at baseline, after 5 weeks on HF/HS diet and after 5 and 7 weeks on experimental diets. The scanner contains a magnetic field that distinguish between fat mass, lean mass and free water. Every Monday, Wednesday and Friday, feed and feed remnants were weighed to calculate feed intake. The wooden bedding was shifted two times throughout the experiment to gather and weigh spilled feed. Wireless running wheels (ENV – 044, Low Profile Wireless Running Wheel for Mice, Med Associates Inc.) were used to obtain information about the mice's physical activity.

2.1.6 Feces collection

After 4 weeks of experimental feeding, feces were collected to measure fat and nitrogen digestibility. The mice were transferred to a clean cage with paper sheets in the bottom, and after one week, feces were collected and further analyzed for fat and nitrogen content. Feed

intake and fat content were noted and subsequently, apparent fat digestibility (AFD) and apparent nitrogen digestibility was calculated by using the following formula:

$$AFD = \frac{\text{amount of fat eaten} - \text{amount of fat excreted}}{\text{amount of fat eaten}} \; x \; 100\%$$

2.1.7 Intraperitoneal glucose tolerance test

To evaluate glucose tolerance, an IPGTT was performed. Prior to testing, the mice were moved to clean cages for 5 hours to fast. Glucose (3 mg/g lean mass) was injected with a needle into the stomach. A small incision was made in the lateral tail vein to measure blood glucose and collect blood for further GSIS analysis. The blood glucose level was measured with a glucometer (Countour, Bayer) before glucose was injected (T0) and after 5, 15, 30, 60 and 120 minutes. Blood was collected at baseline and after 5, 15, 30 and 60 minutes. 20 µl of blood was collected into a minivette coated with EDTA and transferred into a clean Eppendorf tube. The blood samples were centrifuged at 1000G for 8 minutes. Plasma (10 µl) was collected and subsequently stored at -80 °C for further analyses described in section 2.2. At the end of the test, the mice's tail were sterilized with 70% ethanol (EtOH) to prevent infection.

2.1.8 Insulin tolerance test

An ITT was performed to evaluate insulin response. The mice were placed in clean cages prior to the test and a prepared insulin solution (1.00 U/ kg lean mass) was injected into the intraperitoneal space with a needle. A small incision was made in the lateral tail vein to collect blood for measuring blood glucose at T0, 15 min, 30 min, 45 min and 60 min. Blood glucose was measured with an automatic glucometer (Contour, Bayer). To prevent infection, the mice's tail were sterilized with 70% EtOH after finishing the test.

2.1.9 Termination

Prior to termination, all mice were in a randomly fed state. The mice were anesthetized with isofluran (Isoba-vet, Schering Plough, Denmark) by putting them in a Univentor 400 Anesthesia Unit Apparatus (Univentor Limited, Sweden). Euthanasia was performed by cardiac puncture and by using a syringe, blood samples from the heart were transferred to a tube containing an EDTA anticoagulant. To separate plasma from red blood cells, the blood samples were

immediately centrifuged at 2500 x g at 4 °C for five minutes. The samples were stored at - 80 °C foregoing analysis.

2.1.10 Tissue and organ harvesting

Three adipose tissues were dissected during termination: epididymal WAT (eWAT), inguinal WAT (iWAT) and intrascapular BAT (iBAT). Additionally, liver, pancreas and two muscles were excised: *m. tibialis anterior* and *m. gastrocnemius*. Furthermore, feces from colon and cecum was collected. The tissue and organ samples were weighed and divided into bags/cassettes. Samples for quantitative real- time polymerase chain reaction (qPCR) were snap-frozen in liquid nitrogen and stored at -80 °C. Samples for histology were fixed in 4% formaldehyde.

2.2 ELISA Insulin Kit

To quantitatively determine insulin in plasma and evaluate GSIS, the insulin (Mouse) ELISA kit (DRG Instruments, GmbH, Germany) was applied and method was performed according to manufacturer's protocol. Reagents and equipment used are presented in Table A.16 (Appendix VIII).

The ELISA kit and samples were thawed before starting the procedure. 10 µl of each calibrator and sample was transferred into a 96 well microplate. Enzyme Conjugate 1X solution was prepared by mixing Enzyme Conjugate 11X with Enzyme Conjugate buffer. The Enzyme Conjugate 1X solution (100 µl) was added to each well before it was incubated on a plate shaker (700-900 rpm) for two hours at room temperature. During incubation, insulin reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microplate wells. To remove unbound enzyme labeled antibody, the wells were washed six times with 700 µl wash buffer 1X solution using an automatic plate washer (1296-026 Delfia Platewash). Furthermore, 200 µl substrate Tetramethylbenzidine (TMB) was added to each well. The bound conjugate reacts with TMB and is detected by conversion of the uncolored TMB to a colored product. To stop the reaction, 50 µl of Stop Solution was transferred into each well. Optical density was read at 450 nm and 660 nm with a spectophometric plate reader (2030 Multilabel Reader. VIKTOR X5) and results were calculated.

2.3 Histology

2.3.1 Fixation with formaldehyde and phosphate buffer

After dissection, small sections of adipose tissue (eWAT, iWAT, iBAT), liver, pancreas and m. soleus was fixated in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The tissue was stored in fixative overnight and transferred to PB the morning after. As the tissue was stored for one week, a few drops of paraformaldehyde were added to the PB in order to prevent bacterial growth and to preserve the tissue.

2.3.2 Dehydration with ethanol and xylene

To remove fixation solutes and water from the tissue sections, PB was replaced with gradually increasing concentrations of EtOH, as presented in Table A.8 (Appendix IV). When the tissue was completely dehydrated in 100% EtOH, alcohol was substituted with xylene. Xylene was used because it is soluble in both alcohol and paraffin.

2.3.3 Paraffin infiltration and embedding

Paraffin was heated to 60 °C. Samples were placed in liquid paraffin (HI 1210, Leica) and stored overnight. To remove all remnants of xylene, paraffin was replaced twice the following day. Tissues were embedded in paraffin using an EC 350 Paraffin embedding center (Microtom International GmbH, Germany). Furthermore, tissue was placed in the center of a suitable metal mold where the bottom was covered with paraffin. The bed of the cassette was placed over the mold and filled completely with paraffin. It was kept on a cold board until it was completely stiffen, and the mold was removed.

2.3.4 Sectioning and staining

The samples were sent to Molecular Imaging Center at Haukeland University Hospital where professional technicians performed sectioning of the embedded tissue. The thickness of each slide was five µm.

Prior to staining, rehydration of the tissue was necessary. The time schedule in Table A.9 (Appendix IV), illustrates each step in the rehydration, staining and dehydration process. Hematoxylin was used to stain the nucleus and eosin was used to stain the cytoplasm. After staining, the slides were mounted with a xylene- based glue (DPX Mountant for histology, Sigma) and cover glass before drying in the ventilation chamber overnight.

2.3.5 Immunohistochemistry

Immunohistochemestry was performed to evaluate the differences of UCP1 expression in iBAT. As a positive control, iBAT from cold exposed mice in an earlier study conducted on NIFES was included in the procedure.

Table 2.1: *Reagents and time schedule for each step in the immunohistochemistry process.*

Reagent	Time
Citrate buffer (95°C)	2 x 15 min
Cool down	20 min
ddH_2O	10 min
3% H_2O_2 in methanol	10 min
ddH_2O	10 min
PBS + 0,1% Tween	20 min
PBS buffer	15 min
Serum incubation	30 min
Incubation with primary antibody	Overnight
PBS buffer	60 min
Incubation with secondary antibody	60 min
PBS buffer	30 min
ABC	60 min
PBS buffer	30 min
DAB	5 min
Wash with H_2O	2 min
Hematoxylin	1 min
Wash with H_2O	2 min

To make the tissue more reactive, rehydration was necessary. Time schedule and reagents used in the rehydration process are listed in Table A.10 (Appendix V), and contents of the different reagents and prepared solutions used are listed in Table A.12 (Appendix V). Time schedule and solutions used in the immunohistochemistry process are listed in Table 2.1. Phosphate buffer solution (PBS) was applied for "washing" the slides between each treatment. Citrate buffer was used for making the epitopes more available and 3% H_2O_2 in methanol was applied to prevent endogenous peroxidase activity. Incubation with goat serum was necessary to reduce non-specific background staining. The tissue sections were covered with antibodies, which are linked to a marker system so that the reaction between antibody and antigen could be visible in an electron microscope. Avidin – Biotin – peroxidase-complex (ABC) was added to amplify the signal, where peroxidase is bound to the secondary antibody to evoke color in the tissue if

the sample is positive. The final step before staining was completed by dripping Diaminobenzidin tetrahydroklorid (DAB) onto the incision where ABC oxidizes DAB, causing it to precipitate and the location of the antigen in the tissue is colored. Lastly, the tissue was dehydrated according to Table A.11 (Appendix V) and a cover glass was mounted on the slides using the cover glue Histomount (Invitrogen 008030).

2.3.6 Microscopy

The cell morphology of liver and adipose tissue (eWAT, iWAT, iBAT) was examined by using an Olympus BX 51 binocular microscope. An Olympus DP50 3.0 camera was used for photographing a representative field from each section. UCP1 expression was quantified by using Image J.

2.4 Real time qPCR

To identify relative gene expression of certain genes in the liver, ribonucleic acid (RNA) from liver was isolated and several steps were performed before real-time qPCR was conducted.

2.4.1 Homogenization and RNA purification

A small piece of tissue (50 mg) was weighed and transferred to an RNase free-tube together with 1 ml Trizol (Invitrogen, UK) and 4 zinconium beads. The samples were homogenized using a Precellys 24 lysis & homogenization instrument (Bertin Technologies, France) at 6000 rpm for 3 x 10 seconds. Incubation time was 5 minutes before 200 µl chloroform (VWR, Norway) was added to the tubes and shaken vigorously for 20 seconds. Furthermore, samples were incubated for 3 minutes at room temperature and centrifuged (4 °C, 15 min, 1200 rpm). The aqueous phase was transferred to a new tube where 500 µl isopropanol (Arcus Kjemi, Norway) was added. Two incubation steps followed, 10 minutes at room temperature and 45 minutes at 4°C. A new centrifugation was conducted (4°C, 30 min, 1200 rpm), and a pellet was formed. Supernatant was removed from the pellet by using a vacuum suction instrument (IBS Integra Bioscienses, Vacuboy Swizerland) and 1 ml cold 75% EtOH was added. Vortex was conducted before centrifugation (4°C, 15 min, 1200 rpm). Supernatant was once more removed using a vacuum instrument and 50-100 µl ddH2O was added to dissolve the pellet. Finally, RNA concentration was measured with Nanodrop ND-1000 spectrophotometer (Saveen Werner, Sweden) by placing 1.8 µl of sample directly on the measurement pedestal. Additionally, the ND-1000 calculated absorbance and the A260/A280 ratio and A260/A230 ratio, which give an indication of quality and quantity of the RNA samples.

2.4.2 RNA Precipitation

Measurement done on the Nanodrop ND-1000 spectrophotometer did not give high enough 260/230 ratios, and RNA Precipitation was needed. This was done by adding 0.1 times the volume of Sodium Acetat (3 M, Sigma) and 2.5 times the volume of 96% EtOH to each sample before it was frozen at -80 °C overnight. The following day, samples were centrifuged (4°C, 30 min, 1200 rpm). Supernatant was removed and 1 ml of 75% EtOH was added. Furthermore, samples were centrifuged before supernatant was removed again. To make the sample as clean as possible, it was important to remove every drop of EtOH without losing the pellet. Lastly, water was added to the samples and RNA concentration, absorbance and ratio was measured.

2.4.3 Measure of RNA integrity, BioAnalyzer

The quality of the purified RNA samples was analyzed by using BioAnalyzer RNA 6000 and an RNA LabChip kit (Agilent Technologies). The chip constitutes 16 wells, where 12 are filled with RNA samples, three are filled with gel dye mixture and one well is filled with a molecular size ladder (Ambion, USA). RNA is separated due to molecular size in a micro channel system where RNA migration is detected based on the ratio between size and charge of the RNA molecules. Software calculates RNA Integrity Number as well as an image and electropherogram occur, which provides information about the integrity of the RNA samples.

A random selection of 12 samples were used to determine the quality. Before start, reagents were incubated at room temperature for 30 minutes and the heating block was set to 70 °C. To make the Gel-dye mix, RNA Nano dye (0.5 µl) was added to 32.5 µl filtrated gel matrix and centrifuged (4 °C, 10 min, 13000 rpm). Gel- dye mix (9 µl) was added in the G- marked well in the RNA 600 Nano chip. The chip priming station was closed and the plunger was held down. Following 30 seconds of incubation, the piston was released by a “clip mechanism”. After 5 seconds, the piston was dragged carefully back to 1 ml position. Gel-dye mix was supplied to the two other g-marked wells and 5 µl of RNA – 6000 marker was added to each well except wells with added gel. Ladder was applied in the well marked a ladder and furthermore, 1 µl of RNA sample was pipetted into each of the remaining wells. Finally, the chip was placed in the Agilent 2100 Bioanalyzer (Agilent Thechnologies, USA).

2.4.4 Reverse transcription reaction

Before running qPCR, a reverse transcription (RT) reaction from RNA to complementary Deoxyribonucleic acid (cDNA) was necessary. All reagents and samples were kept on ice

throughout the procedure. RNA – samples (n = 60) were thawed on ice and diluted into a concentration equal to 50 ng/µl ± 5%. The concentration of the prepared samples were further measured on the Nanodrop ND-1000 spectrophotometer. A standard curve was made by pooling RNA, where 2 µl of a random selection of 30 samples were added into a new tube. The pooled RNA- samples were used to make a solution with the concentration 100 ng/µl. Furthermore, a double dilution of the 100 ng/µl was prepared in five new tubes (Figure 2.4) and concentration was measured.

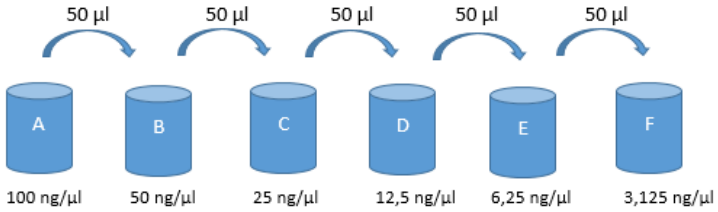

Figure 2.4: Dilution of RNA – samples for standard curve.

The RT reaction mix was prepared as described in Table A.13 (Appendix VI). For quality assessment, two negative controls were included, a non- amplification control (without multiscribe enzyme) and a non-template control (lacking RNA template). The pipetting was conducted in a 96-well RT-plate, where 40 µl of the reaction mix and 10 µl of each RNA – sample was added into each well. A 96-well full plate cover was affixed on top of the wells and it was centrifuged (50 x g, 1 min). Furthermore, the plate went through a thermal cycling RT reaction using a Gene AMP PCR System 9700 PCR machine (Applied biosystems), which program is presented in Table A.14 (Appendix VI). Finally, the 96-well plate was covered with a tape pad and stored at -20 °C.

2.4.5 Quantitative real-time polymerase chain reaction

To quantify gene expression in the liver, qPCR was conducted. Sequence-specific primers are used to determine the relative number of copies of a particular DNA Sequence. qPCR amplifies small DNA sequences encoding a small section of the gene one wishes to analyze. The amplification of DNA is exponential and is accomplished using fluorescent DNA – binding dye. The qPCR instrument measures the fluorescence and increased fluorescent signal is directly proportional to expression of the gene.

The volume of the cDNA plate was doubled by adding 50 µl ddH_2O. Furthermore, the cDNA plate was centrifuged (1000 x g, 1 min) and vortexed (1300 rpm, 3 min) prior to the real time PCR reaction. Real Time PCR Mix was made according to Table 2.2.

Table 2.2: *Reagents and volume used for the SYBR Green reaction mix.*

Reagent	Volume (µl)	Vendor
ddH_2O	335.5	MiliQ BioCel, USA
Primer I	5.75	Invitrogen, UK
Primer II	5.75	Invitrogen, UK
SYBR Green PCR Master Mix	575	Roche - Norge

The prepared qPCR reaction mix (112 µl) was added to an eight -strips tube so that the pipetting could be carried out by a robot (Biome 3000 Laboratory Automation Workstation, Beck Coulter, USA). Reaction mix (8 µl) and cDNA sample (2 µl) was added to each well in a 384 well plate. Furthermore, the plate was covered with an optical adhesive cover and centrifuged for 2 minutes at 1500 x g. Finally, the real-time PCR reaction was performed using the Light Cycler 480 machine.

2.5 Statistical analyses

2.5.1 Microsoft Exel 2013

Processing of raw data and calculation of standard deviation as well as Standard Error of the Mean (SEM) was made in Microsoft Exel.

2.5.2 Graph pad prism 6 and Statistica 12

Graph pad prism 6 was used to identify outliers by applying Grubb's test and normal distribution was tested in Statistica 12. Data in Figure 1 and 2 were tested by a parametric unpaired t- test in Graph pad and Repeated Measurements (RM) one-way analysis of variance (ANOVA) with unequal N HSD post hoc test in Statistica 12. The results from feeding experiment 1 are compared with a previous experiment done on NIFES where the group size varies from our experiment. On the remaining data, a one-way ANOVA with Fisher LSD multiple comparison in Graph pad was used to analyze differences between the six groups. To analyze weight development, accumulated feed intake as well as blood glucose levels during IPGTT and ITT, a RM ANOVA test with LSD multiple comparison post hoc test was applied in Statistica. Data were considered significant when $p < 0.05$. Statistical significance between the experimental groups are marked with different letters. Additionally, a two- ways ANOVA was applied only on data from the LF fed groups, to compare the effect of protein source with exercise. The results of these data are illustrated in a textbox in the figures where p – values up to 0.1 is mentioned. P value < 0.05 is considered statistic significant and marked *.

3.0 RESULTS

To induce obesity, 70 mice were fed a HF/HS diet as presented in Table A.2 (Appendix I) for seven weeks. In order to evaluate how replacement of casein with salmon and entrecôte in a HF/HS diet affected diet-induced obesity (DIO), weight development, feed intake and results from IPGTT and ITT was compared with a previous study done at NIFES, where casein was the only protein source (n = 8).

3.1 Casein, salmon and entrecôte in a HF/HS diet promoted obesity

Despite similar energy intake, replacement of 2/3 of casein with salmon and entrecôte in a HF/HS diet caused a sharp increase in BW and fat mass. As illustrated in Figure 3.1 A-C, a HF/HS diet with casein, salmon and entrecôte as protein source induced obesity after six weeks of feeding. An MRI scan of the mice were performed prior to the feeding experiment and after five weeks to see if the weight gain was due to an increase in fat mass or lean mass. Figure 3.1 A illustrates that the weight gain in the mice fed a HF/HS (Casein, Salmon and Entrecôte) diet is attributable to an increase in fat mass. The BW development during six weeks of feeding was significantly higher in mice fed casein, entrecôte and salmon compared to the mice fed solely casein. As a result, BW gain after six weeks of feeding was significantly higher in the mice fed HF/HS (Casein, Salmon and Entrecôte) compared to the mice fed HF/HS (Casein). Nevertheless, energy intake was virtually the same in the group fed casein, salmon and entrecôte and the group fed only casein (Figure 3.1 D-E). Furthermore, as illustrated in Figure 3.1 F, feed efficiency was significantly higher in mice fed HF/HS (Casein, Salmon and Entrecôte) compared to mice fed HF/HS (Casein), which underscores the fact that the mice who consumed casein and animal protein gained more weight than the mice fed solely casein.

Body weight development, energy intake and feed efficiency

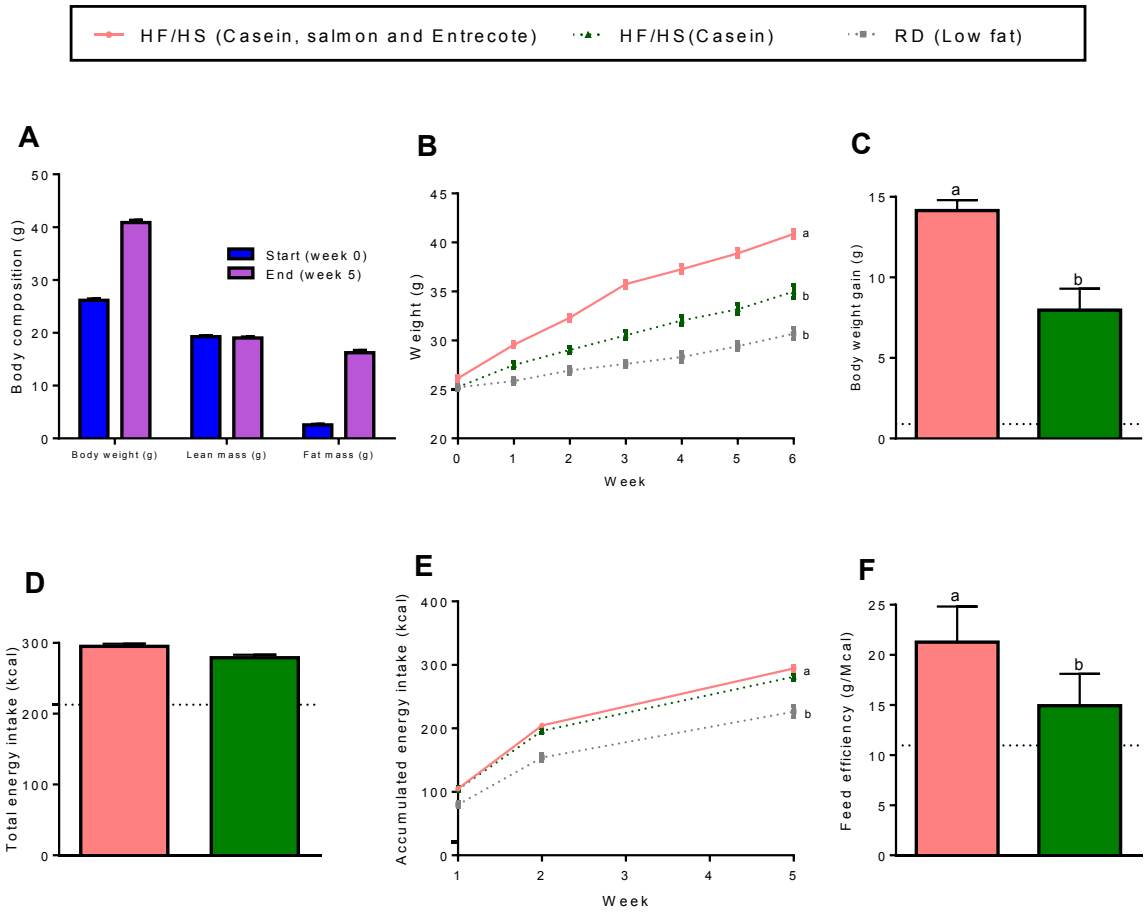

Figure 3.1: Replacing casein with animal protein caused a sharp increase in body weight and fat mass despite similar energy intake. **A**: Body composition before and after five weeks on a HF/HS diet with casein, entrecôte and salmon as protein source. **B**: Body weight development during six weeks of feeding on a HF/HS diet. **C**: Body weight gain. **D:** Total energy intake during three weeks of feeding. **E:** Accumulated energy intake during week 1, 2 and 5. **F:** Calculated feed efficiency. Group variations in body weight gain, total energy intake and feed efficiency were analyzed using a parametric unpaired t- test. Weight development and accumulated energy intake were analyzed by using RM ANOVA with unequal N HSD post hoc test. Different letters denote statistical significance ($p < 0.05$) between the mean in each group. The results are presented as mean ± SEM.

3.2 Glucose tolerance and insulin sensitivity was impaired by HF/HS feeding with casein, salmon and entrecôte

To evaluate the impact of protein source on glucose tolerance and insulin sensitivity, an IPGTT and ITT were performed after six weeks of experimental feeding. Figure 3.2 illustrates glucose levels prior to and throughout the tests and calculated Area Under the Curve (AUC).

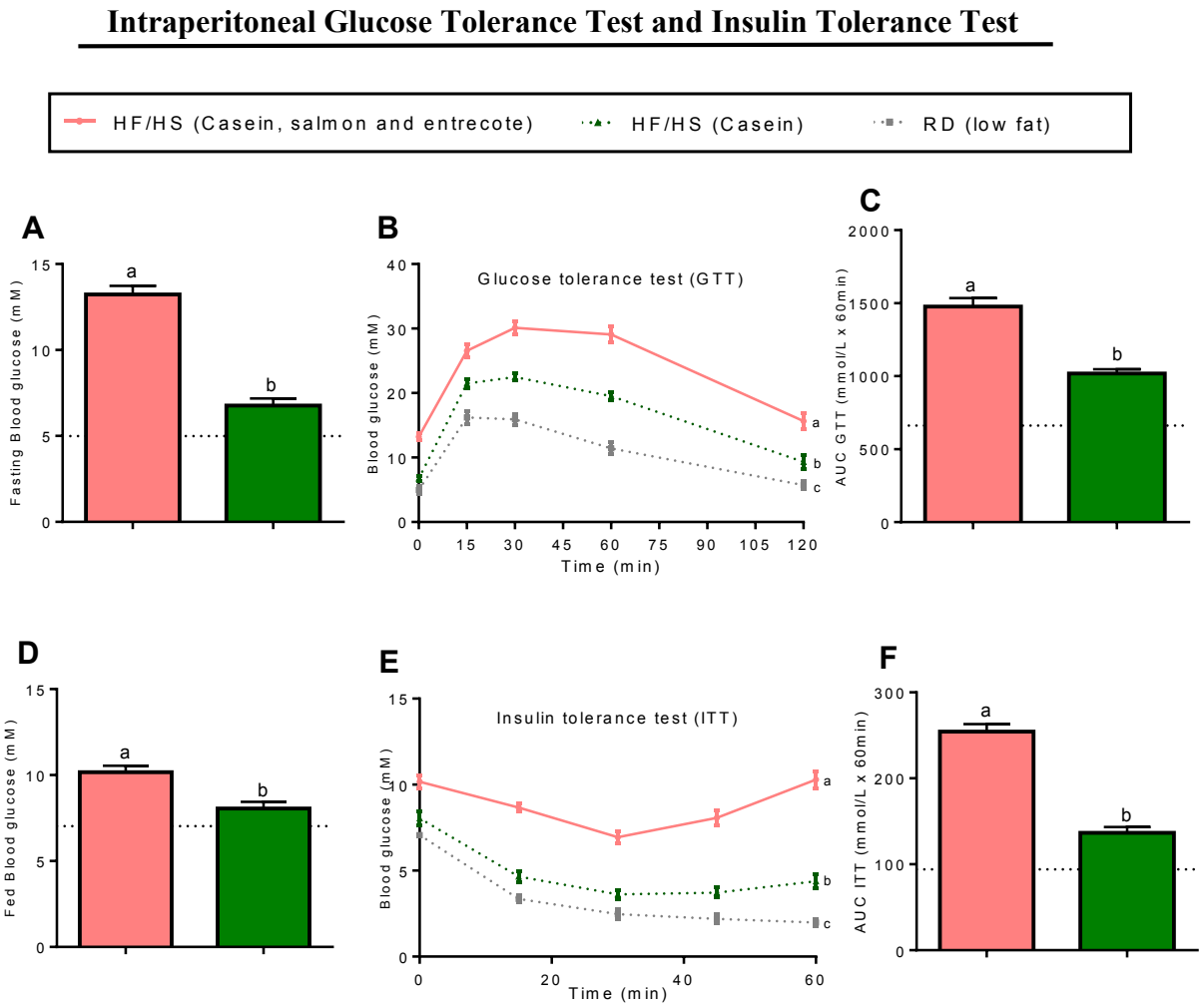

Figure 3.2: Intraperitoneal glucose tolerance test performed on fasting mice (6 h) and Insulin Tolerance Test performed on randomly fed mice after six weeks of feeding diets. **A**: Fasting blood glucose. **B**: Blood glucose levels during IPGTT. **C:** Calculated area under curve during IPGTT. **D:** Fed blood glucose. **E:** Blood glucose levels during ITT. **F:** Calculated area under curve during ITT. Group variations in blood glucose and AUC were analyzed using a parametric unpaired t- test. Blood glucose levels measured during IPGTT and ITT were analyzed by using Repeated Measurement ANOVA test with unequal N HSD post-hoc test. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

The results from IPGTT indicate that there is an association between high BW, high fasting blood glucose and lowered glucose tolerance. Measuring blood glucose after six hours of fasting showed that mice fed casein, salmon and entrecôte in a HF/HS diet had elevated levels of blood glucose compared to mice fed solely casein on the same diet (Figure 3.2 A). Additionally, Figure 3.2 B illustrates that mice fed casein, salmon and entrecôte had an impaired glucose tolerance compared to mice fed casein alone. However, compared to a LF reference diet (RD), the mice fed casein also had an impaired glucose tolerance. To evaluate glucose clearance between mice fed casein, salmon and entrecôte with mice fed merely casein, AUC was calculated. A parametric unpaired t – test of glucose AUC showed that the glucose clearance was significantly better in mice fed casein compared to mice fed casein, salmon and entrecote (Figure 3.2 C).

Results from ITT also demonstrated an association between high BW and impaired insulin sensitivity (Figure 3.2 D-F). The differences in blood glucose prior to test start was statistically significant between mice fed casein, salmon and entrecôte and mice fed only casein, where mice fed casein, salmon and entrecôte had higher fed blood glucose levels than mice fed casein (Figure 3.2 D). Figure 3.2 E demonstrates that mice fed casein, salmon and entrecôte had impaired insulin sensitivity compared to mice fed casein and mice fed a LF diet. There is a minimal response in blood glucose in the mice fed casein, salmon and entrecôte, which mean they cannot be defined completely insulin resistant; however, the insulin sensitivity is clearly impaired. The calculated AUC was significantly lower in the casein- fed mice compared to the casein, salmon and entrecôte – fed mice, which reinforces the fact that the latter group is less insulin sensitive.

3.3 Body weight change due to exercise was greater in mice fed LF diets than in mice fed HF/HS diets.

After being assigned a HF/HS diets for seven weeks, 60 of the most average mice were divided into six groups based on BW, fat mass and lean mass. To investigate if DIO, impaired glucose tolerance and insulin sensitivity was reversible by changing diet, two of the groups remained on a HF/HS diet (Table A.2, Appendix I) as a control, and the four remaining groups switched to a LF diet (Table A.2, Appendix I). To evaluate the effect of different protein sources, two groups were fed entrecôte as main protein source and two groups were fed salmon. Three of the groups (defined as Ex) received running wheels to evaluate the impact of voluntarily exercise.

BW, physical activity and feed intake were measured throughout the period. In the following figures, the mice fed LF diets are termed by the protein source received.

Body weight development, body weight change and exercise

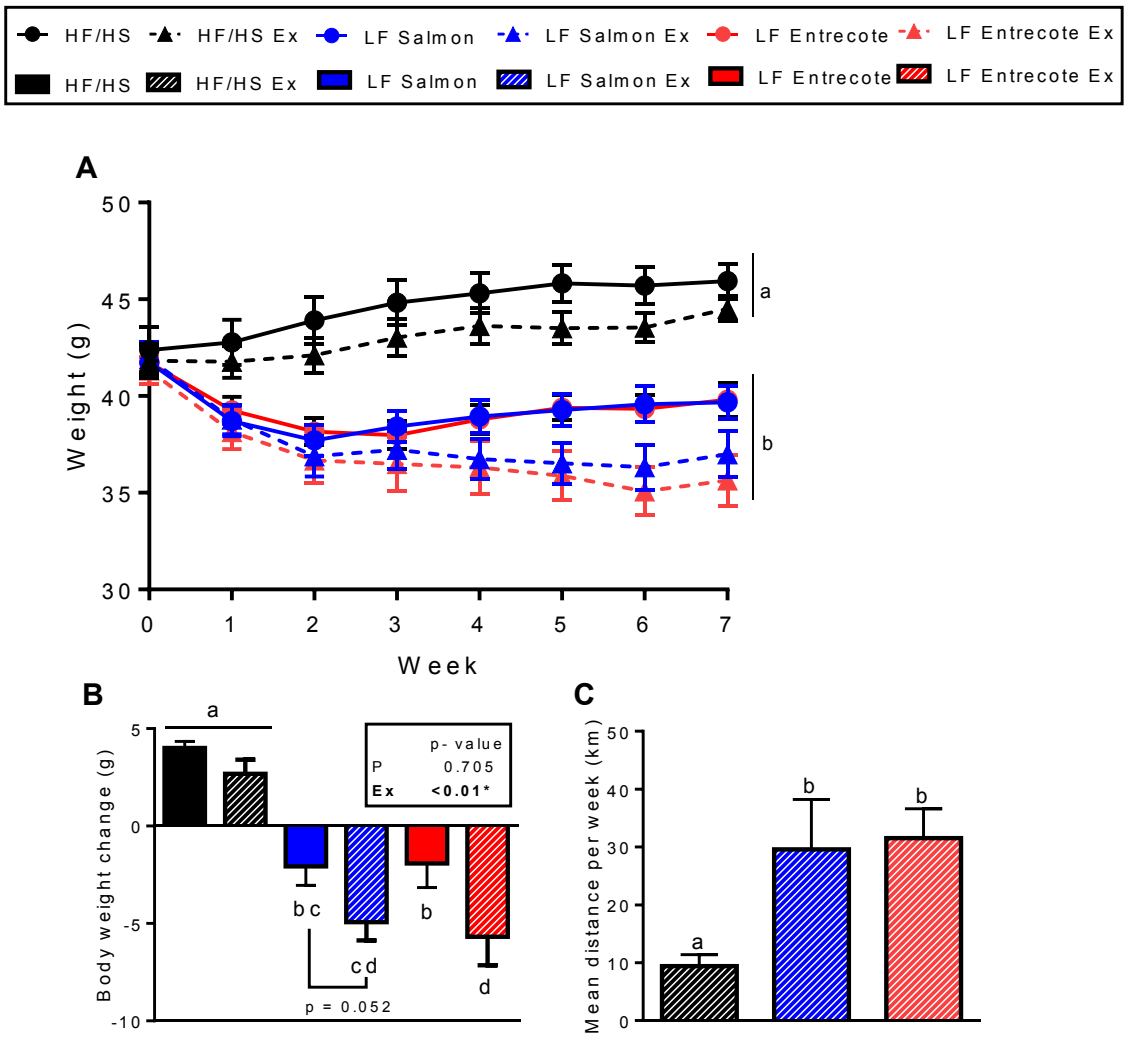

Figure 3.3: The effect of diet and/or exercise on body weight development (**A**), body weight change after seven weeks (**B**) and elapsed mean run distance per week (**C**). Weight development was analyzed by RM ANOVA. Group variations of body weight change and physical activity were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. Additionally, a two way ANOVA was applied to determine whether protein source (P) or exercise (Ex) had an impact on differences in body weight change or exercise, and statistical significance is marked *.

As presented in Figure 3.3A, different diets stimulated weight gain at different rates. However, there was no significant difference between mice fed salmon or entrecôte in the LF diets. The effect of exercise on BW development during seven weeks was not statistically significant in either group. One way ANOVA analyzes of BW change data (Figure 3.3 B) did not show any difference between the Sed and Ex group in the mice fed HF/HS; however, BW change was affected by exercise in the mice fed LF entrecôte. In the LF salmon-fed mice the difference between the Ex and Sed group was not statistically significant, but the p – value was 0.0520. Nonetheless, results from two ways ANOVA comparing exercise with protein source in the LF groups, demonstrated that exercise made a significant difference regarding weight loss in both entrecôte and salmon – fed mice. Figure 3.3 C illustrates the level of mean physical activity in the different groups that received running wheels. The mice fed LF salmon and LF entrecôte run significantly longer than the mice fed HF/HS diet.

3.3.1 Fat digestibility was lower in mice fed entrecôte than mice fed salmon

To evaluate if changes in BW was due to differences in energy intake, feed intake was monitored throughout the experimental period. Total BW change and total energy intake were used to determine feed efficiency (g/Mcal). Furthermore, to evaluate if the changes in BW and feed efficiency was due to differences in fat or nitrogen excretion, feces were collected and their fat and nitrogen content analyzed.

The mice fed HF/HS had the same accumulated energy intake, regardless of exercise (Figure 3.4 A). The mice fed HF/HS had a higher accumulated energy intake than the LF groups, with the exception of the sedentary mice fed LF salmon, they had similar accumulated energy intake as exercised mice fed HF/HS. Nevertheless, sedentary mice fed LF salmon did not differ significantly from the other LF groups when analyzing accumulated energy intake. Figure 3.4 B illustrates that mean energy intake per week is significantly higher in both of the HF/HS fed groups compared to the LF-fed groups. Furthermore, neither training nor protein source seems to determine energy intake. To evaluate the obesogenic or slimming effect of the different diets, feed efficiency was calculated. As expected, feed efficiency was much higher in the HF/HS fed mice than mice fed LF diets. There was however no significantly difference between salmon and entrecôte – fed mice. Both of the LF groups assigned to exercise had lower feed efficiency than the sedentary groups receiving LF diets.

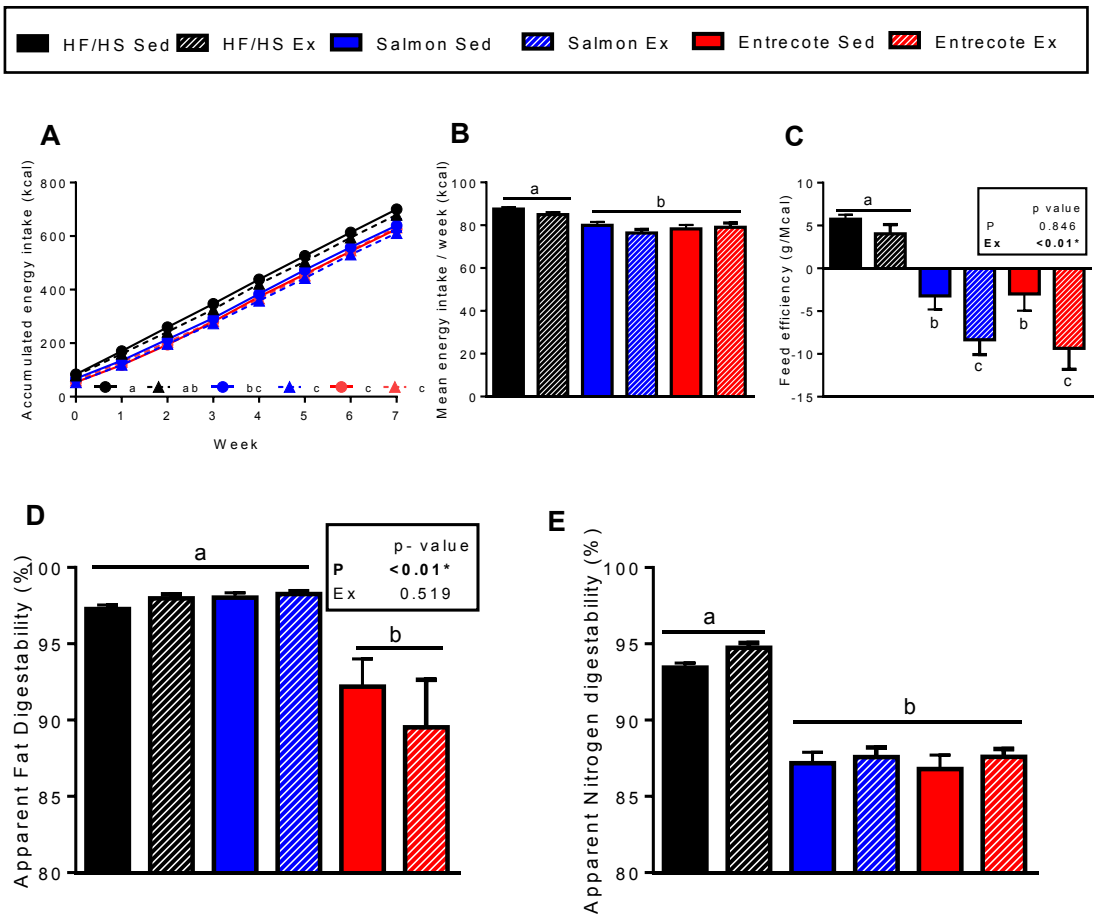

Figure 3.4: Energy intake (**A-B**), feed efficiency (**C**) and apparent fat (**D**) and nitrogen (**E**) digestibility in mice fed a HF/HS (Casein, salmon and entrecôte) and LF diets with salmon or entrecôte as protein source. Accumulated feed intake was analyzed by RM ANOVA. Differences in weekly feed intake, feed efficiency and apparent fat and nitrogen digestibility were analyzed using a one- way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of each group. The results are presented as mean ± SEM. A two way ANOVA was used to determine whether protein source or exercise had an impact on differences in feed intake, feed efficiency or digestibility and statistical significance is marked *.

Interestingly, results from one way ANOVA showed that fat digestibility was significantly lower in the mice fed LF entrecôte compared to the mice fed LF salmon and the mice fed HF/HS. Additionally, a two ways ANOVA of the LF – groups reinforced that the protein source (entrecôte) was a determining factor regarding fat digestibility. As opposed to protein source, Ex did not seem to influence fat digestibility. Figure 3.4 E illustrates that the apparent Nitrogen digestibility was significantly lower in the mice fed LF than in the mice fed HF/HS.

3.4 Exercise combined with low fat diet was beneficial for changes in body composition

To determine whether BW change was attributable to changes in fat mass or lean mass, the mice were MRI scanned prior to feeding experiment 2 and after five and seven weeks. The results from the MRI scan in week 5 as well as the changes in fat- and lean mass from week 0-5 are presented in Figure 3.5. During termination, visceral (eWAT) and subcutaneous (iWAT) adipose tissue depots was excised and weighed to evaluate whether differences in fat mass could be attributed to mass – differences between the various adipose tissue depots. Additionally, to evaluate if the changes in lean mass could be due to changes in muscle weight, *musculus tibialis anterior* and *musculus gastrocnemius* were excised and weighed, and the results are presented in Figure A.7 (Appendix VII).

As illustrated in Figure 3.5 A, after five weeks fed experimental diets, fat mass was significantly higher in the mice fed HF/HS, while exercise did not seem to influence fat mass in this group. The same trend was observed in the mice fed LF salmon; however, the p-value between the Sed and Ex group in the mice fed LF salmon was 0.062 and 0.065 in the mice fed HF/HS. In the mice fed LF entrecôte, the difference in fat mass between Sed and Ex group was statistically significant. Evaluating the changes in fat mass from week 0 – 5, both of the LF groups assigned to exercise had a significantly higher fat – mass reduction than their respective sedentary group. Nevertheless, in the mice fed HF/HS diets, there was no statistical difference between Sed and Ex group, indicating that exercise had no impact on fat mass reduction in mice fed HF/HS. Additional results from the MRI scan showed that after 5 weeks, mice assigned to exercise and fed LF entrecôte, was the only group that had statistically higher lean mass than the mice fed HF/HS. Nonetheless, looking at the change in lean mass, both groups fed LF diets, regardless of exercise had a higher lean mass increase than the mice fed HF/HS; furthermore, the exercised mice fed LF entrecôte had significantly higher lean mass than the sedentary mice fed LF entrecôte. Results from two ways ANOVA demonstrates that the protein source is not crucial for changes in lean mass; however, the p-value for exercise is 0.053, indicating that exercise may increase lean mass in mice fed LF diets.

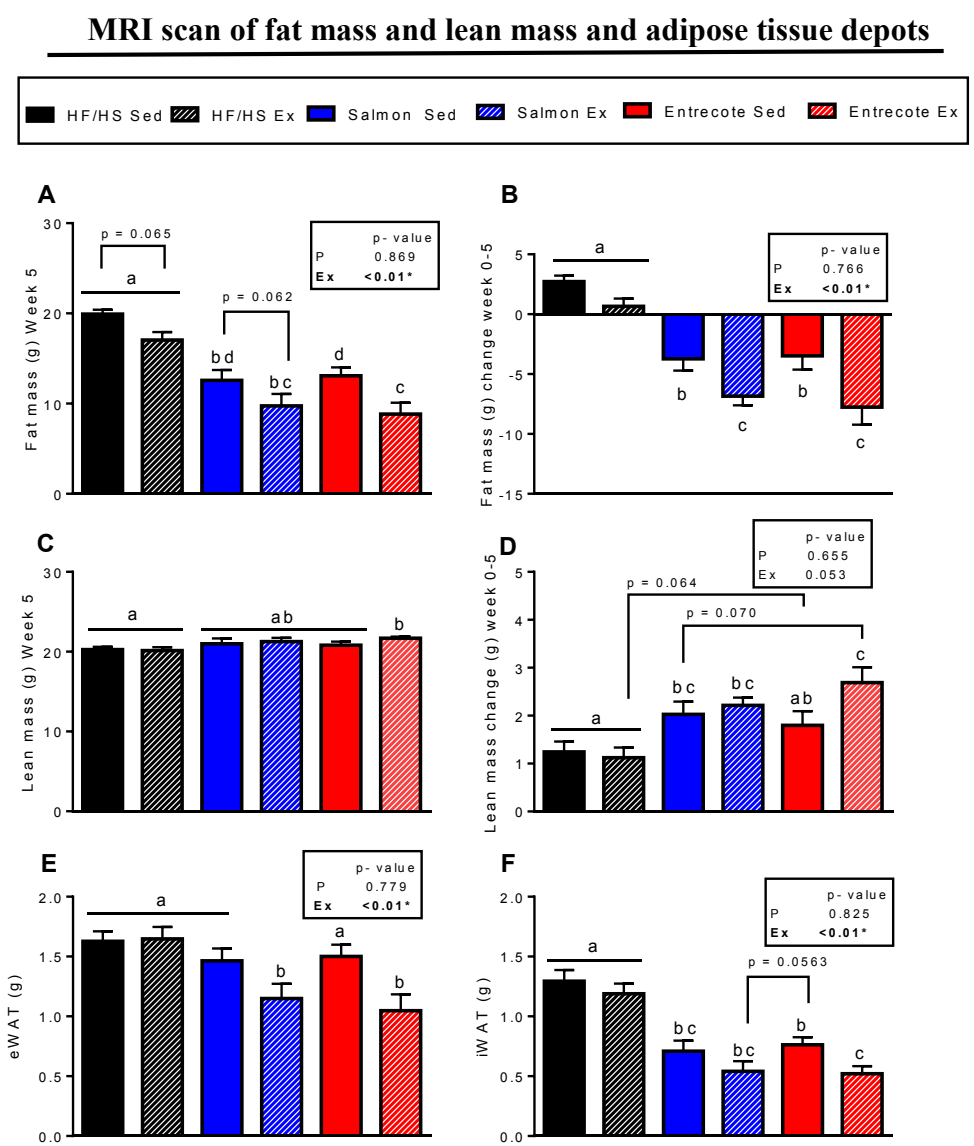

Figure 3.5: Body composition and body composition change in the different groups (n=9-10). **A:** Fat mass after 5 weeks on experimental diets. **B:** Fat mass change during 5 weeks. **C**: Lean mass after 5 weeks on experimental diets. **D:** Lean mass increase after 5 weeks. **E:** Masses of eWAT at termination (Week 7) **F:** Masses of iWAT at termination. Group variations in fat mass, lean mass and adipose tissue mass were analyzed using a one-way ANOVA with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. A two way ANOVA was used to determine whether protein source or exercise had an impact on differences in fat- or lean mass, eWAT and iWAT in the mice fed a LF diet and statistical significance is marked *.

Figure 3.5 E indicates that a LF diet alone does not affect the weight of eWAT; however, combining exercise with a LF diet reduced the weight of eWAT in mice fed salmon and entrecôte. Figure 3.5 F demonstrates that a LF diet alone had an impact on iWAT weight, as the mice fed LF diets had significantly lower weight of iWAT than the mice fed HF/HS. In the

mice fed HF/HS, exercise did not seem to be of importance, and neither in the LF salmon group when evaluating results from one way ANOVA; however, in the mice fed LF entrecôte, exercise made a difference, as iWAT weight was significantly lower in the Ex group than the Sed group. Furthermore, a two-way analyzes on eWAT weight in the LF groups demonstrated that exercise influenced the weight of iWAT.

3.4.1 Adipocyte size in white adipose tissue

The adipocyte size in white adipose depot (eWAT and iWAT) was examined by staining and microscopy of a representative part.

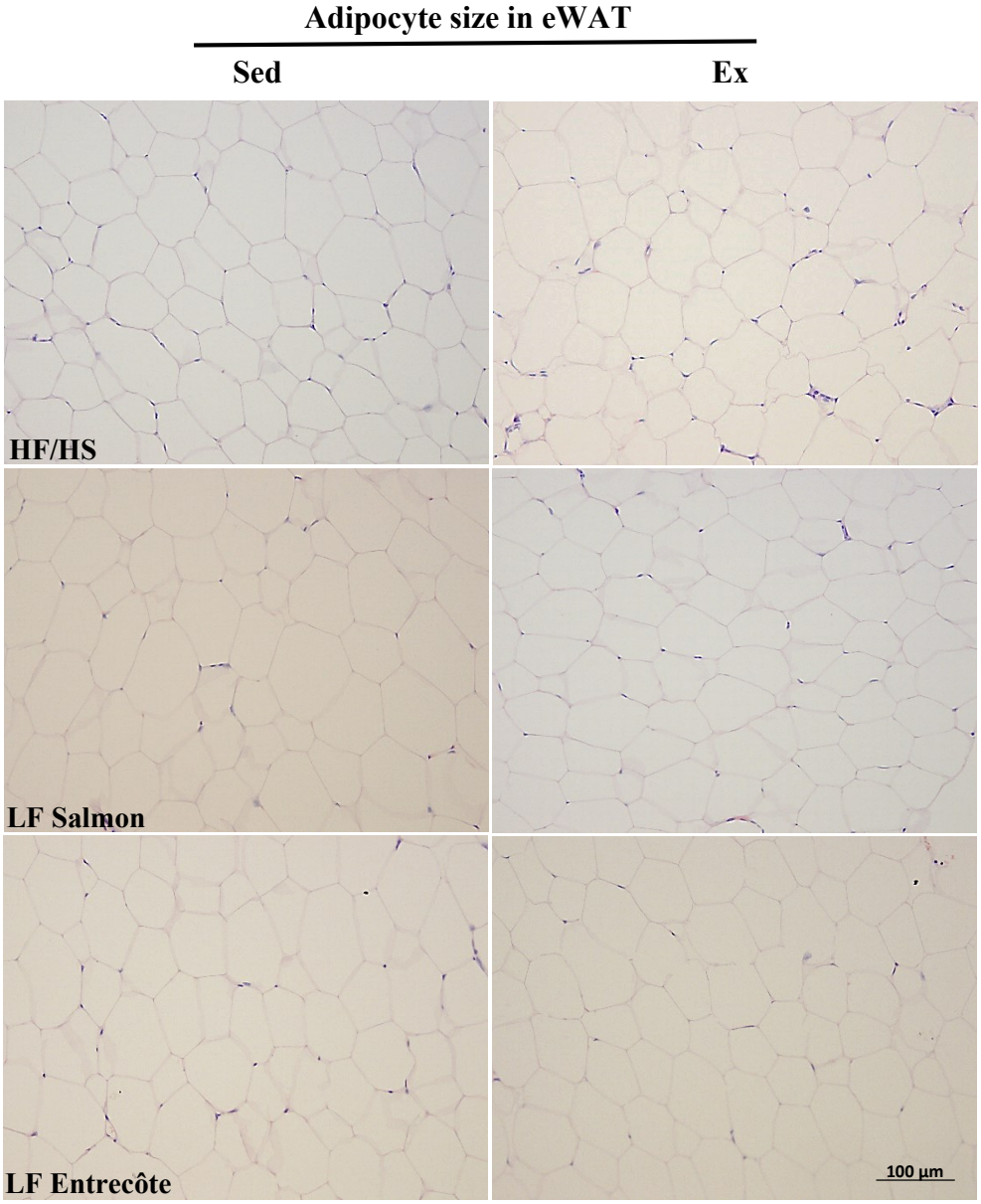

Figure 3.6: Adipocyte micrographs in epididymal adipose tissue in mice fed HF/HS, LF salmon and LF entrecôte, with and without exercise. (Micrographs are magnified x 20)

The micrographs of eWAT (Figure 3.6) supports that neither diet nor protein source seems to influence adipocyte size in eWAT. This is in accordance with Figure 3.5 E, which demonstrate no difference in eWAT weight between sedentary mice fed HF/HS and LF. Figure 3.5 E further illustrates a difference between Ex and Sed group mice fed LF diets; however, the micrographs from Figure 3.6 cannot confirm whether this difference is attributable to differences in adipocyte size.

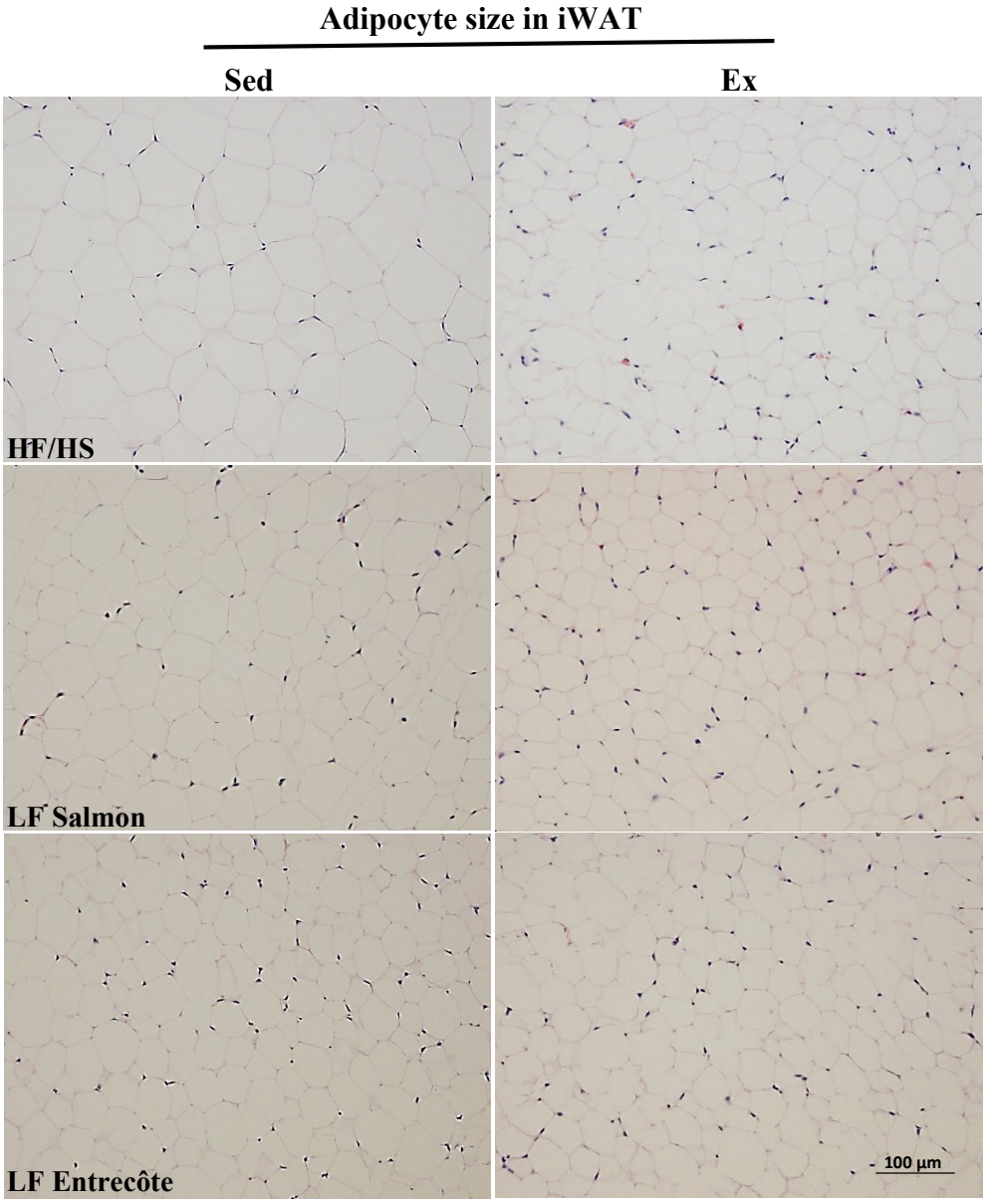

Figure 3.7: Adipocyte micrographs in subcutaneous adipose tissue (iWAT) in mice fed HF/HS, LF salmon and LF entrecôte, with and without exercise (Micrographs are magnified x 20).

The micrographs in Figure 3.7 indicate that exercise makes the size of the lipid droplets in iWAT smaller in mice fed HF/HS. The differences between Sed and Ex are not that obvious in the LF groups; however, the lipid droplets in LF salmon Ex group seems smaller than in the Sed group. In any case, the lipid droplets in the LF groups looks smaller than in the HF/HS group and are consistent with Figure 3.5 F, which indicates that a LF diet reduce adipocyte size in iWAT.

3.5 Brown adipose tissue

To evaluate if diet, exercise or protein source could affect the BAT, iBAT was excised and weighed during termination. The adipocyte size in iBAT was examined by staining and microscopy a representative part.

Figure 3.8 A illustrates that the weight of iBAT is not influenced by exercise in the mice fed HF/HS. All LF groups except LF salmon Sed had significantly lower iBAT weight than both HF/HS groups. Although analyses with one- way ANOVA did not demonstrate significant differences between Ex and Sed group in the mice fed LF diet, analyses with two- way ANOVA indicates that exercise determines the weight of iBAT in the mice fed LF diets. The micrographs of iBAT illustrated in Figure 3.8 B suggest that the differences in iBAT weight between the mice fed HF/HS and the mice fed LF is attributable to reduced lipid accumulation in the mice fed LF. However, whether the adipocytes becomes smaller with exercise in either group, cannot be said by merely considering the micrographs.

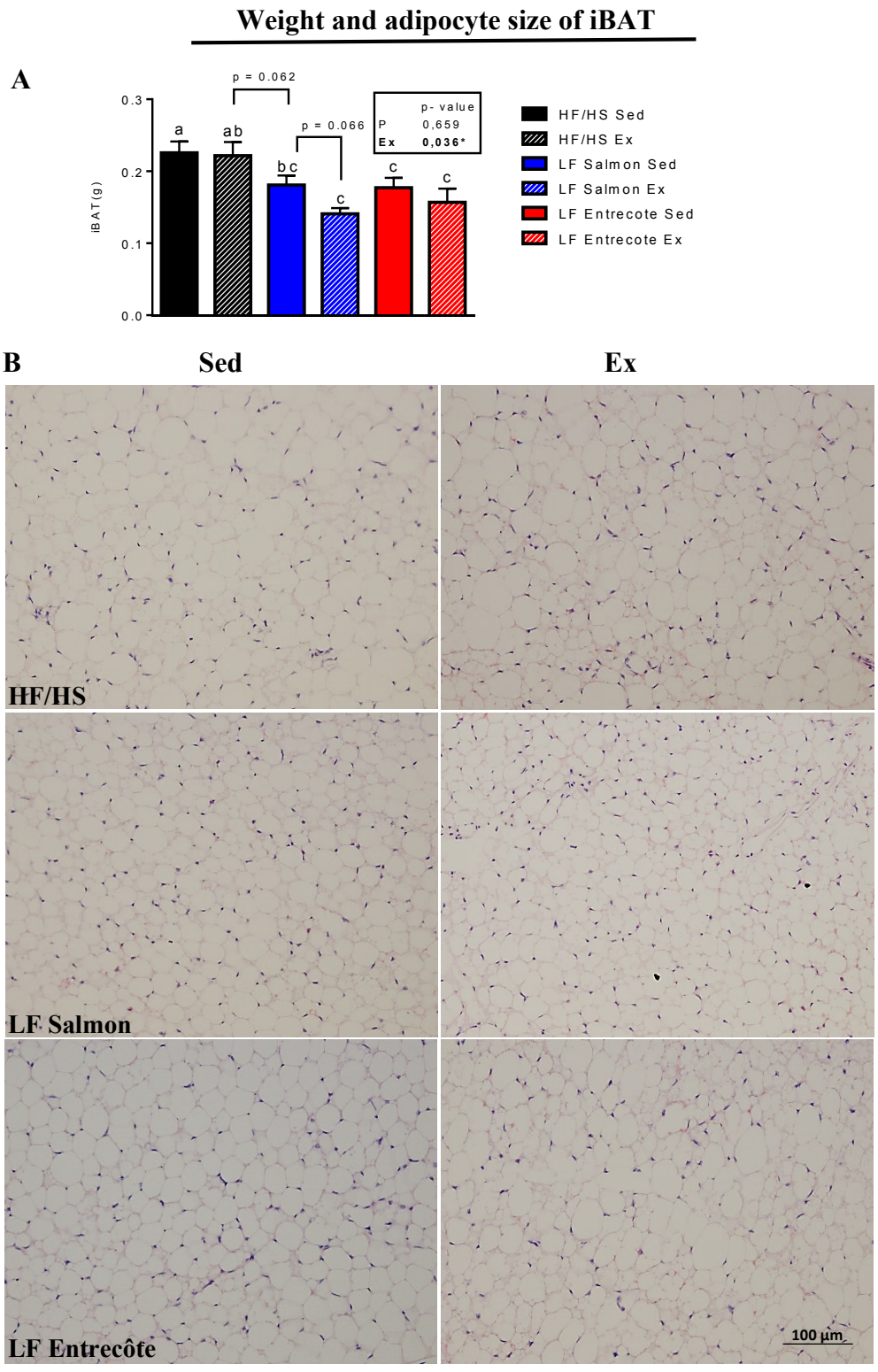

Figure 3.8: Masses of iBAT **(A)** and adipocyte micrographs **(B)** in mice fed HF/HS, LF salmon and LF entrecôte, with and without exercise. Group variations in iBAT were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of each group. The results are presented as mean ± SEM. A two ways ANOVA was used to determine whether protein source or exercise had an impact on differences in iBAT weight and statistical significance is marked *. (Micrographs are magnified x 20)

3.5.1 UCP1 expression in iBAT

To investigate if diet, exercise or protein source influenced energy expenditure trough the expression of UCP1, immunohistochemistry was performed and additional micrographs were taken to quantify UCP1 in iBAT.

Evaluating the micrographs in Figure 3.9 A confirms that diet have an impact on the size of the lipid droplets in iBAT. Furthermore, Figure 3.9 B and micrographs indicate that the expression of UCP1 is slightly elevated in mice fed LF than in mice fed HF/HS diet. In addition, by looking carefully at the micrographs and evaluating Figure 3.9 B, exercise may possibly have an effect on the size of the lipid droplets as well as the expression of UCP1, at least in the mice fed LF salmon. Nevertheless, the expression of UCP1 is impaired in the mice in our experiment, regardless of diet and exercise in contrast to the expression of UCP1 in mice fed high fat – high protein (HF/HP) diets in an earlier study conducted by our research group. By comparing Figure 3.9 A with Figure A.2 (Appendix V), the reduced expression of UCP1 in the mice from our experiment becomes apparent. Figure A.2 illustrates appearance of iBAT in mice fed a HF/HP diet, in addition to after cold exposure. The micrographs in Figure A.2 demonstrates a higher number of multilocular cells, which is a characteristic of BAT. Evaluating Figure 3.9 A, it seems that the low expression of UCP1 may be due to white fat accumulation in iBAT. This further makes it possible to speculate on whether a LF diet or exercise permits elevation of UCP1 expression in iBAT after mice have been obese on a HF/HS diet with salmon and entrecôte as protein source.

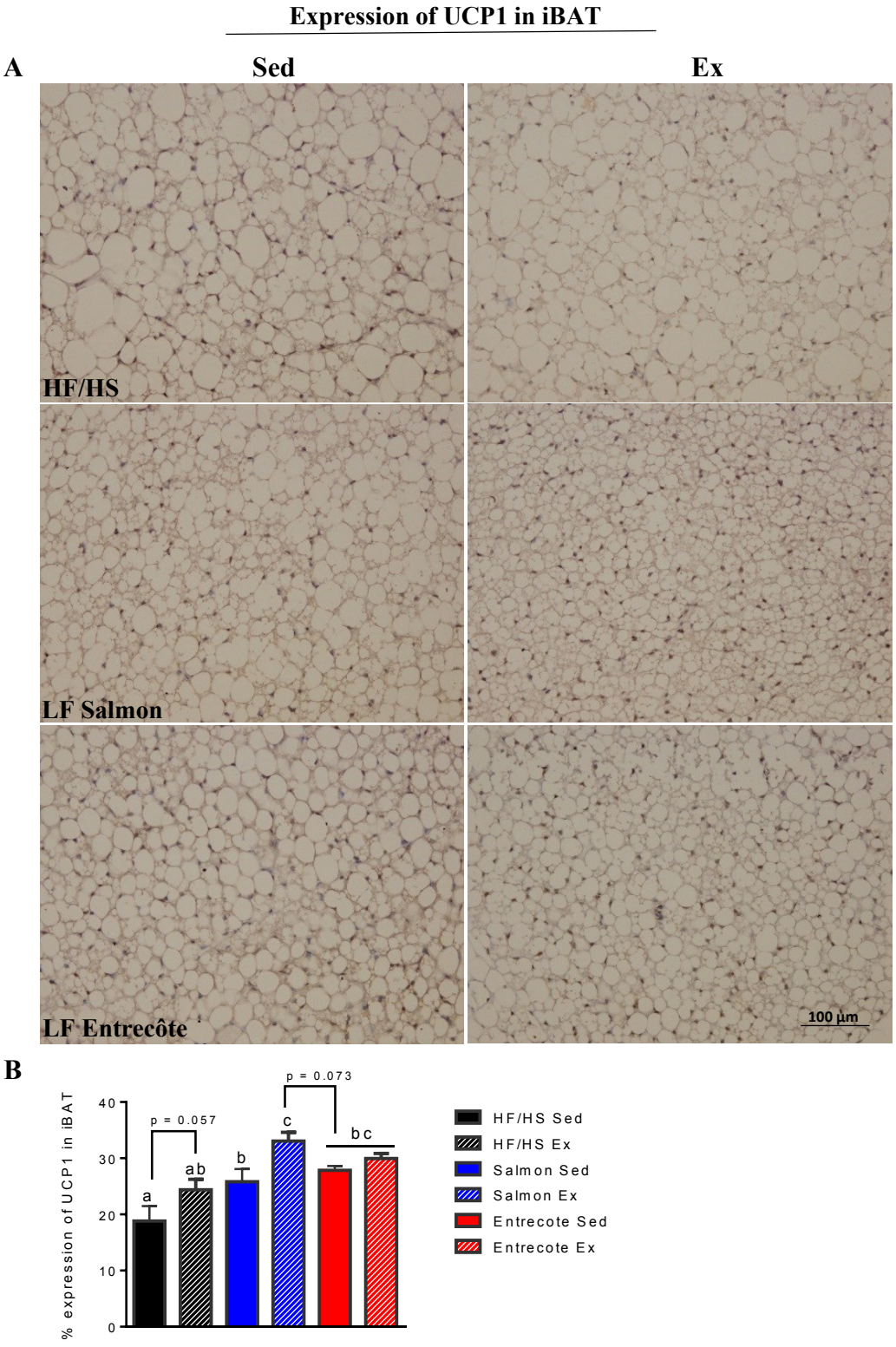

Figure 3.9: Adipocyte micrographs **(A)** and quantified UCP1 expression **(B)** in mice fed HF/HS, LF salmon and LF entrecôte, with and without exercise. Group variations in the expression of UCP1 in iBAT was analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. (Micrographs are magnified x 20)

3.6 Insulin sensitivity was improved by diet and exercise, but only diet influenced glucose tolerance.

To evaluate the impact of diet, exercise and protein source on glucose tolerance and insulin sensitivity, an IPGTT and ITT was performed after six weeks of experimental feeding. The results from the IPGTT are presented in Figure 3.10 and the results from ITT in Figure 3.11.

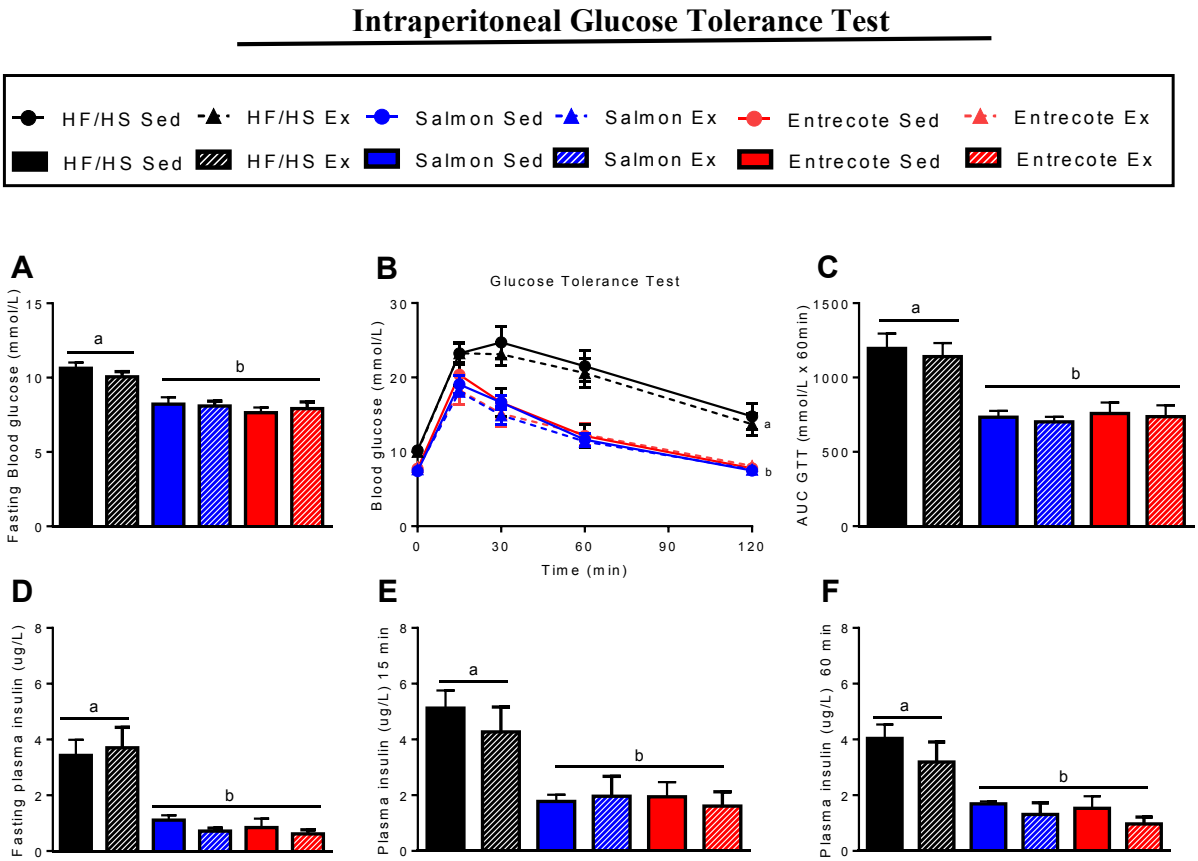

Figure 3.10: Effect of diet on glucose regulation. **A**: Fasting blood glucose **B:** Blood glucose levels during IPGTT performed on fasting mice (6 h) after five weeks on the experimental diets. **C:** Calculated area under curve during IPGTT **D-F:** GSIS at baseline and after 15 and 60 minutes during IPGTT. Group variations in fasting blood glucose, AUC of IPGTT and plasma insulin were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Variations in blood glucose during IPGTT were analyzed using a Repeated Measurement one-way ANOVA with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM.

The results from IPGTT indicates that a LF diet significantly improves glucose tolerance. Figure 3.10 A-C illustrates that mice fed a LF diet, regardless of protein source or exercise,

have a significantly lower fasting blood glucose and improved glucose tolerance than mice fed a HF/HS diet, displayed during the IPGTT and calculation of AUC. Additionally, plasma insulin was analyzed at baseline, after 15 and 60 minutes. The results showed that the levels of insulin were significantly higher in the HF/HS fed mice and that neither exercise nor protein source seemed to be of importance.

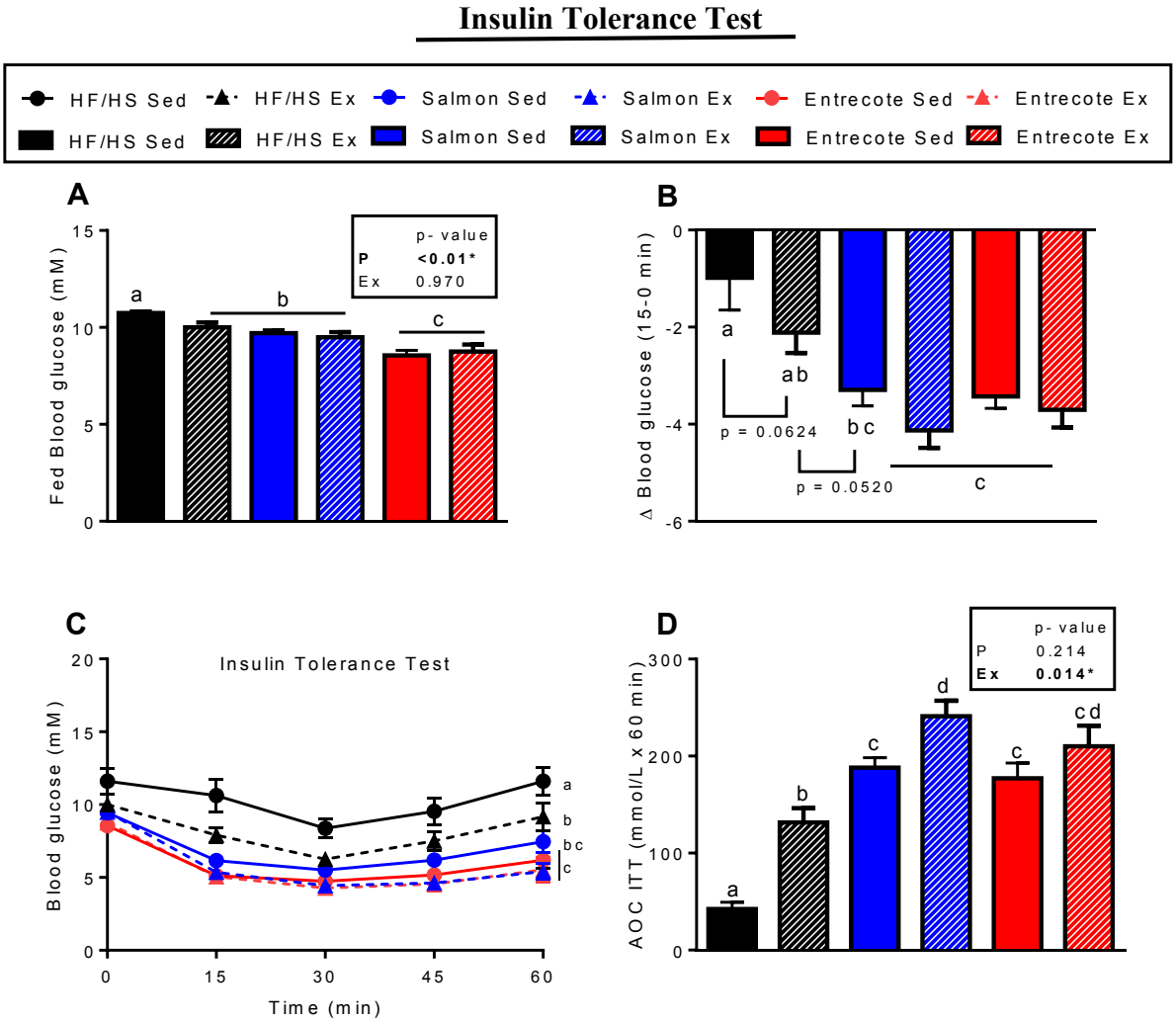

Figure 3.11: Effect of diet, protein source and/or exercise on insulin sensitivity. **A:** Randomly fed blood glucose levels in the different groups (before test start). **B:** Delta blood glucose 15-0 min. **C:** Blood glucose levels during Insulin Tolerance test (ITT). **D:** Calculated area over the curve (AOC). Differences in fed blood glucose, Δ Blood glucose and AOC were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Variations in blood glucose during ITT were analyzed using a repeated measurement one-way ANOVA with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. Additionally, a two ways ANOVA was used to determine whether protein source or exercise had an impact on differences in fed blood glucose, Δ Blood glucose and AOC in the LF groups and statistical significance is highlighted and marked with *.

As presented in Figure 3.11, fed blood glucose was significantly higher in the sedentary mice fed HF/HS than in all other groups. Furthermore, the mice fed LF entrecôte had significantly lower fed blood glucose than mice fed LF salmon. This is further supported by analyzing the data with a two ways ANOVA, which demonstrated that protein source affected fed blood glucose; however, exercise did not seem to be of importance. Changes in blood glucose from 0 – 15 minutes was significantly higher in the LF groups compared to the HF/HS groups with the exception of the sedentary mice fed LF salmon, this group was not significantly different from the HF/HS Ex group; nevertheless, the p – value between the two groups was 0.052. The impact of exercise on changes in blood glucose did not seem to be of importance; however there was a trend towards reduced blood glucose observed in the exercised mice fed HF/HS ($p = 0.062$). Analyzing blood glucose levels during ITT (Figure 3.11 C) with RM ANOVA demonstrates that exercise improves insulin sensitivity in the mice fed HF/HS but not in the mice fed LF. Furthermore, the LF groups demonstrates improved insulin sensitivity compared to HF/HS groups, with the exception of sedentary mice fed LF salmon, which do not have a significantly enhanced insulin sensitivity compared to exercised mice fed HF/HS diet.

In light of the finding that fed blood glucose was elevated in all groups, AOC was calculated to evaluate the response of insulin injection on blood glucose, independent of blood glucose levels at baseline. Figure 3.11 D illustrates that mice fed LF diets have an improved response to insulin compared with mice fed HF/HS diets. Furthermore, Figure 3.11 D demonstrates that in mice fed HF/HS and LF salmon, exercise seems to improve insulin response. The improvement by exercise was however not apparent in the mice fed LF entrecôte. Exercised mice fed LF Salmon had improved blood glucose lowering effect of insulin than sedentary mice fed LF diets. A two-way ANOVA analyzes of AOC indicates that exercise improves insulin sensitivity in mice fed LF diets.

3.7 Switching to a low fat diet reduced liver weight

The liver was excised and weighed during termination. To evaluate whether differences in liver mass were due to fat accumulation or accompanied with any pathological findings, the liver was examined by staining and microscopy of a representative part.

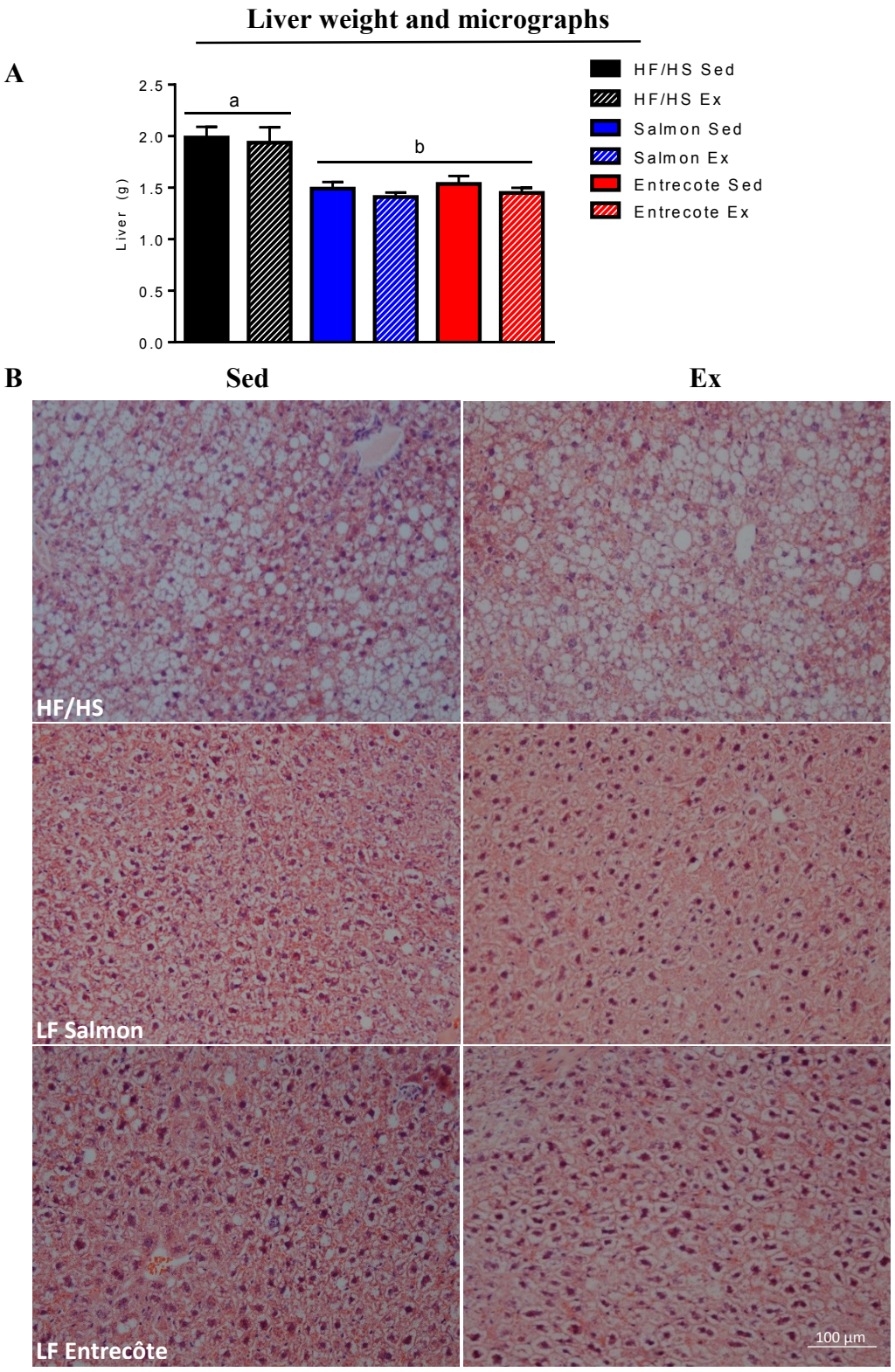

Figure 3.12: Liver weight **(A)** and liver micrographs **(B)** in the different groups. Group variations in liver mass was analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. (Micrographs are magnified x 20)

Figure 3.12 (A) demonstrate an association between the diet and liver weight. All of the mice fed LF diets had significantly lower liver mass than mice fed HF/HS diets. Exercise did not seem to affect the liver size in neither group. The micrographs (B) illustrate that an elevated liver mass in mice fed HF/HS diets is most likely attributable to a higher fat accumulation, as the fat droplets are more evident in these groups compared to the mice fed a LF diet. Consistent with Figure 3.12 A, the micrographs of mice fed HF/HS does not indicate that exercise in this group reduces fat accumulation in the liver. Whether protein sources or exercise influence fat accumulation in the mice fed LF diets, cannot be detected by merely evaluating the micrographs.

3.7.1 Expression of genes involved in fatty acid uptake, synthesis and oxidation

To investigate the effect of a LF diet, protein source and/or exercise on variations in hepatic lipid accumulation, the expression of genes involved in FA uptake, synthesis and oxidation were measured.

Figure 3.13 demonstrates that there were no differences in the expression of *Acc1* in neither group. The expression of *Fasn* was significantly lower in the mice fed LF diets and in the HF/HS Ex group compared to the HF/HS Sed group. However, the expression of *Scd1* was lower in the exercised mice fed LF salmon compared to both the Sed and Ex group fed LF entrecôte. Results from two-ways ANOVA further indicates that protein source alters the expression of *Scd1*, where the mice fed LF salmon had a lower relative expression than the mice fed LF entrecôte. Furthermore, expression of genes involved in FA uptake was lower in the LF groups compared to the mice fed HF/HS, with the exception of *Adrp.* Compared to sedentary mice fed HF/HS, the expression of *Adrp* was only significantly lower in the mice fed LF entrecôte. Analyzing the expression of *Cd36* with a two ways ANOVA in the LF groups demonstrated that protein source influenced the relative expression of *Cd36,* where it was lower in the mice fed entrecôte.

Expression of genes involved in lipid synthesis and fatty acid uptake

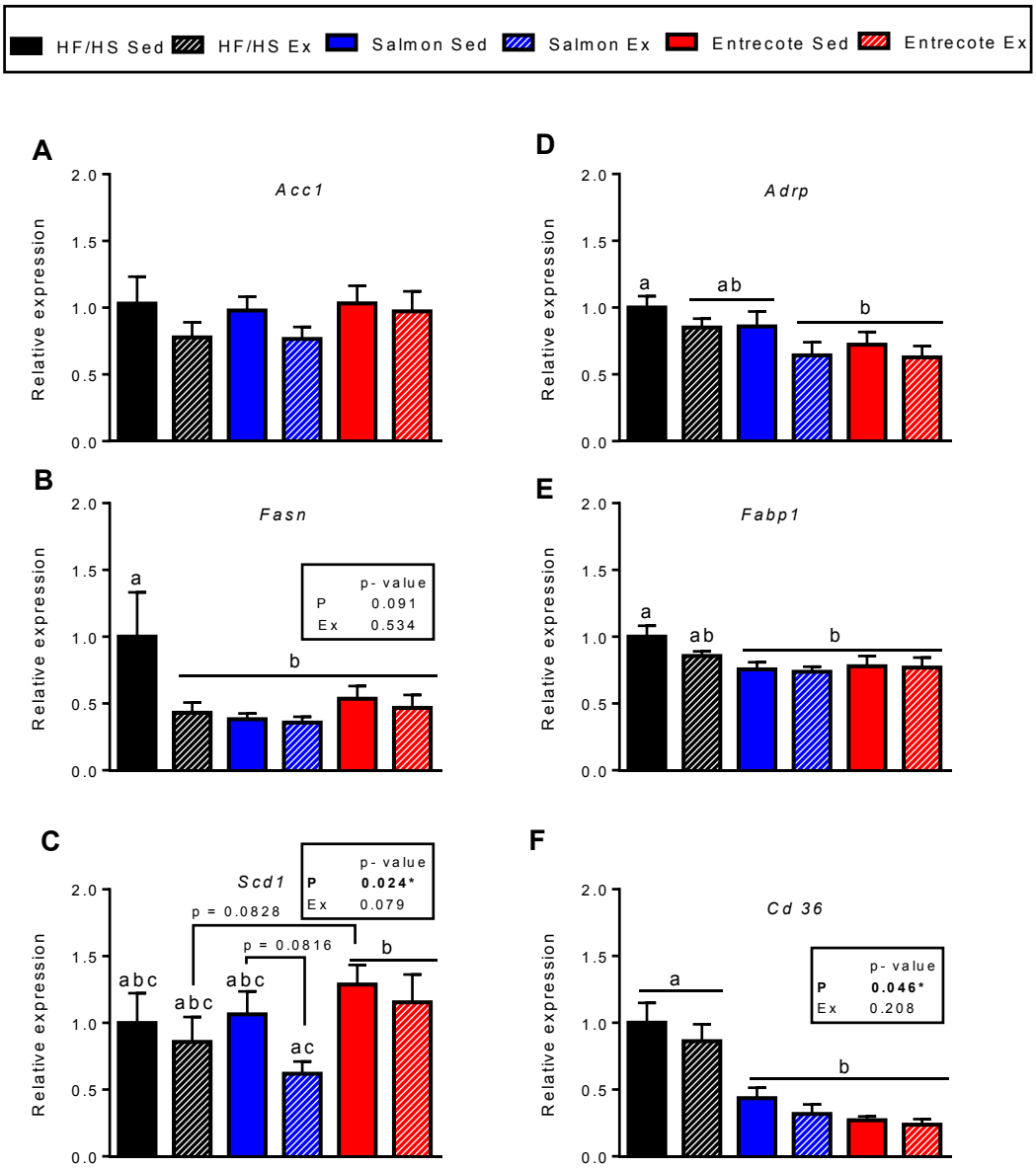

Figure 3.13: Relative expression of genes involved in lipid synthesis **(A-C)** and fatty acid uptake **(D-F)** in the liver. Differences in gene expression were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. To evaluate whether protein source or exercise had an impact on differences in relative gene expression, a two ways ANOVA was applied on the LF groups and statistical significance is marked *.

Expression of genes involved in fatty acid oxidation

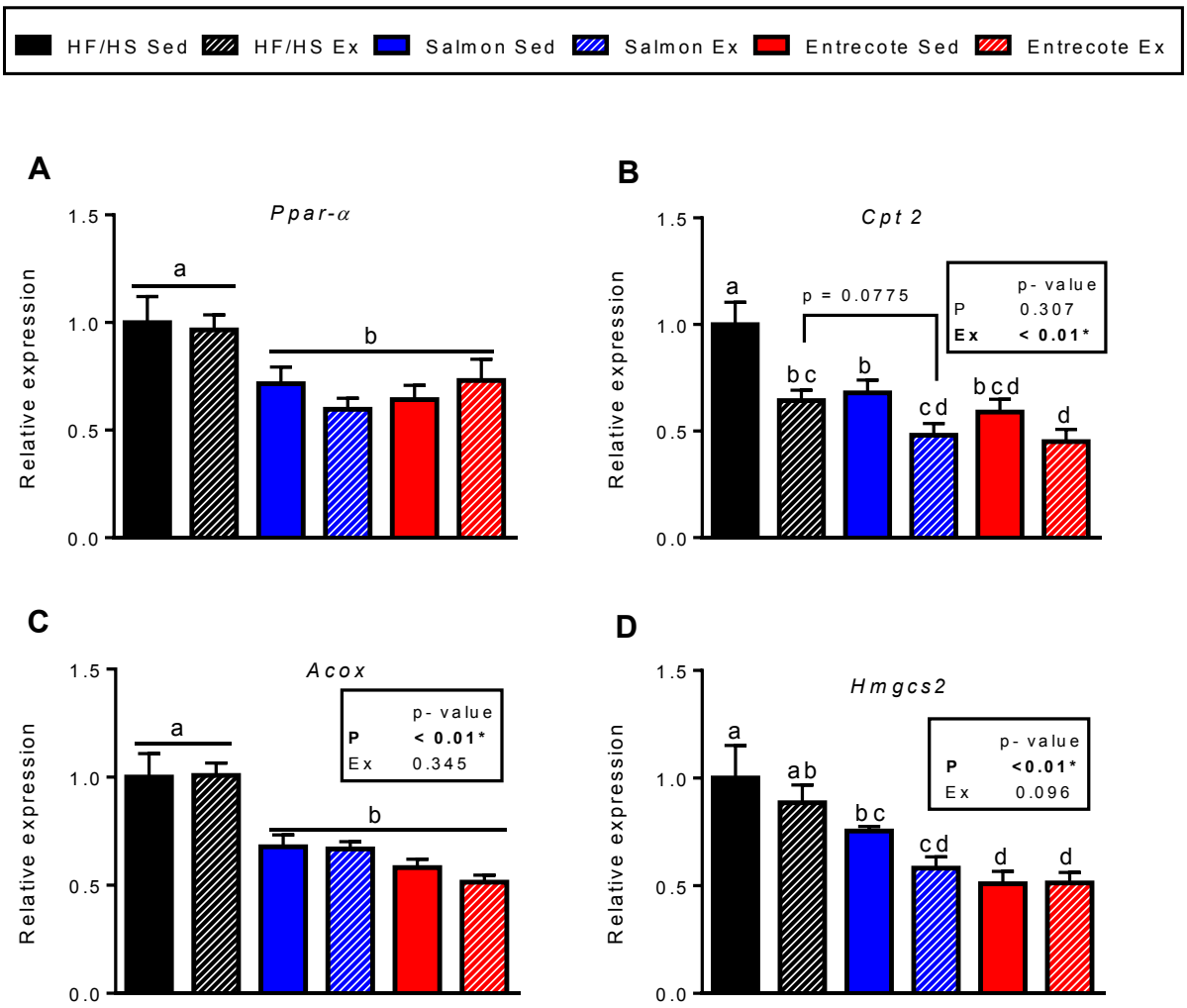

Figure 3.14: Relative expression of genes involved in fatty acid oxidation in the liver. Differences in gene expression were analyzed using a one-way ANOVA test with multiple comparison (Uncorrected Fisher's LCD) of the mean of each experimental group. Different letters denote statistical significance ($p < 0.05$) between the mean of the groups. The results are presented as mean ± SEM. To evaluate whether protein source or exercise had an impact on differences in relative gene expression, a two ways ANOVA was applied on the LF groups and statistical significance is highlighted and marked *.

The relative expression of *Ppar-α* was lower in the mice fed LF diets than the mice fed HF/HS diets, independent of exercise. Figure 3.14 B illustrates that both diet and exercise are of importance in relation to the expression of *Cpt2*. The mice fed LF diets had significantly lower expression of *Cpt2* than the sedentary mice fed HF/HS. In this group, exercise seemed to reduce the expression of *Cpt2*. Although results from one-way ANOVA demonstrated no difference between Sed and Ex group in the mice fed LF entrecôte, results from two-way ANOVA

indicates that exercise impact the expression of *Cpt2* in the mice fed LF diets. The relative expression of *Acox* was lower in mice fed LF diets compared to mice fed HF/HS diets. In addition, the relative expression of *Hmgcs2* was lower in the mice fed LF than in the mice fed HF/HS, with the exception of sedentary mice fed LF salmon. In this group, the expression of *Hmgcs2* was not reduced compared to the exercised group fed HF/HS diet. Nevertheless, there was no effect of exercise in neither group on the expression of *Acox* or *Hmgcs2*. Analyzing the results with a two-way ANOVA indicates that protein source influence the expression of *Acox* and *Hmgcs2,* where mice fed LF salmon had a higher expression than mice fed LF entrecôte.

Relative expression in the lipogenic regulator, *Srebf1* and hepatic genes involved in inflammation and fibrosis as well as endocannabinoid regulated genes and genes involved in endocannabinoid metabolism were measured and are presented in Figure A.3-A.6 (Appendix VI).

4.0 DISCUSSION

In this study, we demonstrate that a HF/HS diet based on casein, salmon and entrecôte as protein sources promote obesity and reduces insulin sensitivity severely compared to a diet with casein as the sole protein source. In agreement with an earlier study, these data suggest that various protein sources in a HF/HS diet have different effects on obesity development (Tastesen et al., 2014). Furthermore, we show that a LF diet, exercise and the combination of these treatments have beneficial effects in reversing diet-induced obesity (DIO), regardless of protein source. Although a LF diet alone had a clear effect on glucose tolerance and the expression of genes involved in hepatic fat accumulation, the combination of a LF diet and exercise seems to have pronounced effect on weight loss, changes in body composition and insulin sensitivity. Thus, our results is in agreement with the finding that exercise and LF diets have great significance in the prevention of obesity-related metabolic disturbances (Vieira et al., 2009). In addition, our results are supported by other studies, which indicate that a LF diet seems to attenuate BW gain in obese mice (Jung et al., 2013) and that exercise improves insulin sensitivity in humans (Perseghin et al., 1996).

4.1 Replacing casein with protein from salmon and entrecôte increase body weight and reduce glucose tolerance and insulin sensitivity

The results from feeding experiment 1 demonstrates that replacement of salmon and entrecôte with casein in a HF/HS diet promotes weight gain, impair glucose tolerance and reduce insulin sensitivity, despite a similar energy intake. These results are supported by a previous unpublished study from our research group, where casein- fed mice had lower energy efficiency than mice fed chicken, cod or pork. Additionally, results from this study demonstrate that mice fed casein had a lower weight gain and an improved glucose tolerance and insulin sensitivity compared to mice fed pork. The beneficial effect of casein remains unknown; however, one possible explanation could be a reduced fat absorption. Casein in a HF background diet has previously been reported to cause a lower fat absorption compared with a HF diet with salmon (Ibrahim et al., 2011). Moreover, in a HF/HS diet, fat excretion is significantly higher in mice fed casein compared to mice fed pork, chicken and cod/scallop (Tastesen et al., 2014). Our results are in accordance with the previous mentioned study, which indicates that fat absorption is higher in mice fed mixed proteins from meat, fish and casein compared to mice fed solely casein. Hence, the elevated fat absorption may further reflect a higher weight gain as demonstrated in our experiment. These findings indicate that casein may not be the most

suitable protein source to use if we want to induce obesity. However, another study comparing different fish proteins with casein showed that body weight gain was significantly lower and insulin sensitivity was improved in rats fed salmon proteins compared with rats fed casein (Pilon et al., 2011). Nevertheless, these findings were not confirmed in our experiment, and possible explanations may be that the amount of salmon protein (1/3) was lower in our diet, or that there appears to be differences in fat absorption between mice and rats. Thus, the contradicting findings related to weight gain and insulin sensitivity indicate that there might be other factors than fat absorption and feed efficiency, which can explain these differences.

4.2 Exercise and low fat diets with salmon or entrecôte reduces body weight and improves body composition in obese C57BL/6J mice

4.2.1 The effect of exercise and a low fat diet on body weight development

Although we did not see any significant difference in BW development between mice fed LF salmon and LF entrecôte during seven weeks of feeding, differences were evident between mice fed LF and HF/HS diets. These results are supported by Vieira et al., who demonstrated that after six weeks on a HF diet, both a LF diet and exercise seemed to attenuate BW gain, but a LF diet had a more pronounced effect (Vieira et al., 2009). These results are further in accordance with a study done with humans, which demonstrated significant weight loss by dieting, but not by exercise (Stefanick et al., 1998). Moreover, evaluating BW change, our results indicate that exercise is of greater importance in the mice fed LF than in the mice fed HF/HS. Nevertheless, it is reasonable to believe that this difference is a result of a significantly higher activity level and lower energy intake in the mice fed LF diets. Another possible additive factor could be the impact of an elevated amount of sucrose in the HF/HS diet, as increased amounts of sucrose dose-dependently increase energy efficiency and WAT mass (Hao et al., 2012). Nevertheless, whether the impact of dietary sucrose has the ability to suppress the effect of exercise is only speculation, and needs to be further investigated.

4.2.2 The effect of protein source on fat digestibility

BW change, energy intake and feed efficiency was not significantly different in mice fed salmon and mice fed entrecôte, and for this reason we did not expect to see great variations in apparent fat digestibility. Surprisingly, our results demonstrated that fecal excretion of fat was significantly higher in the mice fed entrecôte compared to the mice fed salmon. In spite of unexpected results, this finding is in accordance with an unpublished study from our research

group, which demonstrated that in a western diet, seafood- fed mice had a higher apparent fat absorption than mice fed meat. The reason for these differences in fat absorption remains unknown; however, it is tempting to believe that the composition of FAs in the diet (Table A.4, appendix II) may be of importance, as the LF salmon diet consists of more n-3 FAs than the LF entrecôte diet and the content of SFAs is higher in the LF entrecôte diet. Thus, this needs to be further investigated.

4.2.3 The effect of low a fat diet and exercise on fat mass, lean mass and WAT depots

Although mice fed LF entrecôte had a lower fat digestibility than mice fed LF salmon, this was not reflected in fat mass or fat depots. The effect of a LF diet on fat mass was still of great significance, independent of protein source or exercise. This finding is somewhat inconsistent with previous studies, which conclude that exercise have important benefits compared to diet alone (Tsai et al., 2003, Vieira et al., 2009). As there was no significant difference between Sed and Ex group fed HF/HS, and as the sedentary mice fed LF had a significantly reduced BW compared to the mice fed HF/HS Ex, we cannot support this statement. It is however reasonable to believe that the contradicting results may be due to differences in physical activity in our study, as mice fed HF/HS had a significantly lower physical activity than mice fed LF diets. In the previously mentioned studies, the various groups had the same level of physical activity, regardless of diet.

The impact of exercise does, however, seem to have important benefits compared to diet alone in the mice fed LF when evaluating the weight of eWAT. The only groups with lower eWAT mass than the mice fed HF/HS were mice assigned to exercise. The finding that exercise reduces eWAT weight is consistent with findings from Vieira et al. (2009). The observed effect of exercise was, however, not evident in the mice fed HF/HS in our experiment. Contrarily, in a study conducted by Linden et al., the impact of exercise in a very high fat (VHF) diet on eWAT weight was clearly demonstrated (Linden et al., 2014). This could indicate that the amount of dietary sucrose is of importance in regards to eWAT weight, as high amounts of sucrose in the diet is associated with a higher weight of WAT (Hao et al., 2012). Nevertheless, we should not ignore the fact that the mice fed HF/HS diet had a low activity level compared to the mice fed LF diet. The same trend was not observed when evaluating iWAT mass, suggesting that a LF diet alone or a combination of a LF diet and exercise seems to determine the weight of iWAT. Investigating the micrographs of iWAT indicate that both a LF diet and exercise has the ability to reduce the size of lipid droplets in iWAT.

The change in lean mass from week 0-5 was significantly increased in the mice fed LF compared to the mice fed HF/HS diet. This finding suggests that both LF diets alone and in combination with exercise are of great importance to maintain lean mass, independent of protein source. The effectivity of a combined treatment (LF diet and exercise) on preserving lean mass is supported by a study done in humans (Rice et al., 1999). We hypothesized that the increase in lean mass would be greater in the Ex group than the Sed group due to a possible higher muscle mass, at least in the mice fed LF. This hypothesis was rejected, as the weight of both *m. gastrocnemius* and *m. tibialis anterior* did not differ in either group. Nevertheless, as the BW was significantly lower in the mice fed LF compared to the mice fed HF/HS diets and, the *relative* muscle mass is higher in the mice fed LF, this suggests that the mice fed LF diets preserves muscle mass better than the mice fed HF/HS. However, our results indicate that the effect of exercise on lean mass was greater in the mice fed entrecôte than the mice fed salmon. The reason for this remains unknown, but the same trend was observed in BW change and fat mass change in this group. As expected, combining exercise with a LF diet was of greater significance on fat reduction and maintenance of lean mass than either treatment alone. These findings further support the Norwegian national guidelines, which suggests daily amounts of exercise and a intake of saturated fat below 10 E% (Helsedirektoratet, 2014).

4.2.4 The importance of a low fat diet and exercise on UCP1 expression in iBAT

Although fat absorption was significantly higher in mice fed LF salmon than in mice fed LF entrecôte, this was not reflected in BW nor WAT mass. Hence, we hypothesized that the absent increase in BW or WAT mass in mice fed LF salmon could be explained by an increased energy expenditure through the expression of UCP1 in iBAT. However, this hypothesis was rejected, as there was no statistical difference between neither iBAT weight nor expression of UCP1 in mice fed LF salmon and LF entrecôte. Moreover, micrographs of iBAT in any group demonstrated that most of the brown adipose organ consisted of unilocular cells, with a greater diameter in the mice fed HF/HS than the mice fed LF diets. This finding was not surprising, considering that BAT in obese mice is characterized by small, unilocular, UCP1-positive brown adipocytes (Cinti et al., 1997). The abnormal morphology of iBAT in obese mice is most likely attributable to a low sympathetic stimulation of iBAT (Himms-Hagen, 1989). Furthermore, the low sympathetic stimulation is possibly caused by a defect in the receptor-signaling pathway for leptin within the hypothalamus (Chen et al., 1996, Chua et al., 1996). Surprisingly, although the size of brown adipocytes were smaller with a LF diet and exercise, the morphology of BAT still mostly consisted of small, *unilocular*, brown adipocytes, more similar to that of WAT. We

expected that switching to a LF diet or being subjected to exercise would give iBAT back its phenotypic characteristics, namely the multilocular adipocytes. This was, however, not the case and our results suggest that the reverse effect of a LF diet, exercise or protein source on iBAT in obese mice fed a HF/HS diet is not of great significance. Whether the outcome would have been different if the mice had been given a LF diet or exercised over a longer period needs to be further investigated. Moreover, another interesting approach would be to evaluate micrographs from iWAT to quantify expression of UCP1, as it has been proposed that exercise induce browning of subcutaneous adipose tissue (Bostrom et al., 2012). It is hypothesized that the impact of exercise may be explained by metabolites derived from PGC1-α, including the hormone irisin and β-Aminoisobutyric Acid, which both has the ability to stimulate the expression of UCP1 and other thermogenic genes within WAT (Huh et al., 2012, Roberts et al., 2014).

Although not that obvious, our results suggest that a LF diet, exercise and the combination of these factors affect the size of the brown adipocytes and the expression of UCP1 in iBAT. The reduced mass of iBAT in the mice fed LF diet is most likely due to a decreased adipocyte size. A study conducted by De Matteis et al., demonstrated that exercise made the lipid droplets in brown adipocytes shrink, which is in accordance with our findings (De Matteis et al., 2013). Our results further indicate that exercise promote a higher expression of UCP1, predominantly in the mice fed LF salmon. The beneficial effects of exercise on brown adipocytes are poorly understood; however, it is reasonable to believe that the hormone irisin is responsible for some of the beneficial effects seen with exercise (Bostrom et al., 2012). Irisin is regulated by PGC1α, which is secreted from muscle to blood, and has the ability to activate thermogenic functions in adipose tissue, including elevation of UCP1. This hypothesis is further supported by the fact that rats subjected to exercise had an elevated expression of *Ppargc1a* and reduced expression of *Ppargc1b* (De Matteis et al., 2013). Moreover, the expression of MCT-1 was elevated in iBAT of rats assigned to exercise. MCT-1 has recently been detected in iBAT and functions as a transporter for lactate across the plasma membrane (Iwanaga et al., 2009). Lactate is used by BAT as a lipogenic substrate (Saggerson et al., 1988) and is considered the major gluconeogenic precursor in rats exposed to exercise (Brooks and Donovan, 1983, Donovan and Brooks, 1983). Hence, it is hypothesized that increased metabolic activity in iBAT induced by exercise may be related to lactate uptake, as these mice had a higher expression of MCT-1 in brown adipocytes (De Matteis et al., 2013). However, other explanations for elevated expression of MCT-1 is possible, so this needs further investigation.

4.3 The effect of a low fat diet and exercise on glucose homeostasis

4.3.1 The importance of low fat diet is evident in relation to glucose tolerance

Our results demonstrate a clear effect of LF diet in reducing fasting plasma glucose levels and exercise does not seem to improve blood glucose further. These results are supported by other similar experiments done in mice, demonstrating that a switch to LF diet reduced plasma glucose levels to near-euglycemia, and significantly improved glucose tolerance (Jung et al., 2013, Linden et al., 2014). Consistent with other studies (Muurling et al., 2002, Vieira et al., 2009), our results further suggest that a LF diet decrease the level of fasting insulin in plasma. Although the method for measuring insulin levels was not the same in the study by Jung et al (2013), similar observations were made in fasted state, which indicates that a LF diet is more effective in improving glucose tolerance than exercise. Furthermore, these data suggest that metabolic abnormalities induced by HF/HS feeding are reversible upon withdrawal of HF/HS diet. The pathogenesis of obesity- mediated insulin resistance has lately been linked to adipose tissue inflammation (Weisberg et al., 2003, Wellen and Hotamisligil, 2005), as circulating cytokines may negatively affect the insulin signaling pathway (Linden et al., 2014). However, another possible explanation may be due to inflammation in other tissues, including the liver, as weight loss has an obvious effect to attenuate hepatic inflammation (Jung et al., 2013). The reversible effect on hepatic inflammation was associated with lower basal glucose production and a significant improvement in hepatic insulin resistance. The elusive effect of exercise on glucose tolerance in our study may possibly be reflected in the latter study, as in this study, hepatic insulin action was greatly enhanced in the mice submitted to a LF diet but not in the mice submitted to exercise, they remained hepatic insulin resistant. Furthermore, diets high in saturated fat have been shown to directly induce WAT inflammation (Vieira et al., 2009), and this may explain the observed independent effect of LF diet on glucose tolerance.

4.3.2 The importance of diet, exercise and possible protein source on insulin sensitivity

Our findings from ITT indicates that insulin sensitivity is more affected by exercise than what glucose tolerance is. Area over the curve was calculated to see how the mice responded to insulin infusion during ITT. The decrease in blood glucose was higher in the mice submitted to exercise, regardless of diet, indicating a better response to insulin in these animals. However, a diet effect was also observed, whereas the mice fed LF responded better to insulin than the mice fed HF/HS. Our findings are consistent with the statement that a LF diet and/or exercise cause a dramatic improvement in whole body insulin sensitivity in obese mice (Jung et al., 2013). This is further supported in humans, as physical training increased insulin sensitivity in both

patients with impaired insulin sensitivity and normal subjects (Perseghin et al., 1996). The improved insulin sensitivity was explained by a stimulation of insulin mediated muscle glycogen synthesis as well as a reversal of a defect in insulin- stimulated glucose transport-phosphorylation. This explanation is consistent with the fact that one bout of exercise seemed to increase postprandial muscle glycogen synthesis more than threefold in insulin resistant subjects (Rabol et al., 2011). As exercise did not seem to affect changes in hepatic glycogen synthesis in these subjects, it is reasonable to believe that muscle insulin resistance precedes hepatic insulin resistance and that muscle insulin resistance causes postprandial increase in net hepatic triglyceride synthesis, which potentially leads to NAFLD. Thus, the advantageous effect of exercise on insulin sensitivity could be attributable to improved muscle glucose metabolism, as skeletal muscle in mice fed LF diet remained insulin resistant compared to the mice submitted to exercise (Jung et al., 2013). Furthermore, in our experiment, the reduction of WAT size seems to be influenced by exercise, and could possibly be explained by reduced inflammation in WAT (Vieira et al., 2009). In addition, other mechanisms in which exercise may reduce WAT inflammation could be increased blood flow, increased mitochondrial function and facilitated FA oxidation. It is plausible that an increased blood flow to WAT mediated by exercise would ameliorate inflammation by decreasing hypoxia, which is implicated to be a cause of adipose tissue inflammation in obesity (Ye, 2009). However, our results also demonstrate that a LF diet alone has an effect of improving insulin sensitivity, although the effect is greater combined with exercise. Improved insulin sensitivity following weight loss with a LF diet may be associated with reduced content of lipids in the liver (Jung et al., 2013). A possible explanation for this might be that ectopic lipid accumulation and increased levels of intracellular lipid derived metabolites induces hepatic insulin resistance (Kim et al., 2001). Our results from qPCR supports this statement, as for the most part, genes involved in fat accumulation were suppressed in the liver of mice receiving LF diets.

4.4 The expression of genes involved in fat accumulation and fatty acid oxidation

LF diets alone or combined with exercise resulted in a significantly lower liver weight, and micrographs indicates that the elevated liver weight in the mice fed HF/HS is due to hepatic lipid accumulation. This is further compatible with the fact that expression of genes involved in lipid synthesis and FA uptake were mostly lower in the mice fed LF diets, and the significance of exercise was not evident evaluating the micrographs. In our experiment, exercise was of minor importance for regulation of the expression of genes that was measured. One exception was, however, *Fasn*, where exercised mice fed HF/HS diet had a lower expression

than the sedentary mice fed HF/HS diet. However, in the mice fed LF diets, protein source seemed in some degree to determine the expression of certain genes. *Scd1* (involved in lipid synthesis) had reduced expression in the mice fed LF salmon than the mice fed LF entrecôte. The differential effect is most likely linked to the different n-3/n-6 PUFA ratio in salmon and entrecôte, as the ability to suppress expression of genes involved in lipogenesis is a hallmark of fish oil and n-3 PUFAs (Jump et al., 2005). Furthermore, it is reported that in the liver of mice fed a Western diet with salmon as protein source, levels of steorylethanolamide (SEA) were elevated (Midtbo et al., 2015), and administration of SEA is accompanied with down regulation of *Scd1* (Terrazzino et al., 2004). Thus, the lowered expression of *Scd1* may be attributable to elevated levels of SEA, though we did not measure this in our experiment. In addition, it is suggested that n-3 PUFAs reduce hepatic lipid accumulation through increased FA oxidation and suppressed TAG formation (Madsen et al., 1998, Rossmeisl et al., 2014). Our findings support this, as the expression of both *Acox* and *Hmgcs2* (involved in FA oxidation) was elevated in the mice fed salmon compared to the mice fed entrecôte. In light of the beneficial effects of n-3 PUFA on hepatic lipid accumulation, we expected to see the same pattern in the other genes we tested. Nevertheless, expression of *Cd 36* (involved in FA uptake) was lower in the mice fed entrecôte than the mice fed salmon, and in the remaining genes involved in fat accumulation and FA oxidation, there were no difference between the different protein sources. A possible explanation for the unexpected results may be the altered n-3/n-6 ratio of the salmon used in our experiment, which is illustrated in Figure A.1 (Appendix II). Recent research indicates that a reduced ratio of n-3/n-6 PUFA in the fish feed, reflected in the salmon and thus also in the mice subjected to this salmon in the diets, led to a diet- induced accumulation of lipids in the liver (Midtbo et al., 2015). Although the amount of n-3 PUFAs is higher in the LF salmon diet than LF entrecôte, it is possible that the level of n-3 PUFAs is not high enough to identify differences in the expression of certain genes. This is in accordance with the fact that mice exposed to a diet with salmon fed soy- oil and mice fed chicken had a higher expression of genes involved in FA uptake and synthesis compared with mice exposed to salmon fed fish-oil (Midtbo et al., 2015). Thus, an altered n-3/n-6 ratio seems to be of importance due to lipid accumulation in the liver and may possibly explain the expected differences we did not see between salmon and entrecôte.

4.5 Experimental considerations

Although the activity in brown adipocytes and the expression of their hallmark genes are highly influenced by a low temperature, our experiment was conducted under thermoneutral conditions (29.3 ± 0.4 °C). Some may argue that the effect of BAT will disappear when temperature is high; however, a lower room temperature causes chronic thermal stress to mice, which increase their metabolism to defend body temperature. Hence, in order to evaluate if there was an effect of exercise, diet and protein sources on the expression of UCP1, without influence from thermal stress, mice were kept at thermoneutrality. Furthermore, the mice in Ex- groups were assigned a running wheel, making exercise voluntarily. The drawback by voluntarily exercise is that we cannot control the level of physical activity, and there might be great individual differences. However, voluntarily exercise is more convenient by the fact that it is not forced and we have the ability to evaluate whether exercise has an effect on diet and vice versa. Moreover, in our experiment, we had wireless running wheels that detected the level of physical activity and we were thus able to reveal activity level in the different groups. In terms of testing, prior to ITT, we did not remove the running wheels from Ex groups. This makes it hard to distinguish whether the impact of exercise seen in our results is caused by an acute or a long-term effect of exercise. Nevertheless, there is no certainty that the result had been different if we did remove it.

4.5.1 The animal model and relevance to humans

In nutritional research, mice are the most commonly animal model used because of their genetic and physiological similarity to humans. Nevertheless, mice used for research are usually inbred strains with less genetic variation than humans. It is important that the characteristics of a specific strain is not considered general, but as a unique feature for the current strain. The animal model studied in our experiment (C57BL/6J) was used because of its ability to develop DIO, hyperinsulinemia and hyperglycemia (Black et al., 1998). Hence, this specific strain stands out as a representative model to elucidate the pathophysiology of an obesity syndrome similar to that in humans. The Norwegian health authorities recommends increased physical activity, as well as specific amounts of carbohydrates in which added sugar should not exceed 10 E % and fat should not exceed 40 E %. In the present study, we have demonstrated that increased physical activity and reduced amount of fat and added sugar in the diet (8 E %) is beneficial regarding weight loss, supporting the recommendations from National guidelines. The recommendations do however not specify anything about different type of proteins. Our

results supports this, as the protein source in a LF diet did in general not seem to give different effects on DIO. Although the results from our study are in accordance with national guidelines, the same type of study needs to be carried out in humans before we can conclude that they have human relevance.

4.6 Future perspectives

The impact of diet and/or exercise has gained much attention lately as a method for weight regulation. Controversial, some individuals claim that they do not lose weight despite increased physical activity, while others seem to have great benefits from it. The present study demonstrated that in mice fed HF/HS, treatment of DIO was not affected by exercise. In future studies, it would be interesting to investigate if the absent effect of exercise could be attributable to the amount of sucrose in the diet, and if the impact of exercise is modified if we replace sucrose with other carbohydrate sources. Although our results illustrated that the mice fed HF/HS diet had a lower level of physical activity, the basis for this hypothesis is that in mice fed a VHF diet, exercise seemed to be of great importance considering BW development, eWAT weight and fasting plasma insulin levels (Linden et al., 2014). Furthermore, a possible next step for this study could be to evaluate the importance of time perspective in a diet and/or exercise intervention.

In future studies it could also be of interest to reveal explanation for the differences we assessed in fat digestibility between mice fed salmon and entrecôte. Analyses of FA composition in feces would be of interest, to evaluate if some FAs are more easily excreted than others are. It would further be intriguing to explore the effects of gut bacteria on nutrient absorption and excretion in diets with different protein sources. Moreover, as salmon and entrecôte seemed to give various effect on hepatic gene expression, it would have been interesting to see if this reflected the protein level, by applying a method such as western blot.

Another further approach would be to investigate the effect of exercise on expression of UCP1 in iWAT, as the expression was not clearly pronounced in iBAT and a recently published study suggest that exercise may induce beige adipocytes in WAT, possibly attributed to the hormone irisin. Therefore, it would be of interest to measure levels of irisin in plasma.

Lastly, it would be of particular importance to explore if the findings in this animal experiment could be mimicked in humans.

5.0 CONCLUSION

The present study present following findings:

- Mixture of proteins from casein, salmon and entrecôte in a high fat – high sucrose diet promotes obesity and reduces insulin sensitivity significantly compared to a 100% casein based high fat – high sucrose diet.
- Combination of exercise and low fat diet with either salmon or entrecôte have beneficial effects on weight loss and changes in body composition.
- A low fat diet improves glucose tolerance alone and insulin sensitivity is further improved by exercise. The importance of protein source in a low fat diet in relation to glucose homeostasis is not significant.
- The effect of a low fat diet is clear regarding the expression of genes involved in hepatic fat accumulation, where mice fed low fat diet with salmon seemed to have some benefit over mice fed low fat with entrecôte evaluating the expression of genes involved in fatty acid oxidation. The impact of exercise was however not evident in most genes we measured.

Although the results from this animal experiment cannot be directly linked to human nutrition, our findings suggest that a combination of a low fat diet and exercise is of great significance in reversing diet induced obesity and impaired insulin sensitivity, and that overall, salmon and entrecôte exerts the same beneficial effects in a low fat diet. Our results is in accordance with national guidelines; however, further research will be needed to explore these findings in human nutrition.

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APPENDIX

APPENDIX I – Diets

Table A.1: *Diet composition in LF acclimatization diet (ssniff EF R/M Control)*

Crude nutrients	**%**
Dry matter	95.2
Crude protein	20.8
Crude fat	4.2
Crude fibre	5
Crude Ash	5.6
N free extracts	59.4
Starch	46.8
Sugar	10.8
Energy (kJ/g)	18

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

Group	**HF/HS**	**Low fat**	**Low fat**
(g/kg)	Protein	Protein	Protein
Ingredients	**Salmon + entrecôte**	**Salmon**	**Entrecôte**
Entrecôte	111.1	-	145.9
Salmon	138.6	178.2	-
Casein	70.0	103.6	103.6
L-cystein	3	3	3
Starch(normal)	9.5	-	-
Sucrose (melis)	410.5	91.8	91.8
Cellulose	50	50	50
Dextrin	10	524	524
Corn oil	150	2.10	36.7
t-butyl	0.02	0.02	0.02
Mineral mix	35	35	35
Vitamin mix	10	10	10
Cholin	2.5	2.5	2.5
Sum	1000	1000	1003
	Analyzed		
(g/100 g)			
Fat	24.9	8.4	8.4
Protein	21.8	17.3	17.3
Energy (kJ/g)	23.1	18.4	18.3

Table A.3*: Analyzed nutrients in the protein source*

(g/100g)	**Salmon**	**Entrecôte**
Crude fat	43.24	30.29
Crude protein	50.63	63.31

Low fat diets was balanced in fat amount by adding corn oil.

Appendix II – Fatty acid analyses

Table A.4: *Analyzed fatty acid (FA) composition in the experimental LF - diets.*

FAs in diet (mg/g)	Protein **Salmon**	Protein **Entrecôte**
Sum SFA	11.15	28.48
Sum MUFA	42.46	26.09
Sum PUFA	24.73	21.03
LA: 18:2n-6	12.45	19.84
AA: 20:4n-6	0.46	0.13
Sum n-6	14.21	20.05
ALA: 18:3n-3	3.89	0.45
EPA: 20:5n-3	1.68	0.02
DHA: 22:6n-3	2.64	0.00
Sum n-3	10.40	0.77
n-3/n-6 ratio	0.73	0.04
AA/EPA ratio	0.27	6.03
Sum identified	78.34	75.60
Sum unidentified	0.73	1.46
Sum FA.	79.07	77.05
(%)		
n-3	13.14	0.99
n-6	17.97	26.61

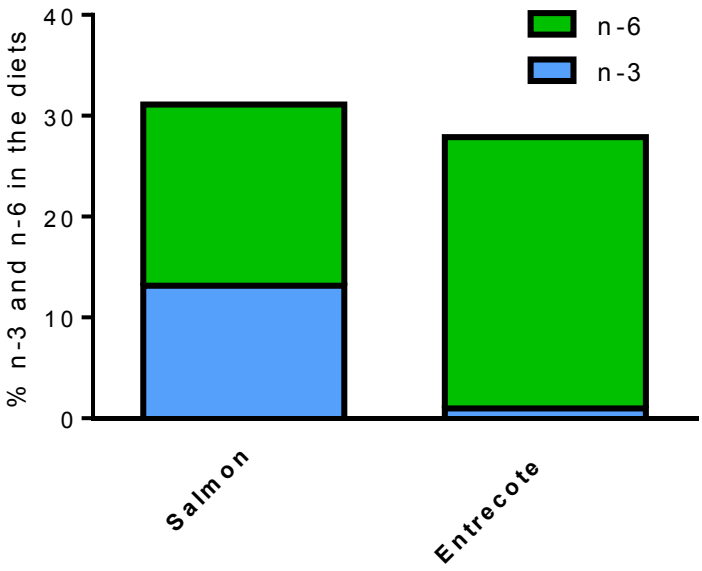

Figure A.1: Percentage distribution of n-6 and n-3 FAs in the LF- diet.

Table A.5 *Fatty acid composition in neutral lipids (NL) in LF- diets.*

Fatty acids in NL (mg/g)	Protein **Salmon**	Protein **Entrecôte**
Sum SFA	11.31	23.37
Sum MUFA	44.29	22.39
Sum PUFA	24.73	19.72
LA: 18:2n-6	12.93	18.92
AA: 20:4n-6	0.45	0.01
Sum n-6	14.73	18.96
ALA: 18:3n-3	4.02	0.38
EPA: 20:5n-3	1.57	0.00
DHA: 22:6n-3	2.12	0.00
Sum n-3	9.86	0.58
n-3/n-6 ratio	0.67	0.03
AA/EPA ratio	0.28	3.61
Sum identified	80.33	65.48
Sum unidentified	1.29	1.09
Sum FA.	81.63	66.58
(%)		
n-3	12,08	0.87
n-6	18,05	28.52

Table A.6: *Fatty acid composition in phospholipids (PL) in LF diets.*

Fatty acids in PL (mg/g)	Protein **Salmon**	Protein **Entrecôte**
Sum SFA	0.57	0.92
Sum MUFA	0.97	0.80
Sum PUFA	1.53	0.88
LA: 18:2n-6	0.30	0.69
AA: 20:4n-6	0.04	0.08
Sum n-6	0.39	0.80
ALA: 18:3n-3	0.12	0.03
EPA: 20:5n-3	0.21	0.01
DHA: 22:6n-3	0.67	0.00
Sum n-3	1.13	0.08
n-6/n-3 ratio	2.90	0.11
AA/EPA ratio	0.18	6.00
Sum identified	3.06	2.61
Sum unidentified	0.04	0.20
Sum FA.	3.11	2.81
(%)		
n-3	36.54	3.09
n-6	12.62	28.98

Appendix III – Amino acid analyses

Table A.7: *Amino acid composition in LF diets*

Amino acids (mg/g)	Protein **LF Salmon**	Protein **LF Entrecôte**
Histidine	4.86	5.09
Serine	8.26	7.94
Arginine	8.14	7.87
Glycine	5.70	5.09
Aspargine	16.15	13.99
Glutamine	33.76	33.49
Threonine	8.00	7.26
Alanine	7.95	6.87
Proline	13.15	13.31
Lysine	15.51	13.64
Tyrosine	6.92	6.83
Methionine	6.12	5.92
Valine	10.97	9.84
Isoleucine	8.52	7.87
Leucine	15.52	14.64
Phenylalanine	8.78	8.43
Sum essential AA	73.41	67.59
Sum non-essential Aas	104.89	100.47
Sum BCAAs	35.01	32.34
Total	178.30	168.07
Hydroxyprolin*	0.14	0.37
Taurine*	0.14	0.00

Appendix IV Histology

Table A.8 *Reagents and time schedule for each step in the dehydration process.*

Reagent	Time (min)	Vendor
50% EtOH	45	Arcus Kjemi. Norway
75% EtOH	45	Arcus Kjemi. Norway
95% EtOH	45 x 2	Arcus Kjemi. Norway
100% EtOH	45 x 3	Arcus Kjemi. Norway
Xylene	45 x 2	Prolabo
Paraffin	Overnight	Histovax. OneMed
Paraffin	15 x 2	Histovax. OneMed

Table A.9: *Reagents and time schedule for each step in the rehydration, staining and dehydration process.*

Reagent	Time (min)	Vendor
Xylene	2 x 10	Prolabo
100% EtOH	2 x 10	Arcus Kjemi. Norway
95% EtOH	2 x 5	Arcus Kjemi. Norway
75% EtOH	5	Arcus Kjemi. Norway
50% EtOH	5	Arcus Kjemi. Norway
ddH2O	5	MilliQ Biocel. USA
Hematoxylin	2	EMS
H2O	Wash	
Eosin	30 sek	Sigma. USA
H2O	Wash	
ddH2O	1	MilliQ Biocel. USA
50% EtOH	2	Arcus Kjemi. Norway
75% EtOH	2	Arcus Kjemi. Norway
95% EtOH	2 x 2	Arcus Kjemi. Norway
100% EtOH	2 x 5	Arcus Kjemi. Norway
Xylene	2 x 5	Prolabo

APPENDIX V – Immunohistochemistry

Table A.10: *Time and reagents used in the rehydration process*

Reagent	Time
Xylene	15 min
100% EtOH	2 x 5 min
95% EtOH	5 min
75% EtOH	5 min
50% EtOH	5 min
ddH_2O	2 x 5 min

Table A.11: *Time schedule and reagents used in the dehydration process.*

Reagent	Time
50% EtOH	10 min
75% EtOH	10 min
95% EtOH	10 min
100% EtOH	10 sec

Table A.12: *Solutions and reagents used in the immunohistochemistry process*

Solution	Contents
PBS buffer	1000 ml ddH_2O + 5 tablets Phosphate Buffered Saline (SIGMA P4417)
Citrate buffer	Tri – Sodium citrate dehydrate + ddH_2O
3% H_2O_2 in MetOH	3% H_2O_2 + MetOH (SIGMA, Lot #SZBD142SV)
PBS + 0,1% Tween	PBS + Technical Tween 20 (Product 28829.296, VWR)
Serum	Vectastain, Goat, normal serum (VECTOR laboratories)
Primary antibody	Anti UCP1 (C4/98), recieved from Prof Jan Kopecky and Dr. Pavel Flachs
Secondary antibody	PBS buffer + Vectastain, Anti-rabbit IgG, antibody (VECTOR laboratories)
ABC	PBS buffer + 2 drops vectastain ABC reagent A + 2 drops vectastain ABC reagent B + H_2O_2
DAB	5 ml ddH_20 + 2 drops buffer stock + 4 drops DAB stock solution + 2 drops H_2O_2 solution

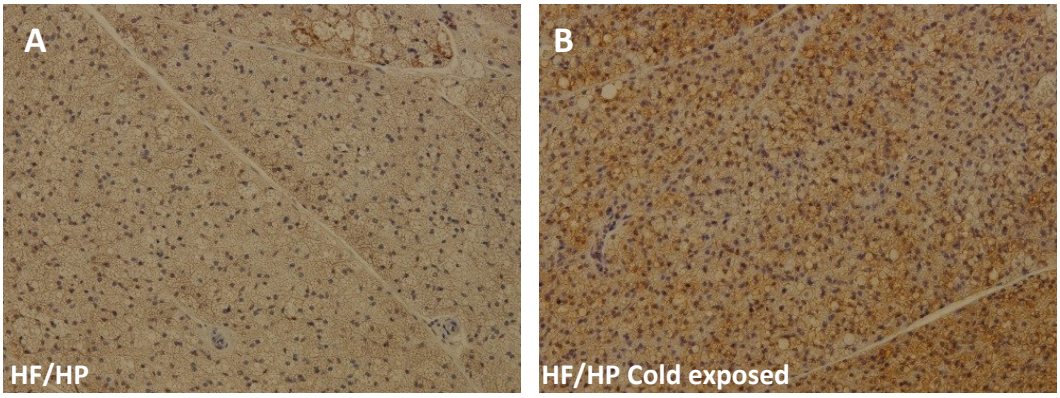

Figure A.2: Immunohistological expression of UCP1 in mice fed HF/HP diet **(A)** and mice fed HF/HP exposed to cold **(B)**.

APPENDIX VI – Real time qPCR

Table A.13: *RT reaction mix for a 50 µl RT reaction*

	Reagents	50 µl	Final concentration	Vendor
Non enzymatic reagents	H2O	890		Applied biosystems
	10x TaqMan RT buffer	500	1 X	Applied biosystems
	25 mM $MgCl_2$	1100	5.5 mM	Applied biosystems
	10 mM deoxyNTPs Mixture (2,5 mM of each dNTP)	1000	500 µM per dNTP	Applied biosystems
	50 µM Random hexamers	250	2.5 µM	Applied biosystems
Enzymes	Rnase Inhibitor (20 U/µl)	100	0.4 U/µl	Applied biosystems
	Multiscribe Reverse Transcriptase (50 U/µl)	167	1.67 U/µl	Applied biosystems

Table A.14: *Instrument setup for Reverse Transcription*

Step	Incubation	RT	Reverse Transcription Inactivation	End
	HOLD	HOLD	HOLD	HOLD
Temperature (°C)	25	48	95	4
Time (minutes)	10	60	5	∞*
Volume (µl)	50			

Table A.15 *Primers used for qPCR*

Oligo name	Sequence (5' to 3')
Aars Forward	GGAGGAACACATCTACGGAA
Aars reverse	CTGTGACAGCAACAATCCTC
Acc1 Forward	TGCTGCCCCATCCCCGGG
Acc1 Reverse	TCGAACTCTCACTGACACG
Acox Forward	GGGTCATGGAACTCATCTTCGA
Acox reverse	GAATGAACTCTTGGGTCTTGGG
Adrp Forward	ACAAGTGCCCTGCCCATC
Adrp Reverse	GGCAACCGCAATTTGTGG
Asns Forward	CCAACCGGTCTTGTCACT
Asns Reverse	CAGGCACTCTGAGCACTA
Cd 36 Forward	AATTAGAACCGGGCCACGTA
Cd 36 Reverse	CGCCAACTCCCAGGTACAA
Cnr2 Forward	ACGGTGGCTTGGAGTTCAAC
Cnr2 Reverse	ACGGTGGCTTGGAGTTCAAC
Cola1 Forward	GCTCCTCTTAGGGGCCACT
Cola1 Reverse	CCACGTCTCACCATTGGGG
Cpt2 Forward	AGCCTGCCCAGGCTGCC
Cpt2 Reverse	AAACCAGGGGCCCTGAGATG
Faah Forward	CCTGGAGACCATGGACAA
Faah reverse	GTCAGGATTCTGCAGCCG
Fabp1 Forward	GGAATTGGGAGTAGGAAGAGCC
Fabp1 Reverse	TGGACTTGAACCAAGGAGTCAT
Fasn Forward	CTTCGCCAACTCTACCATGG
Fasn Reverse	TTCCACACCCATGAGCGAGT
Hmgcs2 Forward	AGCCCAGCAGAGGTTTTCTACAA
Hmgcs2 Reverse	CATACGGGTCTGGCCCAAG
Insig1 Forward	CTGTATTGCCGTGTTCGTTG
Insig1 Reverse	CTTCGGGAACGATCAAATGT
Lpin1 Forward	AAGAGACTGACAACGATCAGGA
Lpin1 Reverse	AAGAGACTGACAACGATCAGGA
Lpin2 Forward	TCCACCAAGGTCACCAAAAG
Lpin2 Reverse	GGGCTCTAGGTTTGGGTTTC
Mgll Forward	CAGACGGACAGTACCTCTTT
Mgll Reverse	ACAAAGATGAGGGCCTTGG
Mmp-2 Forward	TTTGCTCGGGCCTTAAAAGTAT
Mmp-2 Reverse	CCATCAAATGGGTATCCATCTC
Mmp-9 Forward	GGACCCGAAGCGGACATT
Mmp-9 Reverse	CCTTGCCGTCGAAGGGATA
Ppar-α Forward	CGTTTGTGGCTGGTCAAGTT
Ppar-α Reverse	AGAGAGGACAGATGGGGCTC
Psat1 Forward	GTGCTCGAAATGAGTCACAG
Psat1 Reverse	AGCTAGCAATTCCCTCACAA
Scd1 Forward	GATGTTCCAGAGGAGGTACTACAAGC
Scd 1 Reverse	ATGAAGCACATCAGCAGGAGG
Srebf1 Forward	GGAGCCATGGATTGCACATT
Srebf1 Reverse	GCTTCCAGAGAGGAGCCCAG
Tgfb1 forward	AGCAGTGCCCGAACCCCCAT
Tgfb1 reverse	GGGGTCAGCAGCCGGTTACC
Tnf-a forward	CCCTCACACTCAGATCATCTT
Tnf-a reverse	GCTACGACGTGGGCTACAG

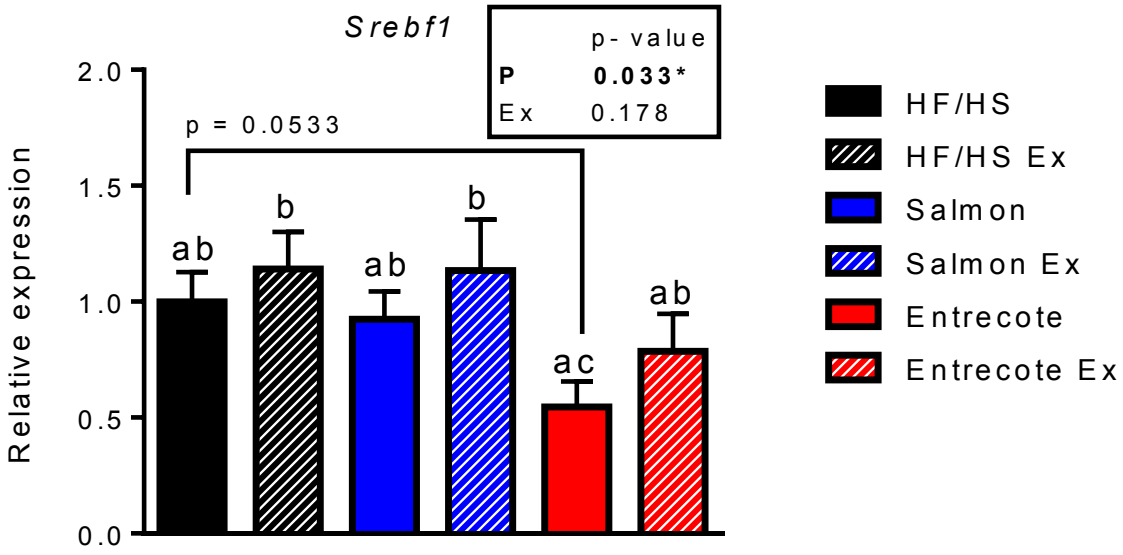

Figure A.3: Relative expression of the lipogenic regulator Srebf1in the liver.

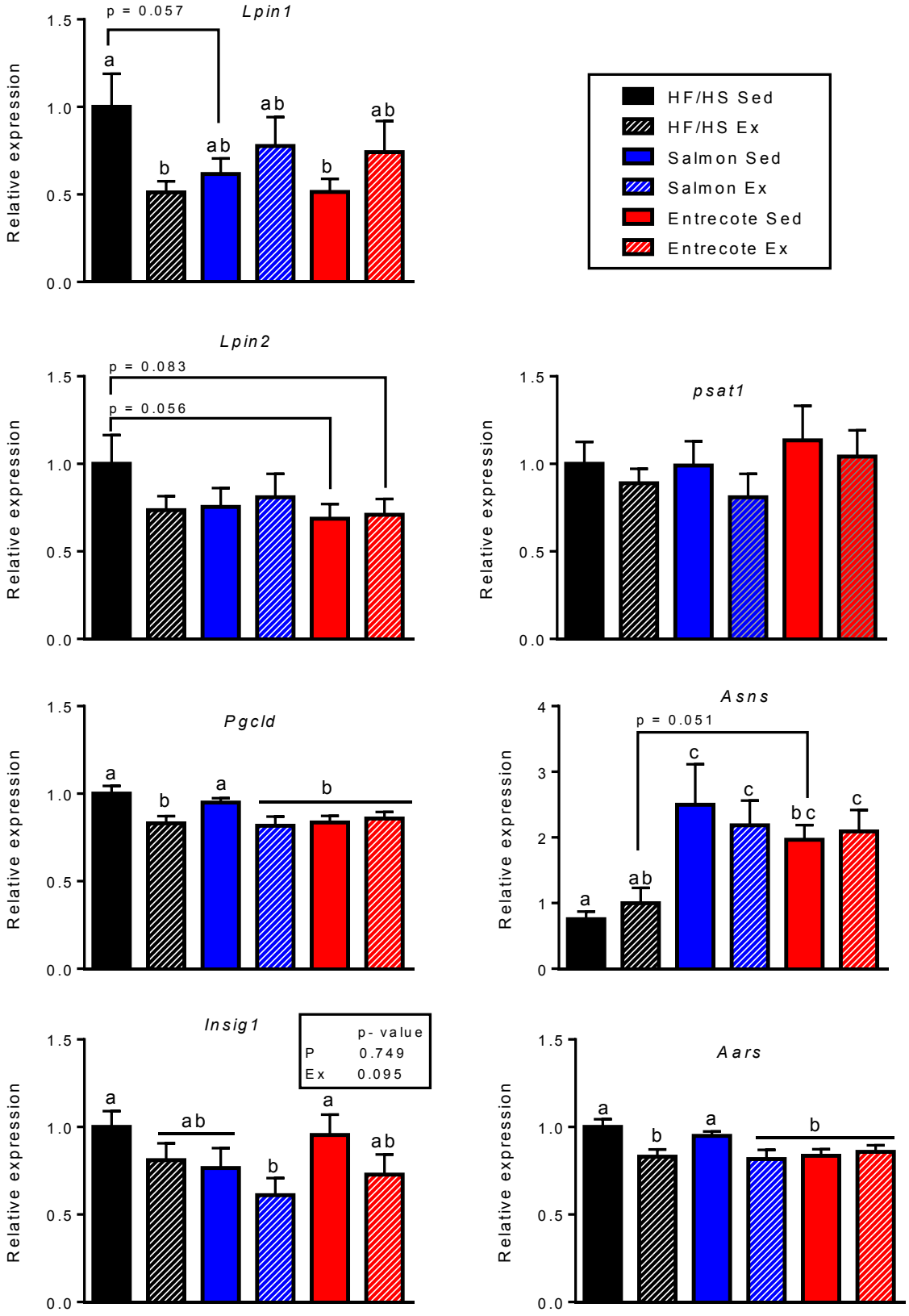

Figure A.4: Relative expression of endocannabinoid regulated genes in the liver.

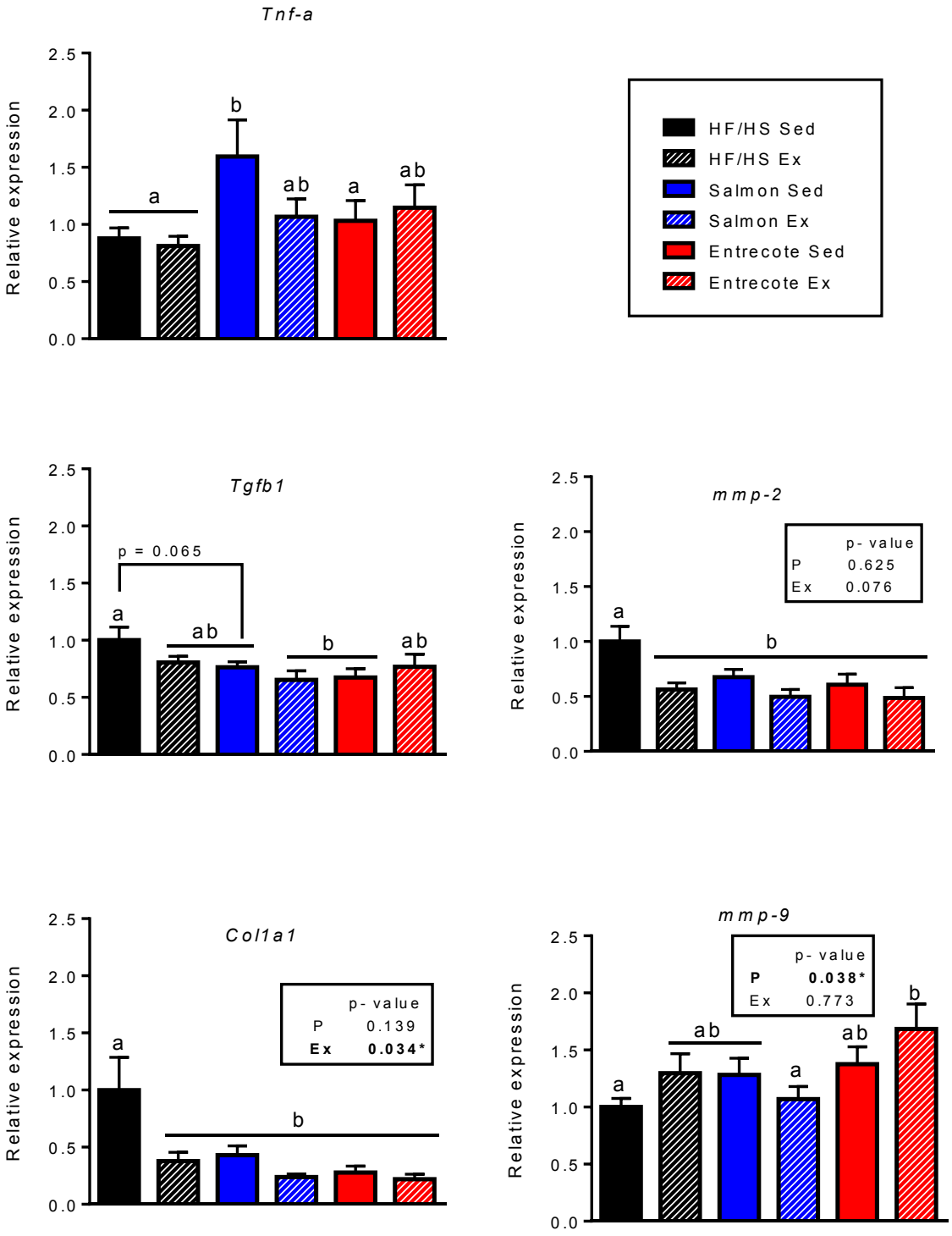

Figure A.5: Relative expression of genes involved in hepatic inflammation, fibrosis and protease inhibitors.

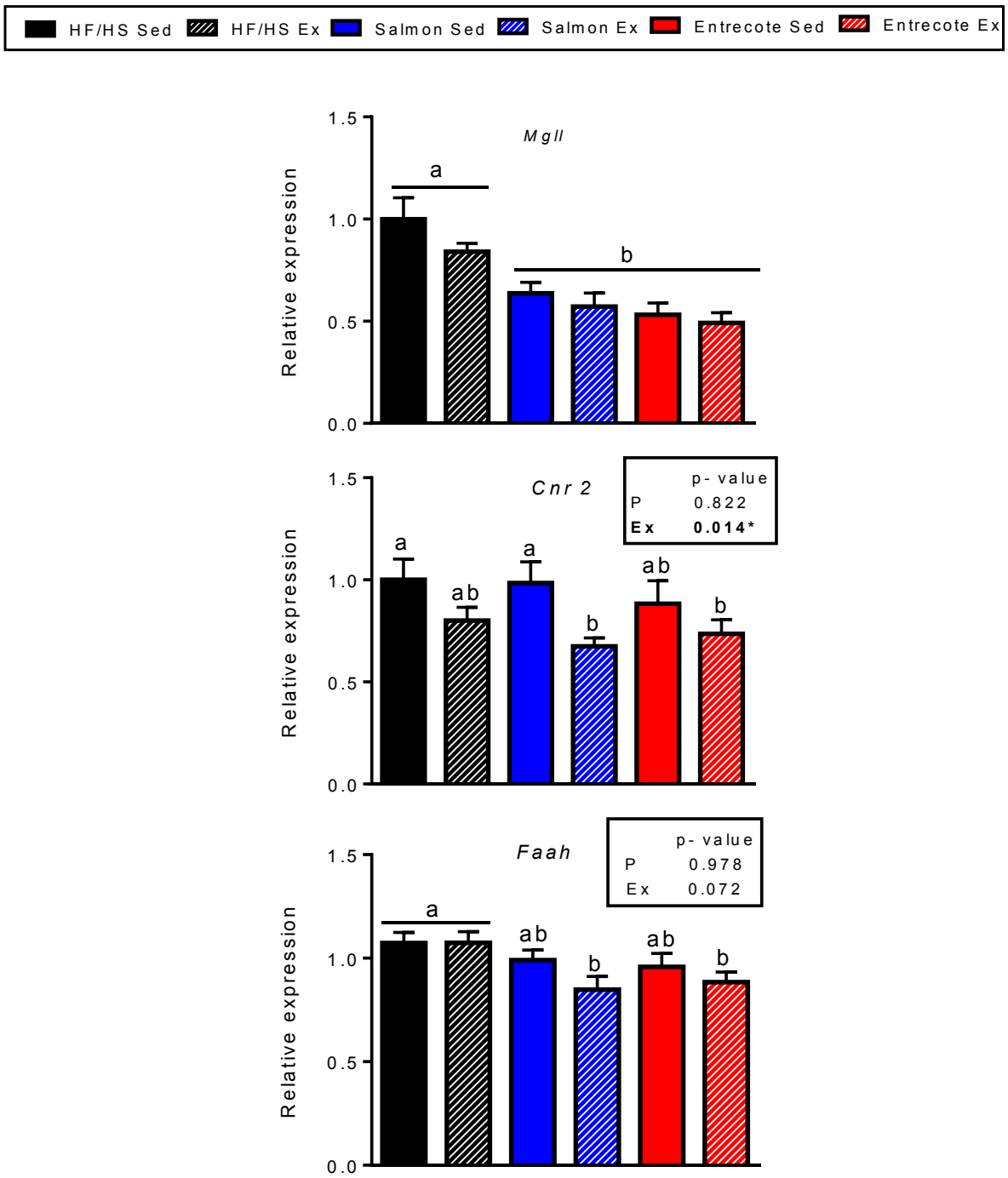

Figure A.6: Relative expression of genes involved in hepatic endocannabinoid metabolism.

Appendix VII – Muscle mass

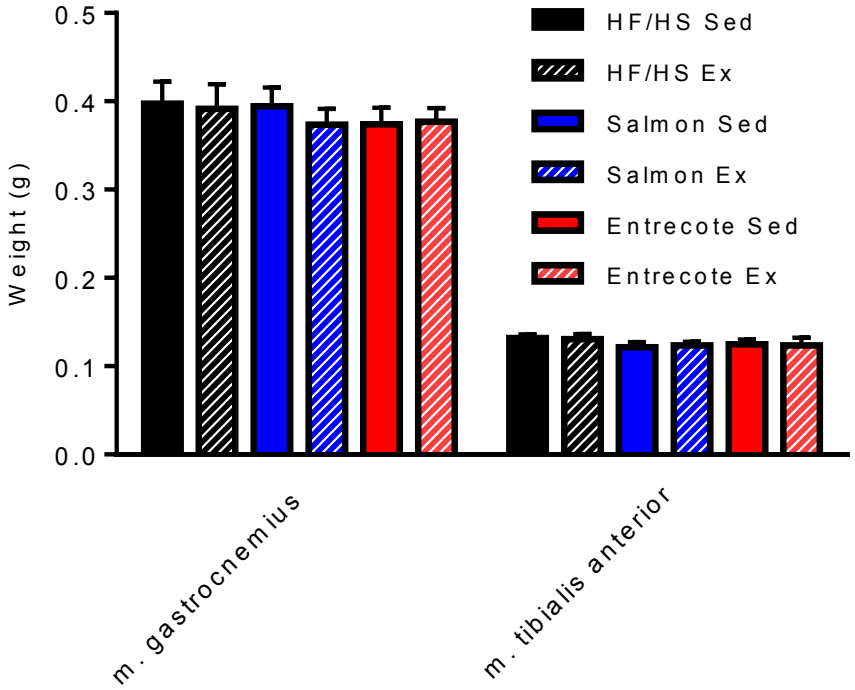

Figure A.7: Weight of *m. gastrocnemius* and *m. tibialis anterior* in the different groups.

Appendix VIII ELISA Insulin Kit

Table A.16: *Reagents and equipment used in ELISA Insulin Kit*

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