PhD thesis
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Effect of various protein sources on body weight development

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Preface

This Phd thesis was funded by “The Danish Council for Strategic Research” (grant no. 10-093539) and the Danish Dairy Research Foundation. The work presented in this thesis was carried during the years 2011-2014 at the National Institute of Nutrition and Seafood Research, Norway and at the University of Copenhagen, Denmark, under the supervision of Professor Karsten Kristiansen, associate professor Lise Madsen, and Dr. Bjørn Liaset.
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Abstract

**Background:** Due to the increasing prevalence of obesity, finding effective dietary strategies for weight loss and weight maintenance is of great interest. High protein diets are reported to protect against diet-induced obesity, however less is known about how different protein sources affect body weight regulation. We aimed to investigate how various protein sources influenced body weight development and glucose metabolism by feeding obesity prone male C57/BL6 mice various protein sources in different background diets.

**Results:** In high fat/high sucrose diets (HF/HS), high fat/high protein diets (HF/HP), and Western diets, consumption of lean meat promoted obesity compared to lean seafood and casein. Consumption of lean meat stimulated accretion of fat mass independent of energy intake when used as the protein source in (HF/HS) diets and most likely due to decreased energy intake when used as the protein source in (HF/HP) diets and Western diets. Consumption of lean seafood increased spontaneous locomotor activity when provided as the protein source in the Western diet and showed a tendency to increase spontaneous locomotor activity when consumed in a (HF/HS) diet compared to lean meat. In comparison to lean seafood, the consumption of lean meat resulted in decreased glucose tolerance when used in both HF/HS- and HF/HP diets. The consumption of lean meat also decreased glucose tolerance when used as the protein source in a Western background. The decreased glucose tolerance associated with the consumption of lean meat in Western background diets was only evident with free access to the diets, most likely due to differences in body composition.

We purpose that the beneficial effects of lean seafood consumption in relation to body weight regulation may be due to an enrichment of the amino acids taurine and glycine.

**Conclusion:** In summary, our results show that consumption of lean seafood is less obesogenic than lean meat. The benefits of lean seafood consumption were associated with increased spontaneous locomotor activity and possible increased satiety.
Sammendrag

Bakgrunn: På grunn av den økende forekomsten av overvekt og fedme, har det blitt viktigere enn noen gang å finne effektive diettstrategier, bade for å redusere vekt og for å forhindre vektøkning. Det har blitt rapportert at høy protein dietter har evnen til å motvirke fedme, men mindre er kjent angående hvordan ulike proteinkilder påvirker kroppsvektregulering. Vi ønsket å undersøke hvordan ulike proteinkilder påvirker kroppsvektutviklingen og glukose metabolisme ved å føre overvekt disponerte C57/BL6 hann mus forskjellige proteinkilder i forskjellige bakgrunnsditter.


Sammenlignet med sjømat resulterte inntak av kjøtt i redusert glukosetolerance i bade HF/HS og HF/HP dietter. Inntak av kjøtt resulterte også i redusert glukosetoleranse i en Vestlig bakgrunnsdiett, men kun ved fri tilgang til diettene, dette skyldes mest sannsynlig forskjeller i kroppssammensetning. Vi foreslår at de observerte forskjellene mellom inntak av mager sjømat og magert kjøtt har en sammenheng med at sjømat er beriket med aminosyrene taurin og glycin.

Konklusjon: Oppsummert viser våre resultater at mager sjømat er mindre fedmefremmende enn magert kjøtt. Fordelene ved inntak av mager sjømat var assosiert med økt frivillig aktivitet og muligens økt metthet.
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## List of abbreviations

- **iWAT**: Inguinal white adipose tissue
- **AUC**: Area under the curve
- **BCCA**: Branched chained amino acid
- **BMI**: Body mass index
- **DAUC**: Decremental area under the curve
- **eWAT**: Epididymal white adipose tissue
- **GI**: Glycemic index
- **GTT**: Glucose tolerance test
- **HF/HP**: High fat and high protein diet
- **HF/HS**: High fat and high sucrose diet
- **ITT**: Insulin tolerance test
- **MRT**: Meal response test
- **MUFA**: Monounsaturated fatty acid
- **RMR**: Resting metabolic rate
- **SFA**: Saturated fatty acid
1. Introduction

1.1 Prevalence of obesity

People are increasingly becoming overweight (body mass index [BMI] > 25 kg/m²) and obese (BMI > 30 kg/m²), and since 1980, the incidence of obesity has nearly doubled globally [1]. In 2008 more than 1.4 billion adults were classified as being overweight and 500 million of them were classified as obese [1]. Especially alarming is the rise in childhood obesity. An estimated 170 million children under the age of 18 are classified as overweight or obese, and over 40 million children under the age of 5 years are estimated to be overweight [1]. In contrast, in 2002 it was estimated that 300 million children were underweight and that 3.4 million died as a consequence of undernutrition [2]. Furthermore, a specific pattern has emerged: in low-income areas, people with high socio-economic status tend to become obese first, whereas in high-income countries/areas, people with low socio-economic status have the highest prevalence of obesity [3].

Figure 1: Prevalence of obesity for both sexes ages 20+ (2008) [1]
1.2 Health impact of obesity
Obesity is associated with numerous health hazards and considered a major public health concern. Increased BMI is a risk factor for several diseases, including type 2 diabetes, hypertension, heart disease, stroke, and various types of cancers [1]. According to the World Health Organisation (WHO), obesity is now the fifth leading cause of global deaths [2].

1.3 Energy balance and evolution of the contemporary diet
In order to gain or lose weight in the absence of an underlying chronic disease, a disruption of energy balance is required. In relation to the regulation of body weight and nutrition, energy balance can be defined as energy storage = energy intake − energy expenditure [4]. That is, in order to lose weight, one needs only to reduce energy intake or increase energy expenditure. Despite the fact that the energy model presented above is an oversimplification, it has formed and continues to form the basic premise of most public health interventions [5]. Generally speaking, a sustained negative energy balance does produce weight loss [6], but this simplistic view fails to acknowledge that body weight regulation is a multifaceted system subject to homeostatic control [7]. For example, following a period with decreased energy intake, there will most likely be a compensatory decrease in energy expenditure due to a reduction in basal metabolic rate and increased hunger [8, 9], and following exercise, one can expect increased hunger [10]. Furthermore, it seems that the energy balance system has a propensity for energy conservation, making it harder to lose weight (and keep the weight off) compared to gaining weight [11]. From an evolutionary perspective and in light of the obesity epidemic, this could very well represent a maladaptation. The authors of the paper “The geometry of human nutrition” have stated that we are stuck in a time lag; the environment has changed rapidly while our physiology has remained unchanged. Looking back to the Paleolithic time up until the late 19th century, the daily need for physical activity matched that of resting metabolic rate (RMR), resulting in daily energy expenditure levels around 3,000 kcal [12], and in the late 20th century, typical total daily energy expenditure was estimated to be 2,000 kcal or less [12]. There has concurrently been an increase in food availability, giving us easy access to processed foods with a low nutrient-to-energy quotient (high energy density), making today’s food habits potentially “obesogenic” [13, 14].
1.4 Macronutrient composition of the diet in relation to body weight regulation

Considerable effort has been put into finding effective dietary strategies both to prevent obesity and to induce weight loss in obese individuals. Still, there is no consensus regarding which diet is most effective for this purpose, and in recent decades different dietary strategies have been recommended for weight loss [6]. Fat-reduced diets have been recommended for several years [6]. Increasing the amount of protein in the diet, at the expense of either fat or carbohydrates, is another popular strategy for weight loss [6]. This is likely due to some of the proposed effects of protein compared to the other macronutrients. Among the macronutrients, protein has the strongest thermic effect, or the highest dissipation of energy as heat, thereby potentially reducing the energy efficiency of high-protein diets [15]. The thermic effect of protein is 20–25% of energy consumed, whereas for carbohydrates it is usually around 5–15% [16]. The thermic effect of fat is not as clear; some claim it is lower than carbohydrates [17], while others claim there is no difference [18]. Beyond the thermic effect, protein is also associated with increased resting energy expenditure and satiety [19].

The present Western diet contains an average of 16% of energy from protein, 35% from fat and 49% from carbohydrates, which represents a major increase in carbohydrate amount at the expense of protein, compared with the diet in the Paleolithic Era [20]. Interestingly, a protein intake of around 16% falls in the range where the thermic effect of protein was shown to be the lowest in a rat study [21]. Human experiments with excess energy intake have actually shown larger weight gain with diets containing 10–15% of energy from protein than with diets containing 3% of energy from protein [15]. In light of this, it is not unlikely that the amount of protein in the diet could be of importance in weight regulation.

In a study by Sacks et al. [22], four different iso-energetic weight-loss diets varying in protein content were compared, but no difference in weight loss over a two-year period was found; weight loss was induced regardless of macronutrient composition. Data from two separate weight-loss intervention studies with iso-energetic diets supported this, and also indicated that high-protein diets resulted in greater conservation of lean mass than high-carbohydrate diets did [23, 24].

The absence of a clear definition of “high-protein diets” (definitions range from 27–68% of energy from protein) might explain the seemingly inconclusive data from human weight-loss intervention studies [19]. Furthermore, during energy restriction, absolute intake of protein could be low and result in a negative nitrogen and protein balance, even when the percentage of calories from protein is increased. This would in turn result in loss of fat-free mass and decreased RMR [25].
Despite the lack of studies demonstrating conclusively that high-protein diets enhance weight loss under iso-energetic conditions, high-protein diets seem to be effective under ad libitum conditions [26-28]. The rationale behind this is based on dietary protein’s ability to increase satiety and potentially lead to decreased energy intake [29, 30]. Several studies reviewed by Halton et al. [31] showed increased satiety and reduced subsequent energy intake after high-protein meals. However, most of these studies were of short duration (1–6 days). Skov et al. [27] reported that a high-protein diet had a greater satiating effect and caused greater weight loss, owing to lower energy intake, than a normal protein diet under ad libitum conditions. Similar results have been reported by Weigle et al. [28], who compared diets containing 30% and 15% of energy from protein under ad libitum conditions. Studies with high-protein diets showing decreased energy intake during ad libitum conditions are in line with the protein leverage hypothesis, which suggests that reduction in protein intake is the main factor driving the obesity epidemic [32]. The basic idea is that appetite is regulated to ensure adequate amounts of protein, resulting in overfeeding when diets are low in protein [29, 30].

**Figure 2**: Proposed effects of increasing the amount of protein in the diet [33]
It is important to note that weight loss as discussed above is not the same as weight maintenance or obesity prevention. When it comes to weight maintenance, diets with moderate to high protein content seem to be the most effective. This is likely due to a combination of increased satiety, and thereby compliance to a weight-maintaining diet [34, 35], and conservation of lean mass, as higher protein intake is associated with suppression of muscle breakdown and increased RMR [36, 37]. Furthermore, several animal studies show that high-protein diets provide protection against diet-induced obesity, due to lower energy intake compared with diets with less protein [38-42].

Altogether, there is compelling evidence indicating that increasing the amount of protein in the diet has the potential to prevent and reduce obesity. Both human and animal studies have shown that increasing the amount of protein at the expense of carbohydrates and/or fat is an effective strategy for weight reduction, short-term weight maintenance, and protection against diet-induced obesity [43-45]. However, evidence supporting the long-term effectiveness of high-protein diets in human subjects is lacking. Another important point is that the above-mentioned studies primarily focused on the composition of the diet with no regard to the quality of the macronutrients.

1.5 Macronutrient quality and weight management
Besides the composition of the diet, quality is also of importance. There are differences between the sources of each macronutrient. Although evidence from intervention trials is inconsistent, diets with a low glycemic index (GI) may prove beneficial in weight management [46]. Studies have shown that high-GI foods could result in overeating and enhance weight gain [47]. Low-GI diets, on the other hand, have been associated with reduced adiposity in animal models and with enhanced satiety, reduced energy intake, and higher weight loss in human studies [45, 48, 49]. Furthermore, low-GI diets could be beneficial for maintaining weight loss. In a study comparing two energy-restricted diets with either high- or low-GI carbohydrates, weight loss was greater in the group consuming the low-GI diet after 8 weeks, and at the one-year follow-up, weight regain was only significant in the high-GI group [50].

Although the major body of research regarding fat sources has focused on cardiovascular health [51], several studies have examined the effects of different fat sources on weight loss and body weight management [52]. Diets enriched with n-3 polyunsaturated fatty acid have been shown to both attenuate and prevent diet-induced obesity in rodents [53-55]. In addition, n-3 polyunsaturated fatty acids from fish have been associated with increased satiety [56] and enhanced weight loss [57].
in overweight subjects. A study from Krebs et al. [58], though, failed to observe increased weight reduction with fish oil supplements. Although some studies show promising results, data from human intervention studies examining the effects of n-3 polyunsaturated fatty acids on body weight regulation are inconclusive, possibly due to the abrogating effects of the background diet [59].

Extensive research has been conducted to elucidate the effects of both dietary protein in general, and protein amount in relation to satiety, thermogenesis, and body weight management. However, scant knowledge exists regarding how different protein sources can influence these parameters. Given that different protein sources provide different amino acids, and that amino acids have varying properties, it is plausible that protein source is of importance. As stated earlier, satiety is a key factor in the efficacy of high-protein diets. Moreover, different proteins may differ in their satiating capacities [60], but data from human studies are inconsistent. The inconsistency from human studies could stem from difficulties related to standardization. Several factors need to be considered when interpreting results from experiments examining the effects of different protein sources on satiety. For instance, how are the meals balanced for protein amount, energy content, and volume? Protein amounts which are too high could mask differences because of the fact that all treatments are very satiating. Different protein sources contain different amounts of endogenous fat, making it hard to separate fat and protein effects. One also needs to discriminate between methods of delivery. Protein as a preload in liquid form could exert different effects as compared to protein ingested in solid form or as part of a meal. The absorption of amino acids will generally be faster after ingestion of protein in liquid form compared to solid foods [61]. This underlines the importance of another factor: timing of measurement due to potential differences in protein kinetics, such as rate of digestion/absorption. Whey, for example, may be digested more quickly than casein [62] and fish more slowly than beef and chicken [63]. Besides this, coingestion of other nutrients can alter the rate of absorption; for instance, fat and fibre may reduce the rate of absorption due to increased gastrointestinal transit time [64]. Despite the issues relating to standardization, several studies have been published regarding the effects of different proteins on appetite; few, however, have accounted for the above-mentioned challenges. In a study by Uhe et al. [63], the satiating effects of fish, beef, and chicken were compared. The study participants were each given a meal consisting of a piece of one protein source (50 grams of protein) and 200 millilitres of water. After finishing the meal, subjects were asked to indicate their level of satiety. The study showed that fish protein was associated with the highest levels of satiety,
and that the satiety produced by fish protein declined more slowly compared to the other protein sources [63]. A similar finding was reported in a study by Holt et al. [65] comparing the satiating effects of 38 food items, using a subjective ranking system with white bread as a reference. Among the protein-rich foods compared (beef, baked beans, eggs, cheese, lentils, and ling fish), ling fish was reported to have the strongest satiating power. However, in the study by Holt et al. [65], the meal with fish had the highest protein content. Since both studies used subjective rating scores, they do not reveal if the perceived increased satiety would lead to decreased subsequent energy intake. In a study from 2006, Borzoei et al. examined if the increased perceived satiety associated with fish protein intake would result in decreased subsequent energy consumption compared to beef protein [66]. Subjects were served an iso-energetic, protein-rich (40% of energy from protein) mixed meal consisting of either fish protein (cod) or meat protein (beef). The meals were also balanced for macronutrients and fibre. Four hours after the test meal, an ad libitum evening meal was served to investigate if intake of either protein source would translate into decreased subsequent energy intake. The results from this study showed that, surprisingly, participants given the fish reduced their energy intake in the following meal by 11% despite the lack of any differences in perceived satiety in the hours following the test meal. Although all of these studies used iso-energetic servings in each meal, only the first [63] and last study [66] had an equal amount of protein in each meal, and none of them had a standardized volume, which may have influenced the results [67]. Furthermore, there were differences in fat sources in all of the above-mentioned studies, making it hard to determine if the observed effects are due to differences in the fat or protein source. Fish protein contains n-3 polyunsaturated fatty acids, which have been associated with increased satiety compared to saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) [68].

A way to circumvent differences in fat content and volume (protein content) is to use preloads. However, testing protein sources in a form that differs from their natural form may provide limited information for use in dietary advice. Furthermore, although different preloads might result in differences in subsequent energy intake, they may fail as a tactic for weight reduction, as total energy intake using preloads could be higher compared to avoiding them [69-71].

1.6 Protein source and body weight regulation
It seems that enhanced weight loss due to increased protein intake is dependent on protein-induced satiety and is apparent only under ad libitum conditions (as discussed in 2.2). Despite the methodological issues in the above-mentioned studies on satiety, these studies indicated that
different protein sources can influence satiety and energy intake differently. This implies that various protein sources may differ in their effectiveness for weight management. Moreover, various proteins could influence energy expenditure differently. Interestingly, protein-induced satiety has been related to increased energy expenditure [16, 72]. Choosing an optimal protein source based on its effect on satiety may therefore have additive effects in the form of increased energy expenditure. For instance, during a stay in a respiratory chamber, energy expenditure was shown to be higher after a high-protein test meal with pork as the protein source compared to a high protein-test meal with soy as the protein source [73]. Whey protein has also been shown to increase energy expenditure after a high-protein test meal compared to both soy and casein protein [74]. Surprisingly, in the latter study, the researchers found an inverse relationship between perceived satiety and energy expenditure, as the intake of casein and soy protein was reported to be more satiating compared to whey. However, subsequent energy intake was not measured. Taken together, the results from human studies on appetite and energy expenditure indicate that consumption of different protein sources can lead to short-term differences in both satiety and energy expenditure. Whether these differences translate into enhanced body weight loss is uncertain.

Rodent studies, which are easier to control or standardize, have provided more consistent and therefore more compelling data: Whey intake has been shown to decrease fat-mass accumulation in mice compared to casein, independent of energy intake [75, 76], and has been found to reduce body fat in rats compared to soy, dependent on energy intake [77]. Furthermore, in a recent study by our group, it was shown that using scallop as the sole protein source provides protection against diet-induced obesity in mice fed a high-fat, high-sucrose diet compared to using chicken, cod, or crab as the protein source [78]. This may have been due to a lower energy intake in the scallop-fed mice, yet there was no difference in energy intake between scallop-fed mice and crab-fed mice.
2.0 Objectives

The primary aims of the thesis were to

- Evaluate the effects of different protein sources on body weight development
- Evaluate the effects of different protein sources on glucose tolerance and insulin sensitivity

The primary aims of the studies are as follows:

1) Evaluate how different protein sources (casein, cod, beef, chicken, and pork) affect energy intake, body weight development, and glucose metabolism using a high-fat, high-protein (HF/HP) background diet, and to compare casein to pork when using either a high-fat, high-sucrose diet (HF/HS) or a HF/HP diet.

2) Evaluate how different protein sources (casein, chicken filet, and a mixture of cod/scallop) affect body weight development and glucose metabolism during equal energy intake using a high-fat, high-sucrose (HF/HS) background diet

3) Evaluate how different protein sources (a mixture of marine protein sources or a mixture of meat protein sources) affect energy intake, body weight development, and glucose metabolism using a Western background diet
3.0 Summary of results

**Paper 1**
The protein source determines the potential of high protein diets to attenuate obesity development.

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The results in this paper are based on two separate animal studies, both using C57BL/6J male mice.

**Experiment 1:** Mice were fed a HF/HP diet containing either casein, soy, cod, beef, chicken, or pork as the sole protein source *ad libitum* for 12 weeks.

**Major findings**
- Meat protein promoted obesity compared to plant and fish protein.
- Chicken- and pork-fed mice exhibited decreased lean mass compared to casein-fed mice.
- Cod-fed mice had the lowest energy intake.
- Casein-fed mice had the lowest energy efficiency.
- Pork-fed mice had elevated levels of insulin in their plasma compared to casein-fed mice.
- Pork- and chicken-fed mice had reduced glucose tolerance and decreased insulin sensitivity compared to casein-fed mice.

**Experiment 2:** Mice were fed a HF/HP diet and a HF/HS diet with either casein or pork as the sole protein source *ad libitum* for 12 weeks. In a follow-up experiment, a separate set of mice were given the same diets while housed in indirect-calorimetry cages for measurements of energy expenditure, spontaneous locomotor activity, and respiration exchange ratio (RER).

**Major findings**
- HF/HS diets were more obesogenic than HF/HP diets regardless of the protein source.
- Pork-fed mice gained more weight, owing to increased fat accumulation, compared to casein-fed mice, independent of the protein:sucrose ratio.
- Pork-fed mice had elevated insulin levels in their plasma, independent of the protein:sucrose ratio.
• Pork-fed mice had reduced glucose tolerance and insulin sensitivity compared to casein-fed mice, independent of the protein:sucrose ratio
• Using pork as the sole protein source induced a reduction in spontaneous locomotor activity compared to casein, independent of the protein amount.

**Paper 2**
A Mixture of Cod and Scallop Protein Reduces Adiposity and Improves Glucose Tolerance in High-Fat, High-Sucrose Fed Male C57BL/6J Mice.

_Hanne Sørup Tastesen, Alexander Krokedal Rønnevik, Kamil Borkowski, Lise Madsen, Karsten Kristiansen and Bjørn Liaset_

C57BL/6J mice were pair-fed a HF/HS diet containing either casein, a mixture of cod and scallop, or chicken as the protein source for 7 weeks. In a separate experiment, mice were given the same diets while housed in indirect-calorimetry cages for measurements of energy expenditure, spontaneous locomotor activity, and RER.

**Major findings**
• Casein- and cod/scallop-fed mice gained significantly less body fat mass compared to chicken-fed mice.
• Casein-fed mice exhibited reduced apparent fat digestibility compared to cod/scallop- and chicken-fed mice.
• Apparent digestibility of nitrogen was higher in cod/scallop-fed mice than in chicken- and casein-fed mice.
• Energy expenditure was increased in cod/scallop-fed mice compared to casein-fed mice during the dark phase.
• Feeding mice cod/scallop protein tended to attenuate the diet-induced reduction in spontaneous locomotor activity observed when switching from a low-fat diet to a high-fat diet during the dark phase.
• Casein-fed animals had reduced glucose tolerance compared to chicken-fed and cod/scallop-fed mice, despite reduced adiposity.
Paper 3
Lean seafood reduces energy intake and attenuate diet induced obesity in C57BL/6J mice.
Alexander Krokedal Rønnevik, Hanne Sørup Tastesen, Kristin Røen Fauske, Ulrike Liisberg Aune, Lise Madsen, Karsten Kristiansen, and Bjørn Liaset

Male C57BL/6J mice were fed Western diets containing a mixture of either lean seafood (ling filet, rosefish filet, cod filet, scallops, and wolf fish filet) or lean meat (chicken, pork, and beef) for 12 weeks. Four separate experiments with separate sets of mice were conducted: *ad libitum* feeding and pair feeding for 12 weeks, acute meal response test and indirect calorimetry measurements at the transition from the low-fat to the Western diet

Major findings
- In an *ad libitum* setting, mice fed lean seafood ate less and gained less fat mass than mice consuming meat
- When pair-fed, there was no difference in weight gain between mice fed seafood and mice fed meat.
- The feeding regime and body composition, rather than protein source, influenced glucose homeostasis.
- The feeding regime and adiposity, rather than protein source, influenced plasma cholesterol.
- Mice fed seafood protein had increased spontaneous locomotor activity compared to mice fed meat protein after switching from a low-fat diet to a high-fat diet during the dark phase
4.0 Discussion of results

4.1 The effects of various protein sources on body weight development
Earlier studies from our group have shown that high amounts of protein in the diet provide protection against diet-induced obesity in mice. However, in these studies, casein was used as the sole protein source [42, 45, 79]. We therefore wanted to examine the effect of protein sources other than casein on body weight development and glucose tolerance. The results from paper I (experiment I) indicate that using beef, pork, or chicken in a HF/HP diet promoted obesity compared to using casein or soy (paper I, figure 1B). The consumption of protein derived from terrestrial animals (chicken, pork, and beef) resulted in significantly more weight gain compared to the intake of casein and soy protein, despite equal energy intake (paper I, figure 1C). Surprisingly, mice receiving either chicken or pork gained more body mass fat than the mice fed the HF/HS diet containing casein (paper I, figure 1B). Furthermore, the chicken and pork fed mice also gained more weight than cod-fed mice, but cod-fed mice consumed less energy (paper I, figure 1C). The results from body composition measurements showed that differences in body mass were due to a higher accumulation of fat mass in mice receiving the meat diets (paper I, figure 1B). Tissue weights from individual adipose tissue depots (rWAT, eWAT, and iWAT) after 12 weeks confirmed differences in adiposity (paper I, supplementary Figs. 1 A-C).

The finding that the consumption of lean meat promoted weight gain is in agreement with an observational study suggesting that the consumption of meat (red meat, poultry, and processed meats) could promote weight gain [80, 81]. In comparison, the consumption of vegetable protein, marine protein, and milk protein (casein) are suggested to have an inverse correlation with body weight gain [81-84].

The obesogenic effect of the consumption of lean meat was confirmed in the follow up experiments in papers I and II, using a HF/HS background diet. Here, we showed that mice fed a HF/HS diet with pork or chicken as the protein source gained more weight than mice fed the same diet with either casein or cod/scallop as the protein source, despite equal energy intake, again owning to a difference in fat accretion (paper I, figures 3A-B and E), (paper II, figures 1A-C).

In paper III, we wanted to examine the effects of a mixture of common protein sources of either marine or terrestrial origin in a Western background diet. After 9 weeks of feeding, mice receiving the meat diet gained significantly more weight than mice fed seafood, due to the increased accumulation of fat mass (paper III, figures 1C and E). However, meat-fed mice had a significantly
higher energy intake (paper III, figure 1A). We therefore did a follow up experiment, where the energy intake for the meat fed mice was restricted according to the energy intake for the mice receiving seafood (4% restriction). Unexpectedly, pair feeding eliminated the discrepancy in weight gain (paper III, figure 3D). This was unexpected because the feeding efficiency was increased for mice fed the meat diet in all of the ad libitum feeding studies (paper I and III). One might expect that decreasing feed availability would increase feed efficiency, but based on the findings in paper III, and from a study specifically examining the effects of reducing feed availability on feed efficiency, it appears that reducing feed availability actually decreases feed efficiency [85]. Differences in feed efficiencies might be explained by differences in the apparent digestibility of fat. In the first experiment in paper I, there were no differences in the apparent fat digestibility between the experimental diets (paper I, figure 1F). In the follow-up, pork had a significantly higher apparent fat absorption compared to casein, independent of protein amount, and independent of protein source, and fat absorption was lower in the HF/HS diets (paper I, figure 3E). Similarly, in paper II, casein had a significantly lower apparent fat absorption compared to cod/scallop (paper II, figure 1F). Moreover, the consumption of casein in a high-fat background diet has previously been reported to cause higher excretion of fat in faeces compared to a high-fat diet with salmon [86]. The lower fat absorption associated with casein could possibly explain the reduced fat-mass accumulation seen in casein-fed mice. However, despite lower feed efficiency in casein-fed mice in paper I (experiment 1), there was no difference in the apparent fat digestibility between the diets (paper I, figure 1F). In addition, in paper III, seafood-fed mice had the highest apparent fat absorption (paper III, figure 3C) despite having lower feed efficiency when the mice had constant access to feed/were fed ad libitum (paper III, figure 1B) than the mice fed meat. Taken together, the inconsistent findings relating to feed efficiency and fat absorption indicate that other factors might be responsible for the observed differences in weight gain.

### 4.1.1 Amino acid composition

Differences in the amino acid composition of the diets could provide a possible explanation for differences in weight gain between the various protein sources. In a meeting abstract from 2009 [87], the results from a study where a high-fat diet was supplemented with one of the 20 proteinogenic amino acids and fed to mice for 4–6 weeks was reported. Several amino acids were reported to have antiobesogenic effects, and lysine was named the most potent. Surprisingly, of all the protein sources used in papers I–III, next to soy, casein actually has the lowest lysine content.
(paper I, table 2). Furthermore, leucine was also reported to have an antiobesogenic effect [87], and several other studies, but not all [88], support this finding [89-91]. Interestingly, casein has a high leucine content and, as shown in our studies, blunts diet-induced obesity. However, the fact that mice consuming the HF/HP diet with meat protein became more obese than mice fed the HF/HS diet with casein (lower total leucine content compared to either of the HF/HP diets) weakens this association. Similarly, in paper II, the cod/scallop diet had a lower leucine content than the chicken diet (paper II, table 2).

Our group has previously shown that scallop protein, with a high endogenous taurine content prevents diet-induced obesity [78]. It has also been shown that supplementation of taurine in drinking water or in the diet prevents obesity in rodents [92, 93]. In paper I, the cod diet had the highest taurine content, and cod-fed mice gained significantly less weight compared to mice fed chicken or pork, but they also had a lower energy intake. Cod-fed mice, however, gained more weight than casein-fed mice. Similarly, but with equal energy intake, in paper II, mice fed a cod/scallop diet gained less weight than mice fed a chicken diet, and did not differ from casein-fed mice. The discrepancy between the results presented in papers I and II might be explained by differences in the taurine content between cod and scallop. In paper III, there was also a difference in the taurine content between the diets (paper III, table 3), with the highest being in the seafood diet. Here, there was no difference in weight gain when mice were fed isoenergetically, however, the background diet was different from the diets utilized in papers I and II.

To summarize, taurine content is higher in marine- compared to terrestrial protein sources, and could be driving the differences in weight gain between the consumption of lean meat and seafood on a HF/HS diet and a HF/HP diet. However, we failed to identify any single amino acid or pattern in our studies that might explain the antiobesogenic effects associated with casein.

4.1.2 Energy expenditure and activity

One of the proposed benefits of increasing the amount of dietary protein is elevated energy expenditure, and this has been shown in several short-term studies [94-96]. In paper I (follow-up experiment 2), we investigated the impact of feeding mice either casein or pork in a HF/HS diet or a HF/HP diet on energy expenditure, using indirect-calorimetry cages. Surprisingly, we observed higher energy expenditure with the HF/HS diets than with the HF/HP diets during the light phase, regardless of the different protein sources (paper I, figures 4C and D). This was unexpected because plasma concentration of urea indicated a higher ureagenesis in casein-fed mice (paper I, figure 3G), which is an energy-demanding process related to increased energy expenditure [97].
explanation for this discrepancy could be the use of an animal model and/or an extreme diet in our experiment, whereas the above-mentioned studies were conducted on human subjects using less extreme dietary treatments. The same set-up was used in papers II and III as well. In paper II, energy expenditure during the dark phase, surprisingly, was higher in cod/scallop-fed mice than in casein-fed mice, and was not significantly different from the chicken-fed mice (paper II, figures 3F and G). In paper III, no difference in energy expenditure was observed (Paper III, figure 6D). Interestingly, in paper I, we found a greater reduction in spontaneous locomotor activity during the dark phase for pork-fed mice compared with casein when switching from a low-fat diet, independent of protein amount (paper I, figure 4B). In paper II, we also observed differences in activity following the switch from the low-fat diet. Here, the cod/scallop diet tended to attenuate the diet-induced reduction in spontaneous locomotor activity compared to both chicken and casein ($p = 0.06$) (paper II, figures 3C-E). A reduction in activity level following the transition from a low- to a high-fat diet has previously been demonstrated in mice and was expected [98]. Intriguingly, in paper III, we found that when switching from a low-fat diet to a Western seafood diet, activity increased (paper III, figure 6B).

Although we observed differences in the activity levels in papers I and III for the different protein sources, there were no differences in energy expenditure. However, our group has previously observed an inverse correlation between activity level and obesity without detecting a difference in energy expenditure [99]. Moreover, in all of the papers, we used a set-up where gas exchange was measured for 1.9 minutes every 30 minutes, while the activity levels were determined continuously. Given that differences in activity are likely to reflect changes in energy expenditure [100], it is possible that potential differences in energy expenditure that, over time, might result in differential fat accretion, were undetectable using our set-up. Taken together, evidence from the experiments involving indirect-calorimetry measurements indicates that intake of lean meat, compared to seafood or casein, may reduce spontaneous locomotor activity in mice and thereby result in lower energy expenditure.

### 4.2 The effects of various protein sources on glucose tolerance and insulin sensitivity

The intake of various proteins and amino acids might result in different effects on insulin secretion and action, and thereby glucose metabolism [101, 102], and high fasting plasma levels of insulin and glucose might indicate reduced insulin sensitivity and increased risk for developing type-2 diabetes [103, 104]. In the first experiment of paper I, we observed impaired glucose tolerance and
elevated fasting insulin levels in mice fed pork and chicken compared to mice fed casein (paper I, figures 2B-D). Furthermore, the same pattern was evident in the second experiment. Compared to HF/HP fed mice, the HF/HS fed mice had higher fasting glucose and insulin levels, impaired glucose tolerance, and decreased response to insulin (paper I, figures 5A-F). Moreover, during a high fat feeding diet, pork-fed mice showed impaired glucose tolerance, reduced response to insulin, and higher fasting insulin compared with casein-fed mice, independent of the dietary protein:sucrose ratio (paper I, figures 5A-F). In paper II, casein-fed mice exhibited reduced glucose tolerance compared with cod/scallop-fed mice when challenged with an oral glucose test, but no differences were found in the fasting insulin or glucose levels between any of the groups (paper II, figures 2 A-F).

The association between the consumption of cod/scallop and improved glucose metabolism is in line with previously published studies showing enhanced glucose metabolism and increased insulin sensitivity in rats fed cod compared with casein [105, 106]. Moreover, the casein-based diets are high in branched-chain amino acids (BCAAs), which have been associated with the development of insulin resistance, as reviewed in [107].

The apparent discrepancy in results presented in papers I and II with regard to glucose tolerance could possibly be explained by differences in the dose of glucose (2 mg glucose/g body mass) and/or delivery method (intraperitoneal injection or oral delivery), which both have been shown to influence the results of the glucose tolerance test [108]. In paper I, the casein-fed mice weighed less than the cod-fed mice and therefore received a lower dose of glucose, whereas in paper II, there were no differences in body weight between the cod/scallop-fed mice and the casein-fed mice. In retrospect, the dose of glucose should perhaps have been calculated based on lean mass, as recommended by Andrikopoulos et al. [108]. In paper III, the dose of glucose was calculated according to lean mass. In the *ad libitum* study, when compared with mice fed the seafood diet, meat-fed mice had increased fasting glucose before the glucose tolerance test in week 9, and significantly higher glucose levels 15 minutes after receiving the glucose load (paper III, figures 2A and B). Moreover, meat-fed mice had elevated levels of glucose 15 minutes after receiving an insulin injection (paper III, figure 2F). However, we did not find any differences in either area under the curve (AUC) during the glucose tolerance test (GTT), or in decremental area under the curve (DAUC) during the insulin tolerance test (ITT), suggesting similar insulin sensitivity between the meat-fed and seafood-fed mice (paper III, figures 2C and F). When pair-fed, the differences for fasting glucose and the initial response 15 minutes after the administration of glucose or insulin
were no longer apparent (paper III, figures 4B and E). This might indicate that the feeding regime and/or body composition may influence glucose metabolism. Two studies showing that dietary restriction has beneficial effects on glucose metabolism and insulin sensitivity in mice [109, 110], as was the case with the meat-fed mice, support the notion that the feeding regime might have influenced glucose regulation in the pair feeding.

4.3 Effects of various protein sources on satiety
As discussed in the introduction of this thesis, different protein sources may differ in their satiating capacities [60, 63, 66]. However, issues relating to the standardization of methodologies complicate separating the protein effects from other factors, such as, the fat source and the volume of the test meal/drink. Therefore, the protein source per se will not reviewed here; instead, the satiating capacity of the diet will be discussed. In paper I, the only difference in energy intake was between the cod-fed mice and the casein-fed mice (paper I, figure 1D). Cod-fed mice consumed less energy than casein-fed mice but did not differ from mice fed any of the other protein sources. Increased satiety after consumption of marine protein has been reported in earlier studies [63, 65, 66]. In these papers, marine protein was compared with terrestrial protein, consistent results showed increased satiety following the intake of seafood compared with meat. In paper I, the energy intake between cod and beef, chicken, or pork was equal, indicating that the diets were equally satiating. The lack of a difference in energy intake might be due to the high amount of protein in all of the diets (33% of energy from protein), making all of the diets quite satiating. The results from paper III support this. In paper III, the association between the consumption of lean seafood and decreased energy intake compared to the intake of lean meat was reproduced using a Western background diet with less protein (16% of energy from protein). Given that energy intake has been related to palpability of food [111], one might argue that differences in feed intake are related to the palpability of the diets. Based on a diet preference test, it seemed that this might be the case in paper III. When given the choice between the seafood and the meat diet, the mice showed no initial preference, but the mice ate more of the meat diet during a six-hour period with access to both diets (paper III, figures 7A and B). Interestingly, mice given drinking water supplemented with taurine exhibited decreased energy intake and were protected against diet induced obesity [92]. Moreover, mice fed a diet with scallop or crab as the sole protein source consumed less energy than mice fed a diet with chicken or cod as the sole protein source. Since mice fed the diets containing the highest amounts of taurine ate
less, one might speculate that the reduced energy intake observed in the seafood-fed mice in paper III was connected to the dietary taurine content.
5.0 Conclusions

When feeding mice a high fat diet with sucrose as the main carbohydrate source:

- Casein was less obesogenic compared to pork and chicken
- Cod/scallop was less obesogenic compared to chicken
- Casein reduced glucose tolerance and insulin sensitivity compared to pork-fed mice
- Casein-fed mice had increased levels of urea in their plasma, suggesting increased ureagenesis
- Cod/scallop preserved glucose tolerance compared to casein

When feeding mice a high-fat, high-protein diet:

- Meat (beef, chicken, and pork) and cod promoted obesity compared to casein protein
- Chicken and pork reduced glucose tolerance and decreased insulin sensitivity compared to casein
- Casein-fed mice had increased levels of urea in their plasma, suggesting increased ureagenesis

When feeding mice a Western diet:

- Meat (chicken, beef, and pork) promoted obesity compared to seafood protein sources (ling filet, rose fish filet, cod filet, scallops, and wolf fish filet) under ad libitum conditions, due to the increased energy intake
- Consuming equal amounts of meat- or seafood protein resulted in equal weight gain and adiposity
6.0 Perspectives

The most intriguing finding in this thesis is the reduced energy intake observed with the consumption of marine protein compared to the consumption of terrestrial meat protein. Furthermore, the ability of casein to blunt the development of diet-induced obesity is quite interesting. The antiobesogenic effects of a casein diet seem to be independent of energy intake, while seafood’s antiobesogenic effects are primarily a consequence of reduced energy intake. It would be interesting to examine the effect of combining seafood protein with casein, to see if there is an additive effect. The consumption of a combination of lean seafood and casein may result in reduced energy intake and reduced weight gain, and in addition, reduced fat accretion independent of energy intake.

It would also be interesting to elucidate the mechanisms behind the reduced energy intake associated with the consumption of diets with marine protein. This might be achieved by measuring plasma levels of various appetite hormones after ingestion of the experimental diets. Preferably, this would be done at first exposure to the diets, when there is no difference in body weight, but a follow up at a later stage would also be informative. This might reveal potential adaptations to the diets. However, measuring plasma hormones is challenging due to a limited sample volume, in particular when using mice as the experimental model.

It would also be of interest to investigate the weight reducing capacity of different protein sources in an energy-restricted diet with mice. Identifying distinct protein sources with increased efficacy for weight loss under hypoenergetic conditions would be of great relevance in the treatment of obesity.
7.0 Bibliography


78. Tastesen, H.S., A.H. Keenan, L. Madsen, K. Kristiansen, and B. Liaset, Scallop protein with endogenous high taurine and glycine content prevents high-fat, high-sucrose-induced obesity and improves plasma lipid profile in male C57BL/6J mice. Amino Acids, 2014. 23: p. 23.


8.0 List of publications

**Paper 1**
The protein source determines the potential of high protein diets to attenuate obesity development. (Manuscript)
*Ulrike Liisberg Aune, Lene Secher Myrmel, Alexander Krokedal Ronnevik, Even Fjære, Susanne Bjelland, Kristin Røen Fauske, Astrid L. Basse, Jacob Bo Hansen, Bjørn Liaset, Karsten Kristiansen, and Lise Madsen*

**Paper 2**
A mixture of cod and scallop protein reduces adiposity and improves glucose tolerance in high-fat, high-sucrose fed male C57BL/6J mice. (Manuscript)
*Hanne Sørup Tastesen, Alexander Krokedal Ronnevik, Kamil Borkowski, Lise Madsen, Karsten Kristiansen, and Bjørn Liaset*

**Paper 3**
Lean seafood reduces energy intake and attenuates diet induced obesity in C57BL/6J mice. (Manuscript)
*Alexander Krokedal Ronnevik, Hanne Sørup Tastesen, Kristin Røen Fauske, Ulrike Liisberg Aune, Lise Madsen, Karsten Kristiansen, and Bjørn Liaset*
9.0 Annex

Manuscripts enclosed
The protein source determines the potential of high protein diets to attenuate obesity development.

Ulrike Liisberg Aune¹,², Lene Secher Myrmel¹,², Alexander Krokedal Rønnevik¹,², Even Fjære¹,², Susanne Bjelland¹, Kristin Røen Fauske¹, Astrid L. Basse², Jacob Bo Hansen², Bjørn Liaset¹, Karsten Kristiansen² and Lise Madsen ¹,²

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Key words: high protein diets, obesity, glucose tolerance, insulin sensitivity, energy efficiency, brown adipose tissue
ABSTRACT

The notion that the obesogenic potential of high fat diets in rodents is efficiently attenuated by increasing the protein:carbohydrate ratio is largely based on studies where casein or whey are used as protein sources. To evaluate to what extent protein source might modulate the effect of high protein diets, we fed mice high fat diets with a high protein:carbohydrate ratio using different protein sources. We observed striking differences in weight gain and accretion of adipose mass. Whereas a high proportion of casein prevented obesity, mice fed a high proportion of soy, cod, beef, chicken or pork protein, gained a substantial amount of adipose tissue and became insulin resistant and glucose intolerant. Using a factorial design, where casein and pork protein were chosen as protein sources, we demonstrated that both protein source and amount influenced feed efficiency as well as development of obesity and insulin resistance. We observed a remarkable difference in response to both protein amount and source in the morphological appearance and UCP1-immunoreactivity of adipocytes collected from interscapular brown adipose tissue. Our data indicate that maintenance of a brown phenotype in the adipocytes in the interscapular region by a high proportion of casein may protect against obesity development. By contrast, adipocytes in the interscapular adipose depot in mice fed pork protein exhibited a clear morphological transformation, acquired larger fat droplets and displayed less UCP1 immunoreactivity. We conclude that diets with high protein:carbohydrate ratio where casein is used as protein source are not representative for all high protein diets. Given the popularity of high protein diets, this warrants further investigations in humans.
INTRODUCTION

High protein diets have become increasingly popular as a tool to prevent obesity development and to lose weight. The efficiency and safety of high protein diets, in particular when combined with a high fat intake, however, are vigorously debated [1, 2]. In rodents, it is evident that obesity development is prevented if a high fat diet is accompanied with an increased protein:carbohydrate ratio [3-10]. Worth noting, however, in rodent experiments where high fat diet induced obesity has been prevented by increasing the protein:carbohydrate ratio, casein or whey has been used as protein sources.

A major consequence of increasing the protein:carbohydrate ratio derives from the ability of sucrose to elicit a rise in blood glucose and thereby stimulate insulin secretion. The important role of insulin secretion and action in adipose tissue in development of diet-induced obesity is underscored by the findings that both Ins1+/−: Ins2−/− mice [11] and fat-specific insulin receptor knockout (FIRKO) are protected against diet-induced obesity [12]. This is in line with our study demonstrating that the glycemic index of the carbohydrate component of the feed determines the obesogenic effect of high fat diets [9].

There are several additional mechanisms by which a high protein:carbohydrate ratio may prevent high fat diet-induced obesity. A high intake of protein is known to have high satiating effect, thereby reducing energy intake [13], but importantly, pair-feeding experiments by us and others have demonstrated that high protein diets also reduce feed efficiency and obesity development independently of feed intake [4, 7, 9]. The reduced energy efficiency may relate to the higher thermic effect of proteins (20-30%) compared with carbohydrates (5-10%) [14]. When the intake of protein is high, increased protein degradation and re-synthesis as well as synthesis of urea lead to loss of ATP. Moreover, if the dietary carbohydrate level is low, ATP is required for gluconeogenesis [15, 16].

Mitochondrial ATP synthesis may be impaired by the uncoupling protein 1 (UCP1) expressed in brown or brown-like, BRITE or beige adipocytes that can be found together with white adipocytes in several fat depots [17]. As UCP1 allows energy to be dissipated in the form of heat, its expression is positively correlated with metabolic inefficiency and UCP1 expression is induced by cold exposure and overfeeding [18]. The number of UCP1-expressing adipocytes in different adipose tissue depot varies between mouse strains and may account for their...
different propensity for diet-induced obesity [19, 20]. A more brown phenotype of typically
white adipose tissue with a concomitant resistance to diet induced obesity can be obtained by
transgenic expression of UCP1 itself [21], as well as by modulation of several key molecules
involved in brown adipocyte differentiation [22]. Given the high capacity of activated brown
adipocytes to take up glucose, browning of adipose tissue has received interest as a strategy to
improve glucose homeostasis [23-26]. Browning of white adipocytes may occur by both
pharmacological and nutritional agents [27]. We have earlier observed increased expression of
Ucp1 in inguinal white, but not in interscapular brown adipose tissue in mice fed high fat diets
by increasing the protein:carbohydrate ratio [7, 9]. However, in rats it has been reported that
increasing the protein:carbohydrate ratio in a low fat diet led to increased expression of Ucp1
in interscapular brown fat [28]. Still, it is not yet known if the protein:carbohydrate ratio can
modulate the number of UCP1-expressing cells.

Different type of proteins may influence both adipose tissue mass and function in various ways,
and few rodent studies have demonstrated that diets with standard levels of different types of
proteins differ in their ability to stimulate Ucp1 expression and accordingly, display different
obesogenic potential [29-31]. Still, the general notion that an increased intake of dietary protein
attenuates obesity development in rodents is more or less exclusively based on studies where
casein or whey was used as the protein source. Casein and whey have a high content of branched
chain amino acids (BCAAs), valine, leucine and isoleucine. In particular, leucine is recognized
as a nutrient signal proposed to mediate, at least in part the effect of high protein diets on
metabolism [10, 32, 33]. Of note, the chronic elevated levels of BCAA in mice with disrupted
mitochondrial branched chain aminotransferase, was associated with increased energy
expenditure. However, the lean phenotype in these mice was accompanied by insulin resistance
[33]. Moreover, metabolic profiling identified elevated BCAA as a signature related to obesity
and insulin resistance in humans [34]. Given the relative high amounts of BCAA in casein and
whey, a high dietary protein:carbohydrate ratio using these protein sources may not be
representative for high protein diets in general. Thus, in this study we aimed to evaluate the
development of obesity and insulin resistance in rodents fed high fat diets with a high
protein:carbohydrate ratio using different protein sources.
MATERIALS AND METHODS

Ethical statement
The animal experiments were performed in accordance with the guidelines of the National Animal Health Authorities (Norwegian approval identification FOTS id.nr 3750). No adverse effects were observed.

Mouse diets
The macronutrient composition of the diets is presented in tables 1-4. A low fat reference diet and regular high fat/high sucrose (HF/HS) diet, both with casein as protein source, were used as reference diets. In the experimental diets, part of the carbohydrate amount was exchanged with protein to prepare high fat/high protein (HF/HP) diets using either casein (Sigma, batch nr. BCBC3986V and 080M0006), soy powder (Ssniff Spezialdiäten, Soest, Germany), cod fillet powder (Seagarden AS), beef tenderloin (H. Bragstad A/S, Bergen), chicken breast fillet (Prior, Norway) or pork sirloin (H. Bragstas A/S, Bergen) as protein sources. Beef, chicken and pork filets were freeze dried and pulverized. The protein sources were analyzed for protein and total fat content as earlier described [35] in order to balance the diets with respect to total protein and fat content. The diets were mixed using a Crypto Peerless EF20 blender, kept at -20 °C, and analyzed for energy, fat, protein and amino acid composition as described [35].

Animals
The data in this paper are based on the results from three separate animal studies using male C57BL/6J Bomta mice (Taconic), 8 weeks of age. In experiment 1 and 2, the mice were kept at thermoneutrality (28-30°C) with a 12-h-light/dark cycle in single cages. The mice were assigned into experimental groups (n=9) by bodyweight and body composition determined by nuclear magnetic resonance (Minispec mq 7.5, NMR analyser, Bruker, Germany) after five days acclimatization. The mice were weighed once a week and fed ad libitum three times a week. After 11 weeks of feeding the mice were fasted for 4 h before they were sacrificed by cardiac puncture under Isoflurane anesthesia (Isoba-vet, Schering-Plough, Denmark). Blood was collected in tubes containing EDTA (Medinor AS, Oslo, Norway), centrifuged at 5000 g at 4°C for 5 min. Plasma was stored at -80°C before further analysis. Liver, muscle and adipose tissue were dissected out, weighed, snap-frozen in liquid nitrogen and stored at -80°C until further analyses. A portion of each adipose depot was fixated for histology. See histology.
section for further details. A third cohort (experiment 3) of C57BL/6J mice were used for indirect calorimetry measurements (see below).

**Insulin and glucose tolerance tests (ITT and GTT)**

After 9 and 10 weeks, respectively, of receiving the experimental diets, GTT and ITT were performed on mice in the conscious state in experiment 1 and 2. Prior to the GTT the mice were fasted for 6 h and then received an intraperitoneal (i.p) injection of 2 mg glucose/g body weight. Blood was collected from the lateral tail vein and glucose levels were measured using a glucometer (Ascensia Contour, Bayer Healthcare, Oslo, Norway) before and 15, 30, 60 and 120 min after glucose injection. Additionally, 20 µl blood were collected at time point 0 and 15 to measure plasma insulin in experiment 2. Before the ITT the mice had free excess to feed, but they were deprived of feed during the test. They received an i.p injection of 0.75 U insulin (Humilin-R)/kg body weight, and blood was collected and glucose measured before as well as after 15, 60, 45 and 60 min.

**Feed efficiency and apparent digestibility**

Feed efficiency was calculated as body mass gain per energy intake (g /MJ). As GTT and ITT may influence on feed intake, data prior to testing (first 8 weeks) were used. After six weeks of feeding the mice were placed in cages with standard wood chip layer replaced by paper lining for the purpose of collecting feces for one week. Feed intake was monitored and feces left behind in cages were collected, weighted and frozen at −80°C. The content of total fat and nitrogen in diets and feces was analyzed as described by [36]. Based on feces measurements and feed intake, apparent digestibility of fat and nitrogen was calculated as follows: 100 × (intake (mg) - fecal output (mg))/(intake (mg)).

**Indirect calorimetric measurements**

In experiment 3, VO₂ and VCO₂ was measured in open-circuit indirect calorimetry cages as described previously [37] using CaloCages (Phenomaster, TSE Systems), equipped with infrared light-beam frames (ActiMot2). The mice were placed in the metabolic cages and fed a low fat reference diet for 72 hours. Gas exchange and beam breaks, as a proxy for activity, were recorded during the last 48 hours. The mice were subsequently fed the experimental diets (n=4) and gas exchange and activity recorded. Diet induced changes for each individual mouse were calculated. Based on two consecutive light (06.00-17.30h) and dark (18.00-05.30h) phases respiratory exchange ratio (RER) was calculated from VO₂ and VCO₂ and spontaneous
locomotor activity was defined as total counts of light-beam breaks. Energy expenditure (EE) was calculated as follows; 16.3 kJ/L × L VO₂ + 4.6 kJ/L × L VCO₂.

**Plasma analyses**

Plasma insulin was determined using a commercial ELISA kit in accordance with the manufacturer’s instructions (Mouse insulin ELISA, DRG, Marburg, Germany). MaxMat PL II analyzer (MAXMAT S.A., Montpellier, France) and conventional kits were used to measure hydroxy-butyrate and non-esterified fatty acids. Free amino acids and urea in plasma were measured using ninhydrin detection on the Biochrom 30+ instrument (Cambridge, UK).

**qRT-PCR**

Total RNA was extracted from mouse adipose tissue using Trizol reagent (Invitrogen). RNA quantity was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and RNA quality was tested on a random selection of samples with BioAnalyzer – RNA 6000 Nano (Agilent Technologies). Reverse transcription and real time quantitative PCR was then performed as described elsewhere [37]. The mRNA expression was normalized to the endogenous housekeeping gene TATA box binding protein (Tbp).

**Histology**

Sections of different adipose tissue depots fixed in 4 % formaldehyde in 0.1 mol/L phosphate buffer (PB) overnight, dehydrated, embedded and stained with eosin and hematoxylin [38]. Immunohistological detection of UCP1-positive cells was performed by an avidin-biotin peroxidase method [39].

**Statistical analyses**

All data are presented as mean ± SEM. Figure preparation as well as some of the statistical analysis were performed using Graph Pad Prism version 6 (GraphPad Software Inc, La Jolla, CA, USA) The data from the first experiment was analyzed using 1 way NOVA analyses followed by Tukey’s multiple comparisons, and group means were considered statistically different at P < 0.05. Data that were repeatedly measured, i.e., growth, energy intake, GTT, RER, activity and EE were analyzed by repeated measurements ANOVA followed by Tukey’s post hoc. In the second and third experiment we used Statistica 9.0, and data were analyzed using a factorial ANOVA test with protein:carbohydrate ratio and protein source as categorical.
predictors. Data that were repeatedly measured, i.e., growth, energy intake, GTT, RER, activity and EE were analyzed by repeated measurements ANOVA followed by Tukey’s post hoc test.

RESULTS

High fat diets with a high proportion of cod, beef, chicken and pork are obesogenic.

In order to investigate if a high protein:carbohydrate ratio is able to attenuate high fat diet-induced obesity when other protein sources than casein are used, we prepared diets where casein was replaced with soy, cod, beef, chicken and pork (Table 1). A casein based low fat regular diet was used as a reference. All protein sources provided the nine indispensable amino acids. However, exchanging casein with other protein sources led to a reduced level of branched chain amino acids (BCAAs) (Table 2). Exchanging casein with cod or terrestrial animal protein also led to a reduced level of phenylalanine. As expected, the soy-based diets contained less methionine and lysine than diets with animal proteins (Table 2), but the sulphur-amino acid requirement (Nutrient Requirements of Laboratory Animals, Forth Revised Edition, 1995) was met. Exchanging casein also changed the composition of dispensable amino acids as the amount of arginine and cysteine increased and the amounts of glutamine, proline and tyrosine were reduced (Table 2).

As expected, the mice fed the high fat diets with a high proportion of casein did not gain more body and fat mass than mice fed the low fat regular diet, whereas mice fed a high fat diet with a high proportion of sucrose gained significantly more body and fat mass (Fig. 1A and B). Of note, only mice fed a high proportion of casein protein had significantly lower fat mass than mice fed the high fat high sucrose (HF/HS) reference diet (Fig. 1B). Fat masses in mice fed a high proportion of soy, cod and beef were comparable to those fed HF/HS (Fig. 1B). Strikingly, compared with mice fed the HF/HS reference diet, mice fed diets with high content of protein from chicken and pork had significantly more fat mass (Fig. 1B) as verified by dissection of different adipose tissue depots (Supplementary Fig. 1). Compared with mice fed the low fat regular diet and mice fed a high proportion of soy, these mice also had reduced lean masses (Fig. 1C).

Replacement of casein with other protein sources led to a reduced feed intake (Fig. 1D). Consequently, compared with casein, feed efficiency was higher with all other protein sources tested, with the exception of soy (Fig. 1E). Based on intake and excretion, the apparent
digestibility of fat in the cod containing diet was lower than in the casein based diet (Fig. 1F), and the apparent digestibility of soy and beef protein was lower than that of casein (Fig. 1G). However, these differences cannot explain the different obesogenic potential of the various high protein diets.

The glucose tolerance and insulin sensitivity was comparable in mice fed the high fat diet with a high content of casein and the low fat reference diet (Fig. 2C-F). Replacement of casein with other protein sources led to a significantly lower glucose tolerance (Fig. 2C and D). Furthermore, compared with mice fed a diet with a high proportion of casein, insulin sensitivity tended to be reduced in mice fed diets with high amount of soy and cod, and insulin sensitivity was significantly reduced in mice fed diets with a high content of beef, chicken or pork (Fig. 2E and F).

**Protein source and protein:carbohydrate ratio modulate the obesogenic effect of high fat diets.**

To further analyse the influence of protein intake and source, we performed a second experiment using a factorial design with protein:carbohydrate ratio and protein source as categorical predictors. As mice fed casein and pork represented the extremes in experiment 1, these were chosen as protein sources. A casein-based regular diet was used as a reference. The dietary compositions are presented in table 3. As expected, body weight gain and adipose tissue mass in mice fed a high proportion of casein were comparable to those mice fed the reference diet (Fig. 3A and B). Both protein level and source influenced on body weight gain and adipose tissue mass (Fig. 3A and B), but lean masses were comparable in this experiment (Fig. 3C). Casein fed mice were less obese than pork fed mice, but increasing the protein:carbohydrate ratio attenuated obesity development independently of the protein source (Fig. 3B). As feed intake was similar in all four groups, calculation of feed efficiency mirrored body weight gain (Fig. 3D). Feed efficiency was, however, not directly linked to digestibility, as both fat- and nitrogen digestibility were reduced with a low protein:carbohydrate ratio (Fig. 3D-F). However, independent of protein amount, fat digestibility was higher in pork than casein fed mice (Fig 3E).

Plasma levels of urea and 4-OH butyrate were higher in mice fed casein than in mice fed pork, suggesting that catabolism of both protein and fat were higher in casein than pork fed mice (Fig. 3G and H). Moreover, plasma levels of free fatty acids (FFA) were higher in casein than in pork
fed mice, suggesting increased lipolysis (Fig. 3I). The protein:carbohydrate ratio did not modulate plasma levels of FFA or 4-OH butyrate, but as expected, plasma levels of urea were higher in mice fed a high proportion of protein.

To evaluate if the apparent differences in metabolism and altered energy expenditure, we utilized indirect calorimetry. As body mass and body composition are strong determinants for both O$_2$-utilization and CO$_2$-production [40], a new set of mice was used in this experiment. The mice were placed in the metabolic cages and fed a low fat reference diet for 72 hours. Gas exchange and beam breaks, as a proxy for activity, were recorded during the last 48 hours. The mice were subsequently fed the experimental diets and gas exchange and activity recorded, and diet-induced changes for each individual were calculated. The source of protein, but not the amount influenced on the activity of the mice. Interestingly, when mice were fed diets containing pork, their activity tended to be reduced during the light period (Fig. 4A) and their activity was significantly reduced during the dark period (Fig. 4B). The type of protein did, however, not affect energy expenditure (Fig. 4C-D). It is well documented that high fat diets increase EE and reduce RER, but unexpectedly, a low proportion of protein relative to sucrose led to a stronger diet-induced EE during both the light- (Fig. 4C) and the dark period (Fig. 4D).

Still, the reduction in RER was more pronounced when the proportion of protein was high (Fig. 4E and F), indicating a lower utilization of carbohydrates.

**Protein source and protein:carbohydrate ratio modulate glucose homeostasis in mice fed high fat diets.**

The protein:carbohydrate ratio did not affect plasma glucose levels, however, insulin levels were higher in mice fed a low protein:carbohydrate ratio. Although only mice fed a high proportion of casein were protected against reduced insulin sensitivity (Fig. 5A and B) and glucose tolerance (Fig. 5C and D), both protein:carbohydrate ratio and the protein source were able to alter these parameters. We also measured insulin levels before and 15 min after glucose injection during the GTT (Fig. 5E and F). At both time points, the insulin levels reflected the state of obesity as insulin levels were higher in pork than casein fed mice, and higher when the dietary protein:carbohydrate level was low.

When dietary fat intake is high, BCAA contributes to development of obesity associated insulin resistance [34]. Compared with pork protein, the levels of BCAA in casein are high (Table 4). Still, analyses of the amino acid profile in plasma collected from mice that were feed deprived
for 4 hours did not reveal higher levels of circulating BCAA in casein compared with pork fed mice. In fact, circulating levels of leucine were higher in pork fed mice (Table 5). Since BCAA metabolism in adipose tissue is able to modulate the circulating BCAA levels [41], we measured the expression of the two first enzymes required for BCAA oxidation, branched chain aminotransferase (Bcat2) and the branched chain ketoacid dehydrogenase complex (BCKDHC) subunits, Bckdha and Dbt, as well as Dld (a subunit of BCKDHC that is shared with pyruvate pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes) in inguinal white (iWAT) and interscapular brown adipose tissue (iBAT). In iBAT expression of Bcat2 was lower in mice fed a high protein:carbohydrate ratio, but expression of these genes was not significantly affected by the type of protein (Fig. 6A). However, in iWAT expression of Dbt mRNA and Dld mRNA were higher in mice fed pork than in mice fed casein, independent of protein amount (Fig. 6B).

**Protein source and protein:carbohydrate ratio influence on iBAT histology.**

To investigate whether an increased protein:carbohydrate ratio lead to an increased amount of BRITE cells in iWAT, histological examinations were performed. Increasing the protein:carbohydrate ratio seemed to lead to smaller adipocytes in iWAT (Fig. 7A). Furthermore, the adipocytes in iWAT from casein fed mice seemed smaller than adipocytes from pork fed mice (Fig. 7A). Expression levels of “brown marker genes” were not significantly altered by either the type or amount of dietary protein (Fig. 7B).

Histological examination of iBAT revealed that only adipocytes from mice fed a high proportion of casein, maintained a classic brown phenotype (Fig. 8A). Adipocytes in mice fed a low protein:carbohydrate ratio, in particular when mice were fed pork, had large “white-like” adipocytes with a single large lipid droplet (Fig. 8A). Expression levels of “brown marker genes” were not significantly altered by either the type or amount of dietary protein (Fig. 8B), but immunohistochemical analyses demonstrated that a higher proportion of adipocytes in casein compared to pork fed mice stained positive for UCP1 (Fig. 8C).
DISCUSSION

The notion that an increase in the protein:carbohydrate ratio efficiently attenuates the obesogenic potential of high fat diets when fed to rodents, is largely based on studies where casein or whey is used as protein sources [3-10]. Thus, in this study we aimed to evaluate development of obesity in rodents fed high fat diets with a high protein:carbohydrate ratio using different protein sources.

We report striking divergence between different protein sources in relation to obesity development. Whereas a high proportion of casein attenuated obesity, mice fed a high proportion of cod, beef, chicken or pork accumulated significantly increased amounts of adipose tissue, became insulin resistant and glucose intolerant. The observed differences in obesity development were not related to energy intake. Thus, we observed large differences in feed efficiency. However, feed efficiency appeared not to be directly related to neither fat nor nitrogen digestibility. Using a factorial design with protein:carbohydrate ratio and protein source as categorical predictors, where casein and pork protein were chosen as protein sources, we demonstrated that both protein source and amount influenced on the development of obesity and insulin resistance. However, different mechanisms may underlie the observed effects.

We utilized indirect calorimetry to evaluate energy metabolism. As both body mass and composition are strong determinants for the gas exchange [40], we subjected the mice to indirect calorimetric measurements before onset of obesity at the transition from a regular chow reference diet to the experimental diets. Of note, we observed no significant difference in EE that could explain the difference in feed efficiency or adiposity. Changing to high fat diets led to an expected reduction in RER, in particular during the dark phase. The reduction in RER was more pronounced when the proportion of protein was high, indicating a lower utilization of carbohydrates. In keeping with higher plasma levels of urea in mice fed diets with a high proportion of protein for 11 weeks, our data support the expectations in terms of dietary substrate availability, i.e., that increasing the protein:carbohydrate ratio in high fat diets led to a higher utilization of amino acids at the expense of carbohydrates. The loss of energy in form of ATPs used in syntheses of urea and by the required conversion of amino acids to glucose [15, 16], may contribute to the reduced feed efficiency when mice are fed diets with high protein:carbohydrate ratios.
The protein source did not modulate RER, but plasma levels of urea and 4-OH butyrate were higher in mice fed casein than pork, indicating that catabolism of both protein and fat was higher in casein than pork fed mice. Interestingly, the source of protein, but not the amount, altered the activity of the mice. When mice were fed diets containing pork, their activity tended to be reduced during the light period and their activity was significantly reduced during the dark period. In line with this observation, we have previously reported an inverse correlation between locomotor activity and development of diet-induced obesity, without being able to detect differences in EE [37]. Lower locomotor activity in mice fed protein from pork may very well over time influence on feed efficiency and obesity development.

Energy may also be lost by conversion to heat by uncoupling of mitochondria in brown adipocytes. An increased number of UCP1 expressing adipocytes protects against diet induced obesity [22], whereas UCP1 ablation augments obesity in mice exempt from thermal stress [42]. White and brown adipocytes are found together in both visceral and subcutaneous fat depots forming a plastic organ [17]. Exposure to themoneutrality, aging and obesity leads to a “whitening” of the adipose organ [43]. However, we have earlier demonstrated that a high proportion of casein in the diets led to increased expression of Ucp1 in inguinal adipose tissue [7-9]. Here we confirm that the proportion of dietary protein seemingly influenced the size of the adipocytes in the inguinal depot [8]. Fasting is known to down regulate Ucp1 expression [44], and the four hour feed deprivation before collection of tissue in this experiment may account for the lack of significant differences in Ucp1 in expression. Furthermore, we were unable to detect UCP1 by immunohistochemistry in these cells. Of note, a lower proportion of UCP1 immunoreactive cells with a more brown like phenotype was maintained in the interscapular region collected from mice fed a low proportion of protein. In particular, a large proportion of the adipocytes from the obese mice fed pork protein at a low protein:carbohydrate ratio was unilocular cells and not UCP-immunoreactive. Adipocytes from the lean mice fed a high proportion of casein on the other hand had a large proportion of multilocular UCP-immunoreactive adipocytes. Thus, maintenance of a brown phenotype in the adipocytes in the interscapular region by a high proportion of casein may protect against obesity development.

The protective effect of intake of high amounts of casein and whey as observed by others [10], on both obesity development and insulin resistance may be related to their high content of BCAA as leucine partially mimics the effect of high protein diets [32, 45]. In our studies, the reduced glucose tolerance and insulin sensitivity in pork fed mice may be directly related to the state of obesity. However, both amino acid composition and amino acid metabolism may
influence directly on glucose homeostasis. On one hand, leucine may directly interact in the insulin signaling pathway, and may furthermore increase the recycling of glucose via the glucose-alanine-cycle [32, 45]. However, metabolic profiling has identified elevated BCAA as a signature related to obesity and insulin resistance in humans [34], and chronic elevated levels of BCAA in lean mice with disrupted mitochondrial branched chain aminotransferase is accompanied with insulin resistance [33]. Despite the higher content of BCAA in casein than pork, mice fed casein did not have higher levels of circulating BCAA. This might be due to modulation of circulating BCAAs in adipose tissue [41].

We conclude that diets with high protein:carbohydrate ratio where casein or whey is used as protein sources may not be representative for high protein diets. These observations are in line with rodent studies demonstrating that diets with standard levels of different types of proteins have different obesogenic potential as well as different effect on insulin sensitivity [29-31, 35, 46, 47]. Given the popularity of high protein diets, this warrants further investigations in humans.
We thank Dr. Pavel Flachs and Prof. Jan Kopecky for kindly providing the UCP1 antibody used for immunohistochemistry and the staff at NIFES for technical assistance and animal care. We would like to specifically acknowledge the early contribution of Vigdis Misje Hagen to this project.

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The authors have no conflicting interests, financial or otherwise.

B.L, K.K. and L.M designed research. U.L.A, A.K.R, E.F, S.B, A.L.B and K.R.F performed experiments and all authors interpreted the results. U.L.A and L.M wrote the manuscript. All authors edited and revised the manuscript. All authors approved the final version. K.K. and L.M have primarily responsibility for the final content.
LITERATURE CITED


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44. Nedergaard, J., V. Golozoubova, A. Matthias, A. Asadi, A. Jacobsson, and B. Cannon, UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and


Table 1. Compositions of the experimental diets used in experiment 1

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<th>Component (g/kg)</th>
<th>RD</th>
<th>Casein</th>
<th>RD</th>
<th>Casein</th>
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<th>Cod</th>
<th>Beef</th>
<th>Chicken</th>
<th>Pork</th>
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**Analyzed**

| Fat (g/kg) | 71 | 252 | 246 | 258 | 246 | 238 | 247 | 270 |
| Crude protein § (g/kg) | 192 | 180 | 360 | 350 | 360 | 380 | 380 | 370 |
| Energy kJ/g | 18 | 23 | 24 | 24 | 23 | 24 | 24 | 24 |

Composition of the regular reference diet (RD), the high fat high sucrose reference diet (HF/HS) and the high fat high protein diets (HF/HP). Analyzed values represents mean of triplicate measurements.* The calculated contribution of fat present in the protein sources. † AIN93G. ‡ AIN93VX NCR95 compliant. § N*6.25
Table 2. *Amino acid compositions of the experimental diets used in experiment 1*

<table>
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<th>HF/HS</th>
<th>HF/HP</th>
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<td>Casein Soy Cod Beef Chicken Pork</td>
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<td>≤ 0.3</td>
</tr>
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<tr>
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<tr>
<td>Σ BCAA</td>
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Values represent mean of triplicate measurements. Σ AA represent the sum of all amino acids. Σ BCAA represent the sum of branched chain amino acids (Leu, Ile and Val).
Table 3. Compositions of the experimental diets used in experiment 2 and 3.

<table>
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* The calculated contribution of fat present in the protein sources. †AIN93G. ‡AIN93VX NCR95 compliant. § N*6.25.

Compositions of the regular reference diet (RD), the high fat high sucrose (HF/HS) and the high fat high protein (HF/HP) using casein and pork as protein sources. Analyzed values represent mean of triplicate measurements.
Table 4. Amino acid compositions of the experimental diets used in experiment 2 and 3

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<thead>
<tr>
<th>AA (mg/ml)</th>
<th>Casein</th>
<th>Pork</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF/HS</td>
</tr>
<tr>
<td><strong>Indispensable</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Hyp</td>
<td>≤ 0.3</td>
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</tr>
<tr>
<td>Ile</td>
<td>9.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Leu</td>
<td>17.5</td>
<td>17.0</td>
</tr>
<tr>
<td>Lys</td>
<td>16.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Met</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>Phe</td>
<td>8.5</td>
<td>8.7</td>
</tr>
<tr>
<td>Thr</td>
<td>7.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Trp</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Val</td>
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<td>11.6</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Ala</td>
<td>5.7</td>
<td>5.5</td>
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<tr>
<td>Ser</td>
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<tr>
<td>Tyr</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Tau</td>
<td>≤ 0.3</td>
<td>≤ 0.3</td>
</tr>
<tr>
<td>Σ AA</td>
<td>196.5</td>
<td>191.0</td>
</tr>
<tr>
<td>Σ BCAA</td>
<td>38.7</td>
<td>37.6</td>
</tr>
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</table>

Values represent mean of triplicate measurements. Σ AA represent the sum of all amino acids. Σ BCAA represent the sum of branched chain amino acids (Leu, Ile and Val).
Table 5. Plasma concentrations of amino acids in mice fed high fat high sucrose (HF/HS) or high fat high protein (HF/HP) diets with casein or pork as the protein source.

<table>
<thead>
<tr>
<th>AA (µmol/ 100 ml plasma)</th>
<th>Casein</th>
<th>Pork</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Indispensable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>5.4 ± 0.3</td>
<td>5.4 ± 0.4</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Ile</td>
<td>8 ± 1</td>
<td>11 ± 2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Leu</td>
<td>16 ± 1</td>
<td>20 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Lys</td>
<td>25 ± 2</td>
<td>32 ± 4</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Met</td>
<td>7.3 ± 0.8</td>
<td>8.0 ± 1.5</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>Phe</td>
<td>9.1 ± 0.6</td>
<td>9.4 ± 0.9</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>Thr</td>
<td>20 ± 2</td>
<td>23 ± 3</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>Trp</td>
<td>4.8 ± 0.2</td>
<td>5.5 ± 0.5</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>Val</td>
<td>26 ± 2</td>
<td>30 ± 5</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Dispensable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>65 ± 6</td>
<td>59 ± 7</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>Arg</td>
<td>5.6 ± 0.6</td>
<td>7.2 ± 0.9</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Asp</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
<td>11 ± 4</td>
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<td>Glu</td>
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<tr>
<td>Ser</td>
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<td>Tyr</td>
<td>12 ± 1</td>
<td>14 ± 3</td>
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</tr>
<tr>
<td>Tau</td>
<td>49 ± 7</td>
<td>55 ± 13</td>
<td>49 ± 7</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=8).
FIGURE LEGENDS:

FIGURE 1 Effect of high fat high protein diets with different protein sources on body mass, body composition and digestibility
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) using casein as the protein source or high fat diets with a high protein:carbohydrate ratio (HF/HP) using different protein sources for 11 weeks. Mice fed a regular chow diet (RD) were used as a reference. Body mass development was recorded (A) and fat mass (B) and lean mass (C) determined after 8 weeks of feeding. Energy intake (D) and feed efficiency (E) was calculated based on data collected during the first 8 weeks of feeding. Apparent fat (F) and nitrogen (G) digestibility was calculated based on feed intake and feces collection during the 6th week of feeding. Data represent mean ± SEM (n=9). Different small letters denote significant differences between the groups (P<0.05).

FIGURE 2 Effect of high fat high protein diets with different protein sources on glucose homeostasis
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) using casein as the protein source or high fat diets with a high protein:carbohydrate ratio (HF/HP) using different protein sources for 11 weeks. The mice were feed-deprived 4h before blood collection and termination. Plasma was prepared and the levels of glucose (A) and insulin (B) were measured. A glucose tolerance test (GTT) was performed (C) and AUC was calculated (D) after 10 weeks of feeding. An insulin tolerance test (ITT) was performed on animals fasted for 6h (E) and area under the curve (AUC) was calculated (F) after 9 weeks of feeding. Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

FIGURE 3 Effect of high fat diets with high and low protein:carbohydrate ratios on body mass gain, body composition and digestibility
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before blood collection and termination. Body mass development was recorded and body weight (BW) gain calculated (A), fat mass (B) and lean mass (C) determined after 8 weeks of feeding. Feed efficiency (D) was calculated based on data...
collected during the first 8 weeks of feeding. Apparent fat (E) and nitrogen (F) digestibility was calculated based on feed intake and feces collection during the 6th week of feeding. Plasma levels of urea (G), hydroxy butyrate (H) and non-esterified fatty acids (NEFA) (I) were measured in plasma collected at termination. Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

FIGURE 4 Effect of high fat diets with high and low protein:carbohydrate ratios on activity energy expenditure (EE) and respiratory exchange ratio (RER)
Male C57BL/6 mice were fed a regular chow reference diet. After three days the diets were switched to high fat diets with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources. Diet induced changes in activity (A and B), EE (C and D) and RER (E and F) for each individual were calculated. Data represent mean ± SEM (n=4). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

FIGURE 5 Effect of high fat diets with high and low protein:carbohydrate ratios on glucose homeostasis
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. An insulin tolerance test (ITT) was performed (A) and AUC was calculated (B) after 10 weeks of feeding. A glucose tolerance test (GTT) was performed on animals fasted for 6 h (C) and area under the curve (AUC) was calculated (D) after 9 weeks of feeding. Blood was collected and serum prepared for insulin measurements before (E) and 15 min after (F) glucose administration. Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with.*
FIGURE 6 Effect of high fat diets with high and low protein:carbohydrate ratios on expression of genes involved in branched chain amino acid transamination in adipose tissue
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before termination. RNA was purified from interscapular brown (iBAT) and inguinal white (iWAT) adipose tissue. cDNA was synthesized and expressions of first branched chain aminotransferase (Bcat2) and the branched chain ketoacid dehydrogenase complex subunits, Bckdhα1, Dbt and Dld were measured in iBAT (A) and iWAT (B). Expression levels were normalized to TATA- box binding protein (Tbp). Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

FIGURE 7 Effect of high fat diets with high and low protein:carbohydrate ratios on histological appearance and gene expression in inguinal white adipose tissue (iWAT)
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before termination. Sections of iWAT (n=4) were stained with eosin and hematoxylin (A) and expressions of uncoupling protein 1 (Ucp1), deiodinase, iodothyronine, type II (Dio2), peroxisome proliferator-activated receptor-γ coactivator 1α (Ppargc1a) and cell death-inducing DFFA-like effector (Cidea) were measured and expression levels were normalized to TATA- box binding protein (Tbp) (B). Gene-expression data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

FIGURE 8 Effect of high fat diets with high and low protein:carbohydrate ratios on histological appearance, protein and gene expression in interscapular brown adipose tissue (iBAT)
Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before termination. Sections of iBAT (n=4) were stained with eosin and hematoxylin (A) and expressions of uncoupling protein 1 (Ucp1), deiodinase, iodothyronine, type II (Dio2), peroxisome proliferator-activated receptor-γ coactivator 1α (Ppargc1a) and cell death-inducing DFFA-like effector (Cidea) were measured and expression levels were normalized to TATA- box binding protein (Tbp). Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.
(Ppargc1a) and cell death-inducing DFFA-like effector (Cidea) were measured and expression levels were normalized to TATA-box binding protein (Tbp) (B). Gene expression data represent mean ± SEM (n=9). Sections of iBAT (n=4) were immunohistologically stained with an UCP1-antibody and the area quantified (C). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with*.

**Supplementary figure 1 Effect of high fat high protein diets with different protein sources on adipose- and lean tissue mass**

Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) using casein as the protein source or high fat diets with a high protein:carbohydrate ratio (HF/HP) using different protein sources for 11 weeks. Mice fed a regular chow diet (RD) were used as a reference. Individual adipose tissue and lean tissue dissected out at termination of the mice (A-D). Epididymal white adipose tissue (eWAT) (A), inguinal white adipose tissue (iWAT) (B), retroperitoneal white adipose tissue (prWAT) (C), intrascapular brown adipose tissue (IBAT) (D), Liver (E), tibialis muscle (F). Data represent mean ± SEM (n=9). Different small letters denote significant differences between the groups (P<0.05).

**Supplementary figure 2 Effect of high fat diets with high and low protein:carbohydrate ratios on adipose- and lean tissue mass**

Male C57BL/6 mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. Individual adipose tissue and lean tissue dissected out at termination of the mice (A-H). Epididymal white adipose tissue (eWAT) (A), inguinal white adipose tissue (iWAT) (B), retroperitoneal white adipose tissue (prWAT) (C), intrascapular brown adipose tissue (IBAT) (D), kidney (E), liver (F), pankereas (G), tibialis muscle (H). Data represent mean ± SEM (n=9). Different small letters denote significant differences between the groups (P<0.05).
Figure 2

A. Fasting (4hrs) glucose (mmol/L)

B. Fasting (4hrs) insulin (µg/L)

C. Blood glucose during GTT (mM)

D. AUC GTT (mmol/Lxh)

E. Blood glucose during ITT (mM)

F. AUC of ITT (mmol/Lxh)

- RD
- HF/HP Casein
- HF/HP Cod
- HF/HP Chicken
- HF/HS Casein
- HF/HP Soy
- HF/HP Beef
- HF/HP Pork
Figure 4

A. Change in activity during light phase (beam breaks)

B. Change in activity during dark phase (beam breaks)

C. Change in EE during light phase (kJ/h*kg BW)

D. Change in EE during dark phase (kJ/h*kg BW)

E. Change in RER during light phase

F. Change in RER during dark phase

Legend:
- HF/HS Casein
- HF/HP Casein
- HF/HS Pork
- HF/HP Pork
Figure 5

A. Blood glucose during ITT (mM)

B. AUC of ITT (mmol/Lxh)

C. Blood glucose during GTT (mM)

D. AUC of GTT (mmol/Lxh)

E. Fasting (6hrs) plasma insulin (pmol/L)

F. Insulin (15 min) during GTT (pmol/L)
Figure 6

A
Gene expression iBAT (relative expression)

Bcat2

Bckdha

Dbt

Dld

B
Gene expression iWAT (relative expression)

Bcat2

Bckdha

Dbt

Dld
Figure 7

A

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<th>iWAT</th>
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<td>High Protein</td>
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B

Gene expression iWAT (relative expression)

- **Ucp1**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Dio2**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Ppargcla**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Cidea**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork
Figure 8

A

iBAT

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<tr>
<th></th>
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<tbody>
<tr>
<td>High sucrose</td>
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<tr>
<td>High Protein</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
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</tbody>
</table>

B

Gene expression iBAT (relative expression)

- **Ucp1**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Dio2**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Ppargc1a**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Cidea**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork
Figure 8

C

<table>
<thead>
<tr>
<th>Casein</th>
<th>Pork</th>
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<tr>
<td>High sucrose</td>
<td>High sucrose</td>
</tr>
<tr>
<td>High Protein</td>
<td>High Protein</td>
</tr>
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</table>

**iBAT**

![High sucrose Casein](image)

![High Protein Casein](image)

![High sucrose Pork](image)

![High Protein Pork](image)

**UCP-1 staining iBAT (%)**

- RD
- HF/HS Casein
- HF/HP Casein
- HF/HS Pork
- HF/HP Pork

- a
- b

* *
A Mixture of Cod and Scallop Protein Reduces Adiposity and Improves Glucose Tolerance in High-Fat, High-Sucrose Fed Male C57BL/6J Mice

Hanne S. Tastesen, Alexander K. Rønnevik, Kamil Borkowski, Lise Madsen, Karsten Kristiansen and Bjørn Liaset

This study was part of the ‘Lean seafood in the prevention of the metabolic syndrome’ project which is financially supported by the Norwegian Research Council (200515/I30) and the National Institute of Nutrition and Seafood Research. Parts of this work were also financially supported by the Danish Council for Strategic Research (project No 2101-08-0053), the Danish Dairy Research Foundation, the Danish Natural Science Research Council, the Novo Nordisk Foundation and the Carlsberg Foundation.


Supplemental Table 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org

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National Institute of Nutrition and Seafood Research, Bergen, Norway

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Running title: Reduced adiposity in mice fed seafood protein
Word count: 6862; Number of figures: 3; Number of tables: 3; Online Supporting Materials: 1
Author list for indexing: Tastesen, Rønnevik, Borkowski, Madsen, Kristiansen, Liaset
Abstract

Background: Low and high protein diets regulate energy metabolism in animals and humans.

Objective: We aimed to evaluate whether different dietary protein sources modulate energy balance when ingested at ‘normal’ levels.

Methods: Obesity-prone male C57BL/6J mice were pair-fed high fat (67 energy percent), high sucrose (18 energy percent) and normal protein (15 energy percent) diets with casein, chicken filet or a mixture of cod and scallop (1:1 on amino acid content) as protein sources. Effects on metabolism were evaluated by indirect calorimetry performed before onset of diet-induced obesity and glucose tolerance test after six weeks of feeding.

Results: At equal energy intake, the casein- and cod/scallop-fed mice had lower feed efficiency than chicken-fed mice, which translated into significantly reduced adipose tissue mass after seven weeks of feeding. Concomitantly, the chicken-fed mice had elevated hepatic TAG and 4h feed-deprived plasma total cholesterol concentrations relative to casein- and cod/scallop-fed mice. The reduced adiposity in the casein-fed, compared to the chicken-fed mice, was likely related to the three percent lower apparent fat digestibility in casein-fed compared to chicken- and cod/scallop-fed mice. Spontaneous locomotor activity decreased in casein- and chicken-fed mice when shifting from low-fat to high-fat diets, but cod/scallop-feeding tended ($P = 0.06$) to attenuate this decrease. Moreover, at transition from low-fat to high-fat feeding, energy expenditure decreased in all groups, but was decreased to a greater extend in the casein-fed than in the cod/scallop-fed mice, indicating that protein sources regulated energy expenditure differently. Despite their lean phenotype, the casein-fed mice became more glucose intolerant compared to the chicken- and cod/scallop-fed mice.

Conclusion: Protein from different sources modulates energy balance in C57BL/6J mice when given at normal level. Ingestion of a mixture of cod/scallop protein prevented diet-induced development of obesity compared to intake of chicken filet and preserved glucose tolerance, compared to casein-intake.

Key words: casein, chicken, cod, diet-induced obesity, energy expenditure, glucose tolerance, indirect calorimetry, protein sources, scallop, seafood.
Introduction

Identifying nutritional strategies to alleviate the obesity pandemic are of great interest. Diet-induced thermogenesis, i.e. the regulated liberation of energy in the form of heat [1], could lower food efficiency, and thereby act preventive against obesity development. Already in 1939, induction of adaptive thermogenesis by feeding rats very low (4-8 wt%) or very high (54 wt%) protein diets was described [2]. Later, the increment in thermogenesis by low protein diets was verified in rats [3, 4], in baby pigs [5], and similar effects were observed in young human subjects [6]. Thus, intake of low-protein diets induces thermogenesis, but instead of resulting in decreased body mass, the reduced food efficiency is compensated for by a higher food intake [7].

Whereas low-protein diets may increase energy intake, high-protein diets are more satiating than an isoenergetic amount of carbohydrates or fat [8, 9]. Moreover, high-protein intake induces higher post-prandial thermogenesis than high-carbohydrate ingestion does [10, 11]. It is likely that both reduced energy intake and elevated thermogenesis might be underlying mechanisms explaining, at least in part, the reduction in body mass observed in mice [12-15] and humans [9, 16, 17] by replacing carbohydrates with protein.

Despite the known effects of low- and high protein diets on thermogenesis, limited information exists on whether varying protein sources affect body mass and composition differently [18]. From studies in rodents, we know that consumption of hydrolyzed rather than intact proteins reduces body mass gain, adipose tissue mass and hepatic and plasma lipid concentrations [19-21]. Moreover, whey ingestion decreases fat mass relative to casein intake in mice [22-24], and an intervention study with free-living overweight and obese subjects indicated that intake of whey protein, but not soy protein (both ~56g protein/day for 23 weeks) resulted in a significant reduction in body mass, fat mass and waist circumference, relative to the carbohydrate (maltodextrin) control treatment [25]. In a randomized, double-blinded intervention study with cross-over design, ingestion of a liquid test meal consisting of 50% whey protein, 40% carbohydrate and 10% fat, induced a higher postprandial thermic effect than equal amounts of casein and soy protein [26]. Thus, studies in both rodents and humans indicate that protein sources might differently affect body weight gain and adiposity.
Recently, we showed that obesity-prone C57BL/6J mice exhibited distinct metabolic responses to intake of various dietary protein sources in connection with a high fat, high sucrose (HFHS) background diet [27]. Mice fed scallop muscle as the sole protein source were protected against diet-induced obesity, enlarged liver mass and hyperlipidemia as compared to mice fed chicken- or cod-filets. However, the scallop-fed mice also had lower ad libitum feed intake, indicating different satiating effects of the protein sources [27]. Therefore, the present study was undertaken in order to elucidate whether the protein sources casein, chicken breast filet or a mixture of cod filet and scallops muscle, would affect diet-induced obesity during equal energy intake (pair-feeding) in HFHS-diets fed to male C57BL/6J mice for seven weeks.

Materials and methods

Ethical statement  The animal experiments were approved by the Norwegian National Animal Health Authorities (permit number 3421, Expt. 1) and the Danish National Animal Experiments Inspectorate (permit number 2012-15-2934, Expt. 2) and care and handling complied with the ethical standards of the 1964 Helsinki Declaration, as revised in 1983. No adverse events were observed.

Experimental diets  Low-fat (LF) diet (OpenSource Diet no D12450B, Research Diets, NJ, USA) was used to feed mice during acclimatization period and as a reference diet (Table 1 and Table 2). Three isoenergetic experimental HFHS-diets were made with protein from different sources; casein sodium salt from bovine milk (casein) chicken breast filets (chicken) and a mixture of wild caught cod filets and Canadian scallop muscles (cod/scallop) as previously described [27, 28] with the modification that 3 g cystine/kg diet were added to all diets in the present study. The final compositions of the diets are shown in Table 1 and Table 2. Feed efficiency was calculated as body mass gain per energy intake (g BM/MJoule).

Animal studies  Male C57BL/6JBomTac mice (Taconic, Ejby, Denmark) weighing approximately 25 g at arrival were housed individually at thermoneutrality (28 ± 1°C) under a 12h light-dark cycle. The mice were pair-fed to obtain equal energy intake. The mice were fed low fat reference diet (LF) while acclimatizing to the animal facility before switching to the experimental diets. After the feeding period the mice were anaesthetized by inhalation of isoflurane (4%, Isoba Vet) and euthanized by exsanguination by cardiac puncture. The blood was heparinized (20.2 units sodium heparin/mL blood), centrifuged (4°C, 2500g, 5 min)
and plasma fractions were stored at -80°C until analysis. Two experiments were carried out as follows;

Experiment 1 (Expt. 1) encompassed 32 mice (n = 8/group) which were assigned into experimental groups by bodyweight after five days acclimatization and fed either LF or HFHS-diets (Table 1 and 2) for seven weeks. In Expt. 1 LF was used as a reference group and not included in the statistical analyses unless specifically stated. At week six the mice were subjected to an oral glucose tolerance test (O-GTT). After seven weeks the mice were terminated and 4h feed-deprived plasma as well as epididymal white adipose tissue (eWAT), perirenal/retoperitoneal white adipose tissue (p/rWAT), inguinal white adipose tissue (iWAT), and interscapular brown adipose tissue (iBAT) were collected and frozen at -80°C. Experiment 2 (Expt. 2) included 30 mice (n = 10/group). After seven days acclimatization the mice were placed in indirect calorimetry cages for 72 hours for baseline indirect calorimetry and activity measurements while still on LF. Based on body weight and baseline measurements of total activity and RER in light and dark phases the mice were divided into three groups and fed the experimental high fat, high sucrose-diets for another 72 hours of measurements and subsequently terminated.

Diet composition analyses Diets were analyzed as previously described [27]. In short; Energy contents were determined by bomb calorimetry (Parr Instruments, Moline, IL, USA). For total amino acid analysis norvaline was added to samples as internal standard, samples were hydrolyzed (6 M HCl, 110±2°C, 22h) and derivatized (AccQ-Tag Ultra Derivatization Kit, Waters, MA, USA). Amino acids were separated and detected on the ACQUITY UPLC System (Waters, MA, USA), identified using Pierce Amino Acid Standard H (Thermo Fisher Scientific Inc., IL, USA) to which norvaline, taurine and hydroxy-proline were added and finally quantified by internal and external standard regression. For tryptophan analysis the samples were hydrolyzed (Ba(OH)₂, 110±2°C, 20h), pH adjusted to 6.2, separated by HPLC (Shimadzu 6A/6B) equipped with a SUPELCOSILTM LC-18 HPLC-column, detected in UV-spectrophotometer (Shimadzu SPD 6A) at 280 nm and quantified using a standard curve of L-Tryptophan (T-0254, Sigma-Aldrich). Total cysteine was determined, at the Norwegian Institute of Food, Fishery and Aquaculture, after oxidation of cysteine/cystine (9:1 performic acid (88%): H₂O₂ (30%) (v/v)) to yield cysteic acid.

Feces collection After six weeks the mice were placed in cages with standard wood chip layer replaced by paper lining for the purpose of collecting feces for one week. Feces left behind in cages were collected, weighted and frozen at -80°C until analyses for nitrogen and total fat content. Based on feces
measurements and diet-intake data apparent digestibility of fat and nitrogen was calculated as follows: 100 × (intake (mg) - fecal output (mg))/(intake (mg)).

**Nitrogen and fat content in diets and feces** Nitrogen content was determined by the Dumas method using Leco FP-528 nitrogen analyzer (Leco Corp, MI, USA). The crude protein content in the diets was calculated as nitrogen content multiplied by 6.15 for casein and 5.6 for chicken filet and cod/scallop [29]. Total fat content was determined gravimetrically after extraction with organic solvents before and after acidic hydrolysis as described previously [27].

**Plasma measurements** MaxMat PL II analyzer (MAXMAT S.A., Montpellier, France) and conventional kits were used to measure 4h feed-deprived plasma lactate [Sentinel Diagnostics, Italy], TG, total cholesterol, LDL-cholesterol and glucose [MaxMat, France] and HDL-cholesterol and total bile acids [Dialab, Austria] concentrations. 4h feed-deprived plasma insulin concentrations were analyzed using DRG mouse insulin ELISA kit (DRG Diagnostics, Germany).

**Liver lipid analysis** Total liver lipids were extracted with chloroform:methanol (2:1, v:v). Lipid classes were analyzed via automated Camaq HPTLC system and separated on HPTLC silica gel 60 F plates as previously described [30].

**qRT-PCR** was performed as described previously [21]. In short, total RNA was isolated from tissue samples with TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Qualities and concentrations of the purified RNA were assessed using NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Using GeneAmp PCR 9700 (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA), TaqMan RT buffer, dNTP, oligo(dT)primers, RNase inhibitor, Multiscribe Reverse Transcriptase (N808-0234, Applied Biosystems) and RNase-free water RT reactions were performed for 60 min at 48°C. The produced cDNA was subject to qRT-PCR in LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Indianapolis, IN, USA) using SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche Applied Sciences) and gene-specific primers (Supplemental Table 1). Data were analyzed as a ratio between gene of interest and reference gene by taking into account the PCR efficiencies of the different genes and normalizing to both TATA box binding protein (*Tbp*) as reference
gene and LF as control samples as follows; Ratio = \((E_{\text{Target}})^{\Delta CT \text{ Target (control - sample)}} / (E_{\text{Ref}})^{\Delta CT \text{ Ref (control - sample)}}\),

where E is efficiency of the appropriate PCR reaction, Target is the gene of interest, Ref is the reference gene (Tbp), sample is the sample of interest, control is LF and \(\Delta CT\) is the difference in CT-values between control and sample [31, 32].

**Oral glucose tolerance test** After six weeks on experimental diets mice were subjected to an 6h feed-deprived oral glucose tolerance test (O-GTT). Early in the morning of the test day mice were placed in cages without feed and after six hours feed-deprived blood glucose was measured in whole blood, taken from the tail vein by a Bayer Contour glucometer and glucose test strips (Bayer, Germany). Glucose was administered by oral gavage (2 mg glucose/g body mass) and blood glucose concentration was measured 15, 30, 60 and 120 minutes after glucose administration. Blood glucose incremental area under the curve (iAUC, mmol/L/h) was calculated as AUC above baseline value, i.e., 6h feed-deprived blood glucose, by applying the trapezoid rule to a plot of group mean blood glucose concentration versus time of measurements [33, 34].

**HOMA-IR and QUICKI** Based on 4h feed-deprived plasma glucose and insulin Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as follows: \(1/(\log(\text{insulin [mU/l]})+\log(\text{glucose [mg/dl]}))\) [35] and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated as follows: Glucose (mmol/l)×insulin (µU/ml)/22.5 [36].

**Indirect calorimetry and spontaneous locomotor activity** VO\(_2\) and VCO\(_2\) was measured in open-circuit indirect calorimetry cages as described previously [21]. In short, the mice were housed in CaloCages (Phenomaster, TSE Systems), equipped with infrared light-beam frames (ActiMot2). VO\(_2\) and VCO\(_2\) was measured for each cage, i.e., mouse, for 1.9 min once every 30 min, while light-beam breaks were measured continuously. Measurements were performed for a total of 72h on LF and consecutively for 72h on HFHS experimental diets. Of the 72h measurements the first 24h were regarded as an adaptation period and only the subsequent 48h were used for analyses; Based on two consecutive light (06.00-17.30h) and dark (18.00-05.30h) phases respiratory exchange ratio (RER) was calculated from VO\(_2\) and VCO\(_2\) and spontaneous locomotor activity was defined as total counts of light-beam breaks. Energy expenditure (EE) was calculated as follows; 16.3 kJ/L × L VO\(_2\) + 4.6 kJ/L × L VCO\(_2\) [37].
Statistical analyses The data represent group means ± SEM. After homogeneity of variances was established by Levene’s test the data were subjected ANOVA analyses followed by Tukey’s pair-wise comparisons and group means were considered statistically different at $P < 0.05$. Data that were repeatedly measured, i.e., growth, energy intake, O-GTT, RER, activity and EE were analyzed by repeated measurements ANOVA followed by Tukey’s post hoc. Data for which the variances were not homogenous were transformed, and after homogeneity of variances was established the transformed data were subjected to ANOVA followed by Tukey’s Post Hoc. Raw data are shown in all tables and figures with notes in the respective legends specifying which data were transformed before statistical analysis.

Results

Reduced body mass gain and feed efficiency in casein- and cod/scallop-fed mice

Casein- and cod/scallop-fed mice gained significantly less body mass compared to chicken-fed mice during six weeks (Fig. 1A-B). Energy intake was equal between groups (Fig. 1C) and the feed efficiency thus reflected body mass (Fig. 1D). The dietary fat intake was equal between groups, but significantly more fat was excreted in the feces in the casein-fed mice ($P < 0.001$). Thus, apparent fat digestibility was lower in this group than in chicken- and cod/scallop-fed mice (Fig. 1F). Nitrogen content differed between the diets causing slightly lower nitrogen intake in casein-fed than in chicken- and cod/scallop-fed mice ($P = 0.002$). The fecal excretion of nitrogen was lower in cod/scallop-fed than in chicken-fed mice ($P = 0.006$) and apparent nitrogen digestibility was thus higher in cod/scallop-fed than in casein- and chicken-fed mice (Fig. 1H). The lower body mass gain seen in casein- and cod/scallop mouse was reflected in lower iWAT, eWAT, p/rWAT and iBAT masses than in chicken-fed mice (Fig. 1E). No differences were seen in soleus muscle and heart tissue between groups, but liver mass was increased in chicken-fed compared to casein-fed mice and kidney mass was increased in cod/scallop-fed compared to casein-fed mice (Fig. 1G).

Elevated plasma and liver lipids in chicken-fed mice

Obesity is associated with dysregulation of plasma lipids and ectopic fat accumulation, and thus, we measured plasma and liver lipids. Plasma metabolites and liver lipids measured after 4h feed-deprivation are listed in Table 3. Chicken-fed mice had increased plasma total cholesterol compared to casein- and cod/scallop-fed mice. Furthermore, chicken-fed mice had increased plasma HDL cholesterol, LDL cholesterol
and TG concentrations compared to casein-fed mice and a tendency towards increased plasma HDL cholesterol \((P = 0.07)\), LDL cholesterol \((P = 0.06)\), and TG concentrations \((P = 0.07)\) compared to cod/scallop-fed mice. Casein-fed mice had increased HDL/total cholesterol ratio compared to chicken- and cod/scallop-fed mice and increased plasma total bile acids compared to chicken-fed mice. No differences were seen in 4h feed-deprived plasma FFA, glycerol, \(\beta\)-hydroxybutyrate or alanine aminotransferase between the groups (Table 3). Liver TG and total neutral lipid concentrations were higher in chicken-fed than in casein- and cod/scallop-fed mice, while no differences were seen in liver free cholesterol, steryl ester or diacylglycerides between groups (Table 3).

Hepatic expression of genes involved in de novo lipogenesis and gluconeogenesis is modulated by dietary protein source

Based on the increased plasma and liver lipids in chicken-fed mice we analyzed hepatic mRNA expression of genes involved in lipogenesis and gluconeogenesis. Hepatic expression of phosphoenol pyruvate carboxykinase-1 \((Pck-1)\), the rate limiting enzyme controlling gluconeogenesis by catalyzing the formation of phosphoenolpyruvate from oxaloacetate, was higher in cod/scallop-fed than in casein- and chicken-fed mice (Table 3). Hepatic expression of stearoyl-CoA desaturase-1 \((Scd-1)\), an enzyme catalyzing the conversion of SFA to MUFA, important for targeting FFA to either incorporation into lipoproteins (VLDL) or storage as TG in lipid-droplets, was higher in casein-fed than in cod/scallop-fed mice (Table 3). Furthermore, expression of sterol regulatory element-binding transcription factor 1 \((Srebf1)\) an enzyme initiating transcription of genes required for de novo lipogenesis, tended to be higher in cod/scallop-fed than in casein-fed mice. No difference in expression of the lipogenic genes Acetyl-CoA carboxylase \((Acaca)\), fatty acid synthase \((Fasn)\), Diacylglycerol acyltransferase 1 \((Dgat1)\) or 3-Hydroxy-3-methylglutaryl-CoA reductase \((Hmgcr)\) was observed (Table 3).

Decreased glucose tolerance in casein-fed and increased insulin resistance-score in chicken-fed mice

As obesity, visceral adiposity, and hepatic steatosis have been shown to associate with impaired glucose and insulin homeostasis, we subjected the mice to 6h feed-deprived O-GTT after six weeks of feeding. Casein-fed mice had higher blood glucose concentrations compared to chicken- and cod/scallop-fed mice 30 minutes after glucose administration, and compared to cod/scallop-fed mice 60 minutes after administration (Fig. 2A) despite equal feed-deprived blood glucose concentrations in the groups at the beginning of O-GTT
The glucose was administered according to body mass (2 mg glucose/g BM) and thus chicken-fed mice received a greater load of glucose than casein- and cod/scallop-fed mice (Fig. 2C). The calculated iAUC blood glucose (Fig. 2D) tended to be higher in casein-fed mice compared to chicken- and cod/scallop-fed mice ($P = 0.09$). In 4h feed-deprived plasma collected at the termination of the mice after seven weeks, lactate and glucose concentrations were higher in chicken-fed than in casein-fed mice (Fig. 2E-F) while insulin concentration tended to be increased in chicken-fed mice ($P = 0.09$, Fig. 2G). HOMA-IR insulin-resistance-score was higher in chicken-fed than in casein-fed animals and tended ($P = 0.07$) to be higher in chicken-fed than in cod/scallop-fed mice ($P = 0.07$, Fig. 2H). QUICKI insulin-sensitivity-score was higher in casein-fed than in chicken-fed mice and tended ($P = 0.08$) to be higher in cod/scallop-fed than in chicken-fed mice ($P = 0.08$, Fig. 2I).

**Difference in RER between light and dark phases abolished by HFHS-feeding**

To elucidate whether altered EE was an underlying mechanism behind differences in fat accretion, we utilized indirect calorimetry. During the 48h of indirect calorimetry measurements that were analyzed, LF-fed mice had higher RER in dark than in light phases ($P < 0.0001$, Fig. 3A-B). After the shift to HFHS-diets, RER decreased in both light and dark phases and the difference between light and dark phases was no longer evident (Fig. 3A-B). The different protein sources caused no differences in RER between the groups neither in light nor in dark phases (Fig. 3B).

**Increased EE and a tendency towards increased activity in cod/scallop-fed mice**

Similarly to RER, activity level differed between light and dark phases in LF-fed mice with higher activity levels during dark phases ($P < 0.0001$, Fig. 3C-D). The initial activity levels measured while the mice were fed the LF-diet were similar (Fig. 3D-E) and changing to HFHS-diet did not change the activity during the dark phases (Fig 3D). However, feeding the HFHS-diets decreased activity level during light phases ($P = 0.018$) (Fig 3D). Total activity tended to decrease with the shift from LF to HFHS-diets ($P = 0.068$, Fig. 3E). In dark phases cod/scallop-fed mice tended to be more active than casein- and chicken-fed mice ($P = 0.09$, Fig. 3D) and a strong tendency towards higher total activity was seen in cod/scallop-fed compared to casein- and chicken-fed mice ($P = 0.06$, Fig. 3E). Consistent with activity, EE was higher in dark than in light phases in LF-fed mice. With the shift to HFHS-diets, EE decreased in dark phases while no difference was seen between LF- and HFHS-feeding in light phases (Fig. 3F-G). No difference was seen between groups in light...
or dark phases while on LF-diets, whereas EE tended to decrease \((P = 0.08)\) in casein-fed compared to chicken- and cod/scallop-fed mice in light phases and increased in cod/scallop-fed compared to casein-fed mice during dark phases.

**Discussion**

An increasing body of evidence supports a preventive role of high protein-diets against development of obesity. Less is known as to whether different protein sources consumed at normal dietary levels may differently affect energy balance. In the present study, we fed obesity-prone male C57BL/6J mice high fat (67 energy %), high sucrose (18 energy %), normal protein (15 energy %) diets with casein, chicken filet or a mixture of cod filet and scallop muscle as the protein source. At equal energy intake, chicken-fed mice had a higher feed efficiency as compared to the casein- and cod/scallop-fed mice, which after seven weeks feeding translated into increased body and adipose tissue masses. Concomitantly, the chicken-fed mice were hyperlipidemic and had enlarged liver mass with elevated hepatic TAG levels, relative to the casein- and cod/scallop-fed mice. Thus, we demonstrated that different protein sources affected diet-induced obesity and associated co-morbidities in C57BL/6J mice when given at normal levels in a HFHS background diet.

Body fat accretion was reduced, evident as lower adipose tissue masses and reduced liver TAG, in the casein- and cod/scallop-fed compared to the chicken-fed mice. Interestingly, the apparent fat digestibility was reduced from an average of about 98% in the chicken- and cod/scallop-fed mice, to an average of about 95% in the casein-fed mice. Assuming that the apparent fat digestibility was constant for the entire seven-week period, the casein-fed absorbed approximately five g less fat than the chicken- and cod/scallop-fed mice. In mice, intake of a high fat (HF) casein diet has previously been reported to cause higher fecal fat excretion and a leaner phenotype as compared to intake of a HF-salmon diet [38]. Hence, it is likely that the reduced apparent fat absorption was a contributing factor to the reduced fat accretion in casein-fed mice in the present study.

The cod/scallop-fed mice maintained a lean phenotype, relative to the chicken-fed mice, without reduction in fat absorption. To elucidate whether the protein sources modulated energy metabolism, we subjected the mice to indirect calorimetric measurements before onset of obesity at the transition from low-fat (LF) to
HFHS-feeding. HF-diets disturb feeding pattern and behavioral circadian rhythm in mice [39], such that the
LF induced fluctuations in RER between dark and light phases, reflecting different feed intake and substrate
oxidations, is completely abolished after a switch to a casein-based HF-diet [40]. Accordingly, the RER was
promptly reduced, and the differences in RER between light- and dark phases disappeared after the switch
to HFHS-diets in the present study. There was no protein source-effect on the RER. However, following the
transition from LF to HF diets EE decreased less in the cod/scallop-fed compared to the casein-fed mice, but
we observed no significant difference in EE between chicken-fed and cod/scallop-fed mice that could explain
the difference in adiposity. Our indirect calorimetry setup monitored gas exchange of each mouse for 1.9
minutes every 30 minutes, and it has been argued that the monitoring frequency has to be considerably
higher in order to detect the 2-5% changes in diet-induced EE sufficient to elicit long term alterations on
energy balance [41]. A decrease in spontaneous locomotor activity has previously been demonstrated at the
transition from LF to HF-diets [39], which was also observed in the casein and chicken-fed mice in the
present study. Importantly, cod/scallop-feeding tended ($P = 0.06$) to attenuate this decrease in activity at the
transition from LF to HF-diets in the present study. In line with this notion, we have previously observed an
inverse correlation between locomotor activity and development of diet-induced obesity, without being able
to detect differences in EE [21]. Indeed, whereas gas exchange was quantified at intervals (i.e. 1.9 min every
30 min), beam breaks were detected continuously, increasing the sensitivity of this measure as an indicator
of EE. Therefore, differences in locomotor activity that nearly reached statistical significance ($P = 0.06$), are
likely to reflect changes in EE that over time could explain the divergent fat accretion between the chicken-
and cod/scallop-fed mice.

We have previously used another casein-based HF (47 energy percent), high sucrose (36 energy percent)
diet to precipitate obesity and glucose intolerance in mice [12-14]. By increasing the fat content to 67 energy
percent and reduce the sucrose content to 18 energy percent, the casein-fed mice in the present study
remained lean. Despite their lean phenotype, the casein-fed mice became glucose intolerant, relative to the
cod/scallop-fed mice, when challenged in an oral glucose test after six weeks feeding. Cod protein intake
has previously been associated with improved glucose metabolism in rats due to better peripheral insulin
sensitivity as compared to casein-feeding [28, 42, 43]. Moreover, in a randomized controlled intervention
study with crossover design, insulin-resistant subjects exhibited improved insulin sensitivity [44] and reduced
levels of the inflammatory marker high-sensitivity C-reactive protein after intake of a cod-based relative to a
meat and dairy-based diet for 4 weeks [45]. Therefore, both in the present study, as well as in studies with
rats and humans, intake of cod as compared to casein is associated with improved glucose metabolism.

During HF-feeding, metabolic adaptations to the elevated fat load occurs by increasing mitochondrial content
and oxidative capacity in liver [46, 47] and skeletal muscle [40, 48]. As a strong regulatory interaction exists
between lipid and carbohydrates oxidation [49], HF-feeding represses the use of glucose as an energy
substrate (i.e. glycolysis) [40, 46], a condition that could promote glucose intolerance. Based on the
improved glucose clearance in the cod/scallop-fed mice in the present study as well as in HF cod-fed rats
reported by others [28, 43], it is possible that glycolysis it better maintained in rodents fed cod (or cod/scallop)
based HF-diets compared to those fed casein-based HF-diets.

The present study was not designed to identify underlying mechanisms, merely to elucidate whether diets
with casein, chicken filet or a mixture of cod filet and scallop muscle modulate diet-induced obesity. As
locomotor activity can be stimulated [50, 51] and EE increased [52] by dietary taurine it is possible that the
high taurine concentration of the cod/scallop-diet contributed to the observed modulation of energy balance
in these mice. In addition, altered metabolism of branched-chain amino acid (BCAA) is likely associated with
glucose dysregulation and the development of insulin-resistance [53]. In line with this notion, BCAA-
supplementation in a casein-based HF-diet impaired glucose tolerance in rats [54]. In the present study, the
BCAA-content was 39% higher in the casein-diet than in the cod/scallop-diet, which may have contributed to
the observed differences in glucose tolerance. However, further studies are needed to clarify if varying amino
acid content contributed to the observed differences in the present study.
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Statement of Authors’ Contributions to Manuscript
B.L. and H.S.T. designed research; H.S.T., B.L., K.B. and A.K.R. conducted research; H.S.T. and B.L. analyzed data and conducted statistical analysis; H.S.T. and B.L. wrote the paper; and H.S.T., B.L., L.M. and K.K. had primary responsibility for the final content. All authors read and approved the final manuscript.
Figure legends

**Figure 1** Growth curve for mice fed the experimental diets for six wk (A), body mass gain (B), cumulative and total energy intake (C), feed efficiency (D), adipose tissue masses (E), apparent fat digestibility (F), lean tissue masses (G) and apparent nitrogen digestibility (H) in male C57BL/6J mice fed the experimental diets for six wk. Data (Expt. 1) represent group means ($n = 8$) ± SEM analyzed by one-way ANOVA followed by Tukey’s pair-wise comparisons. Body mass development and cumulative energy intake was analyzed by repeated measurements ANOVA followed by Tukey’s post hoc. Means that do not share a letter are significantly different ($P < 0.05$). * indicates significantly higher body mass in chicken-fed than in casein-fed mice. # indicates significantly higher body mass in cod/scallop-fed than in casein-fed mice. ¤ Indicates significantly higher body mass in chicken-fed than in cod/scallop-fed mice.

**Figure 2** Blood glucose measured before and at 15, 30, 60 and 120 minutes after oral administration of glucose (gavage, 2mg/g body mass) during 6h feed-deprived oral glucose tolerance test after six wk on the experimental diets (O-GTT, A), 6h feed-deprived blood glucose (B), glucose dose administered by oral gavage (C), incremental blood glucose AUC (D). Plasma lactate (E) plasma glucose (F) and plasma insulin (G) measured in 4h feed-deprived plasma collected at the termination of the mice after seven wk on the experimental diets. HOMA-IR (H) and QUICKI (I) scores calculated based on 4h feed-deprived plasma glucose and insulin levels. Data (Expt. 1) represent group means ($n = 7-8$) ± SEM analyzed by one-way ANOVA followed by Tukey’s pair-wise comparisons. O-GTT curve was analyzed by repeated measurements ANOVA followed by Tukey’s post hoc. Means that do not share a letter are significantly different ($P < 0.05$). # indicates significantly higher blood glucose in casein-fed than in cod/scallop-fed mice. ¤ Indicates significantly higher blood glucose in casein-fed than in chicken-fed mice.
Figure 3 RER in mice fed LF for 72h and HFHS experimental diets for 72h in open-circuit indirect calorimetry cages (A), average respiratory exchange ratio (RER) during 48h on LF and HFHS diets in light and dark phases (B), spontaneous locomotor activity during 72h on LF and 72h on HFHS diets (C), spontaneous locomotor activity in light and dark phases during 48h in mice fed LF and HFHS diets (D), total spontaneous locomotor activity during 48h in mice fed LF and HFHS diets (E) energy expenditure (EE) during 72h on LF and 72h on HFHS diets (F) Average EE during 48h in light and dark phases in mice fed LF and HFHS diets (F). Data (Expt. 2) represent group means ($n = 9-10$) ± SEM analyzed by ANOVA followed by Tukey’s pairwise comparisons. RER, activity and EE data were analyzed by repeated measurements ANOVA followed by Tukey’s post hoc. Means that do not share a letter are significantly different ($P < 0.05$).
Table 1. Composition of the experimental diets

<table>
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<th>Composition (g/kg)</th>
<th>LF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Casein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chicken&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cod/scallop&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
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<td>Butylated hydroxytoluene</td>
<td>–</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>DiCalcium Phosphate</td>
<td>12.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>5.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Potassium Citrate, 1 H₂O</td>
<td>15.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyzed (g/kg)</th>
<th>Crude protein&lt;sup&gt;h&lt;/sup&gt;</th>
<th>Ash</th>
<th>Fat</th>
<th>Gross energy kJ/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>170</td>
<td>31</td>
<td>44</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>48</td>
<td>390</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>60</td>
<td>400</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>75</td>
<td>390</td>
<td>25.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> OpenSource diet no. D12450B (Research Diets, Inc. NJ, USA)
<sup>b</sup> Casein (cat. no. C8654, lot BCBC 3986, Sigma-Aldrich, MO, USA)
<sup>c</sup> Chicken breast fillets (Kyllingfilet naturell, Ytterøykylling AS, Norway)
<sup>d</sup> Cod fillets (Wildcaught in the Northeastern Atlantic) and Canadian scallops, (Wild North Atlantic scallops, 20-30 ct, Placopecten magellanicus, Clearwater Seafoods Limited, NS, Canada)
<sup>e</sup> LF: soybean oil. Casein, chicken and cod/scallop: corn oil
<sup>f</sup> Mineral Mix S10026
<sup>g</sup> Vitamin Mix V100001
<sup>h</sup> Crude protein, N × 6.15 for casein; N × 5.6 for chicken filet and cod/scallop
### Table 2 Amino acid composition of experimental diets

<table>
<thead>
<tr>
<th>mmol/kg</th>
<th>LF</th>
<th>Casein</th>
<th>Chicken</th>
<th>Cod/scallop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>60</td>
<td>68</td>
<td>138</td>
<td>119</td>
</tr>
<tr>
<td>Arg</td>
<td>31</td>
<td>36</td>
<td>66</td>
<td>73</td>
</tr>
<tr>
<td>Asx</td>
<td>100</td>
<td>109</td>
<td>154</td>
<td>149</td>
</tr>
<tr>
<td>Cys</td>
<td>28</td>
<td>36</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>Glx</td>
<td>274</td>
<td>307</td>
<td>212</td>
<td>191</td>
</tr>
<tr>
<td>Gly</td>
<td>40</td>
<td>47</td>
<td>109</td>
<td>192</td>
</tr>
<tr>
<td>His*</td>
<td>29</td>
<td>34</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Ile*</td>
<td>66</td>
<td>77</td>
<td>75</td>
<td>61</td>
</tr>
<tr>
<td>Leu*</td>
<td>120</td>
<td>142</td>
<td>126</td>
<td>107</td>
</tr>
<tr>
<td>Lys*</td>
<td>99</td>
<td>112</td>
<td>136</td>
<td>116</td>
</tr>
<tr>
<td>Met*</td>
<td>29</td>
<td>34</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>Phe*</td>
<td>51</td>
<td>59</td>
<td>48</td>
<td>41</td>
</tr>
<tr>
<td>Pro</td>
<td>153</td>
<td>185</td>
<td>61</td>
<td>47</td>
</tr>
<tr>
<td>Ser</td>
<td>94</td>
<td>107</td>
<td>78</td>
<td>73</td>
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<tr>
<td>Thr*</td>
<td>63</td>
<td>70</td>
<td>78</td>
<td>64</td>
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<tr>
<td>Trp</td>
<td>9</td>
<td>11</td>
<td>11</td>
<td>8</td>
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<tr>
<td>Tyr</td>
<td>36</td>
<td>44</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>Val*</td>
<td>94</td>
<td>111</td>
<td>88</td>
<td>69</td>
</tr>
<tr>
<td>Hyp</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Tau</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>EAA</td>
<td>551</td>
<td>640</td>
<td>630</td>
<td>512</td>
</tr>
<tr>
<td>BCAA</td>
<td>280</td>
<td>330</td>
<td>290</td>
<td>237</td>
</tr>
<tr>
<td>Total AA</td>
<td>1376</td>
<td>1590</td>
<td>1540</td>
<td>1494</td>
</tr>
</tbody>
</table>

Asx: sum of Asp + Asn  
Glx: sum of Glu + Gln  
* essential amino acids  
EAA: sum of essential amino acids  
BCAA: sum of branched-chain amino acids  
Total AA: total sum of amino acids
Table 3 4h feed-deprived plasma metabolites, liver lipids and liver relative gene expression in male C57BL/6J mice fed the HFHS diets with differing protein sources for seven wk (Expt.1)

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>Casein</th>
<th>Chicken</th>
<th>Cod/scallop</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma metabolites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.24 ± 0.18</td>
<td>3.54 ± 0.18b</td>
<td>4.53 ± 0.19a</td>
<td>3.88 ± 0.15b</td>
<td>0.003</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>2.68 ± 0.13</td>
<td>3.14 ± 0.13ab</td>
<td>3.72 ± 0.17a</td>
<td>3.23 ± 0.13ab</td>
<td>0.022</td>
</tr>
<tr>
<td>HDL:total cholesterol ratio</td>
<td>0.27 ± 0.01</td>
<td>0.89 ± 0.01a</td>
<td>0.82 ± 0.01b</td>
<td>0.83 ± 0.01b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.87 ± 0.05</td>
<td>0.97 ± 0.08a</td>
<td>1.42 ± 0.08a</td>
<td>1.16 ± 0.07ab</td>
<td>0.002</td>
</tr>
<tr>
<td>Total bile acids (mmol/L)</td>
<td>3.0 ± 0.29</td>
<td>2.7 ± 0.23a</td>
<td>1.9 ± 0.17b</td>
<td>2.4 ± 0.19ab</td>
<td>0.027</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.75 ± 0.07</td>
<td>0.40 ± 0.03b</td>
<td>0.65 ± 0.05a</td>
<td>0.50 ± 0.06ab</td>
<td>0.003</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>0.46 ± 0.04</td>
<td>0.32 ± 0.04</td>
<td>0.27 ± 0.03</td>
<td>0.33 ± 0.07</td>
<td>0.74</td>
</tr>
<tr>
<td>Glycerol (mmol/L)</td>
<td>0.32 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.80</td>
</tr>
<tr>
<td>β-hydroxybutyrate (mmol/L)</td>
<td>0.42 ± 0.11</td>
<td>0.34 ± 0.04</td>
<td>0.21 ± 0.08</td>
<td>0.25 ± 0.05</td>
<td>0.27</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>24 ± 2.75</td>
<td>28 ± 8.79</td>
<td>26 ± 1.53</td>
<td>50 ± 17.08</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Liver lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mg/g)</td>
<td>29.3 ± 6.51</td>
<td>25.8 ± 2.61ab</td>
<td>48.6 ± 8.56a</td>
<td>24.7 ± 2.82b</td>
<td>0.009</td>
</tr>
<tr>
<td>Total neutral lipids (mg/g)</td>
<td>35.2 ± 6.90</td>
<td>30.0 ± 2.62ab</td>
<td>52.9 ± 8.72a</td>
<td>28.7 ± 2.84b</td>
<td>0.009</td>
</tr>
<tr>
<td>Cholesterol (mg/g)</td>
<td>2.9 ± 0.09</td>
<td>2.4 ± 0.09</td>
<td>2.5 ± 0.06</td>
<td>2.7 ± 0.15</td>
<td>0.43</td>
</tr>
<tr>
<td>Steryl ester (mg/g)</td>
<td>3.0 ± 0.44</td>
<td>1.6 ± 0.16</td>
<td>1.6 ± 0.19</td>
<td>1.2 ± 0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>Diacylglycerides (mg/g)</td>
<td>0.1 ± 0.04</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>0.1 ± 0.02</td>
<td>0.43</td>
</tr>
<tr>
<td><strong>Liver relative mRNA expression</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pck-1</td>
<td>n/a</td>
<td>0.24 ± 0.06b</td>
<td>0.40 ± 0.09a</td>
<td>1.06 ± 0.36a</td>
<td>0.041</td>
</tr>
<tr>
<td>Scd-1</td>
<td>n/a</td>
<td>0.03 ± 0.01a</td>
<td>0.02 ± 0.01ab</td>
<td>0.004 ± 0.001b</td>
<td>0.028</td>
</tr>
<tr>
<td>Srebf1</td>
<td>n/a</td>
<td>0.79 ± 0.21</td>
<td>1.45 ± 0.37</td>
<td>2.10 ± 0.46</td>
<td>0.07</td>
</tr>
<tr>
<td>Acaca</td>
<td>n/a</td>
<td>1.53 ± 0.50</td>
<td>0.98 ± 0.26</td>
<td>1.30 ± 0.41</td>
<td>0.65</td>
</tr>
<tr>
<td>Dgat-1</td>
<td>n/a</td>
<td>0.75 ± 0.01</td>
<td>0.89 ± 0.19</td>
<td>0.67 ± 0.16</td>
<td>0.58</td>
</tr>
<tr>
<td>Fasn</td>
<td>n/a</td>
<td>0.52 ± 0.20</td>
<td>0.94 ± 0.25</td>
<td>0.91 ± 0.23</td>
<td>0.42</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>n/a</td>
<td>2.07 ± 0.60</td>
<td>2.89 ± 0.61</td>
<td>3.77 ± 1.06</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Data represent group means (n = 8) ± SEM analyzed by one-way ANOVA followed by Tukey’s Post Hoc. Means that do not share a letter are significantly different (P < 0.05). Scd-1 data were transformed before statistical analysis.
Figure 1

A. Growth curve

B. Body mass gain

C. Cumulative and total energy intake

D. Feed efficiency

E. Adipose tissues

F. Apparent fat digestibility

G. Lean tissues

H. Apparent N digestibility
Figure 2

A. Oral Glucose Tolerance Test

B. Feed-deprived blood glucose

C. Glucose dose

D. Glucose iAUC

E. Plasma lactate

F. Plasma glucose

G. Plasma insulin

H. HOMA-IR

I. QUICKI

Legend:
- LF
- Casein
- Chicken
- Cod/Scallop
Figure 3

A. Respiratory Exchange Ratio (VCO₂/VO₂)

B. RER, average 48h

C. Spontaneous activity, beam breaks

D. Activity, 48h

E. Activity, 48h, total

F. Energy expenditure (kJ/h/kg)

G. EE, average 48h

Legend:
- LF (Casein)
- LF (Chicken)
- LF (Cod/Scallop)
- Casein
- Chicken
- Cod/Scallop
### Supplemental Table 1 Genes and corresponding primer sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Gene name</th>
<th>5'prime</th>
<th>3'prime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pck-1</td>
<td>Phosphoenol pyruvate carboxykinase-1</td>
<td>CCACACCATGGCAATTATGC</td>
<td>CATATTTCCTCAGCTTGCGG</td>
</tr>
<tr>
<td>Scd-1</td>
<td>Stearoyl-CoA desaturase-1</td>
<td>GATGTTCAGAGGAGGTACTACAAG</td>
<td>ATGAAGCACTACAGGAGGAGG</td>
</tr>
<tr>
<td>Sreb1</td>
<td>Sterol regulatory element-binding transcription factor 1</td>
<td>GGAGCCATGGATTGCACATT</td>
<td>GCTTCAGAGGAGGAGCCAG</td>
</tr>
<tr>
<td>Acaca</td>
<td>Acetyl-Coenzyme A carboxylase alpha</td>
<td>TGCTGCCCCATCCCCGGG</td>
<td>TCGAACTCTCACTGACAG</td>
</tr>
<tr>
<td>Dgat-1</td>
<td>Diacylglycerol acyltransferase-1</td>
<td>GGTCGCACTCGTCACAGA</td>
<td>CCACAGGATGCCATCTGA</td>
</tr>
<tr>
<td>Fasn</td>
<td>Fatty acid synthase</td>
<td>CTTGCCAACTCTACCATGG</td>
<td>TTCCACACCCATGAGGAGT</td>
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<tr>
<td>Hmgcr</td>
<td>3-Hydroxy-3-Methylglutaryl-Coenzyme A reductase</td>
<td>ATCATCTGACGATAACCGC</td>
<td>GCCGCAATACCCAGATGT</td>
</tr>
<tr>
<td>Tbp</td>
<td>TATA-box binding protein</td>
<td>ACCCTTCACCAATGACTCTATG</td>
<td>ATGATGACTGACGAAAACTGC</td>
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</table>
Lean seafood reduces energy intake and attenuates diet-induced obesity in C57BL/6J mice

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¹Department of Biology, University of Copenhagen, Copenhagen, Denmark. ²National Institute of Nutrition and Seafood Research, Bergen, Norway.

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ABSTRACT

Background: High-protein diets are reported to protect against diet-induced obesity. However, it remains to be established if and to what extent different protein sources affect body weight regulation when they are consumed at normal dietary levels. Here we investigated how a mixture of lean seafood and a mixture of lean meat provided as the protein source in a Western background diet modulated body weight development and metabolism.

Methods: Obesity-prone male C57BL/6J mice were either fed ad libitum or pair-fed Western diets with a mixture of lean seafood or a mixture of lean meat as the protein source for 12 weeks. Glucose and insulin tolerance tests were performed after 9 and 10 weeks, respectively. Before the onset of obesity, a meal response test was performed to evaluate postprandial glucose and C-peptide, and energy metabolism was measured by indirect calorimetry.

Results: When given free access to the experimental diets, mice fed a Western diet with lean seafood consumed less energy and accumulated less body fat mass compared to mice fed a Western diet with lean meat. During pair feeding, the difference in body fat mass accumulation was equalised, suggesting that differences in energy intake was a main factor determining adipose mass accumulation and weight gain in mice fed the meat diet. Overnight fasted glucose was elevated in meat-fed mice determined prior to the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). Yet no differences in glucose tolerance or insulin sensitivity were observed by OGTT and ITT. Pair feeding prevented the difference in fasting glucose. Furthermore, meat-fed mice had higher plasma levels of LDL cholesterol, HDL cholesterol and total cholesterol than seafood-fed mice. However, in the pair-fed mice, no differences were observed. Spontaneous locomotor activity was increased in seafood-fed mice at the transition from a low-fat diet whereas it decreased for meat-fed mice, indicating that the protein source may modulate activity level and thereby drive differences in body fat mass gain in an ad libitum setting.

Conclusion: In an ad libitum setting mice fed lean seafood consumed less energy and gained less body fat compared to mice fed lean meat suggesting that intake of lean seafood increased satiety. In the ad libitum setting feed efficiency was higher in the lean meat fed mice than in the lean seafood fed mice. By contrast, in the pair-feeding experiment we observed no differences in feed efficiency between lean seafood fed and lean meat fed mice. However, we observed a striking increase in spontaneous locomotor activity upon intake of the lean seafood.
Western diet, which together with the decreased feed intake would contribute to the diminished weight gain in lean seafood fed mice compared to mice fed the lean meat Western diet.
INTRODUCTION

Human and animal studies have shown that increasing the amount of protein at the expense of carbohydrate and/or fat is an effective strategy for weight reduction, short-term weight maintenance and protection against diet-induced obesity [1-3]. Compared to fat and carbohydrate, protein is reported to induce a greater sense of satiety and to suppress appetite. Meals high in protein could therefore reduce both acute and later energy intake [4-6]. Furthermore, protein strongly augments diet-induced thermogenesis [7-11]. However, prolonged adherence to high-protein diets can be challenging [12, 13], and evidence supporting the long-term effectiveness of high-protein diets in human subjects is limited. Moreover, it is still debated whether high-protein diets might cause any adverse health effects [14].

In addition to the amount of dietary protein, it is also likely that protein quality may influence diet-induced obesity. Although research into the area of protein quality and source is scarce [15], some findings indicate that addition of seafood protein to an existing diet may promote weight loss. Inclusion of a daily fish meal into a caloric-restricted diet is associated with higher weight loss compared to control diets without fish in young adults [16]. Another study reported that intake of fish resulted in a higher satiety score than intake of beef protein [17]. A more recent study showed that subjects consuming a fish meal had a lower energy intake during the following meal compared to subjects given a meat meal [18].

Our group has previously shown that a high-fat/high-sucrose diet with a mixture of cod/scallop resulted in a lower energy intake in mice compared to using chicken as the protein source [19]. However, to date, few studies have compared the effects on diet-induced obesity of lean seafood and lean meat when consumed as the sole protein source in a Western diet over time. We hypothesised that a lean seafood diet would be more satiating and therefore would lead to a lower energy intake and less weight gain compared to a lean meat diet. We tested this hypothesis by feeding obesity-prone C57BL/6J Western diets with a mixture of lean seafood (ling, rosefish, wolf fish, cod and scallop) or a mixture of lean meat (chicken, beef and pork) for 12 weeks under both ad libitum and pair-fed conditions.
MATERIALS AND METHOD

Ethical statement

The animal experiments were approved by the National Animal Health Authority (Norwegian approval identification 4057 and 5358).

Animal studies

Male C57BL/6J Bom Tac mice 7 weeks of age were obtained from Taconic Europe (Ejby, Denmark). The mice were housed individually and kept on a 12/12-h light/dark cycle at thermoneutrality, 28±2°C. Fresh water and feed were provided three times a week, and body mass was recorded once a week. Before starting the experiments, the mice were given a low-fat diet for 1 week to acclimate. After the acclimatisation period, the mice were divided into three diet groups (low-fat, seafood and meat) in a manner to equalise group means of lean, fat and body mass. The feeding trials lasted for 12 weeks and consisted of 30 mice (n=10/group) per experiment. In the first trial, the mice were given free access to the diets. In the second experiment, meat-fed mice were restricted to the amount consumed by the seafood group to ensure equal energy intake. Data on energy intake, body composition and body mass are based on the first 9 weeks of the studies, prior to oral glucose tolerance- and insulin tolerance testing. In week 12, fasted mice (6 hours) were anaesthetised with isoflurane (Isoba-vet, Schering-Plough, Denmark) and euthanised by cardiac puncture. Liver, kidneys, heart and white adipose tissues were quickly dissected out, weighed, snap frozen and stored at −80°C. Whole blood drawn from the heart during cardiac puncture was collected in EDTA tubes and immediately centrifuged at 4°C at 2500g for 5 min. EDTA plasma was separated and stored at −80°C.

Experimental diets

The seafood and meat diets were based on a Western diet (5TJN, Western diet for rodents, TestDiet) and made by completely exchanging casein with either lean seafood powder or meat powder. The seafood powder consisted of equal amounts of cooked and freeze-dried skinless filets from ling, rosefish, cod, wolf fish and muscle from Canadian scallop. The meat powder consisted of equal amounts of cooked and freeze-dried skinless chicken breast, pork striploin and beef striploin. Differences in endogenous fat from the protein powders were balanced for
by reducing the amount of lard added to the diets. One group of mice was fed a casein-based low-fat reference diet (5TJS, TestDiet). The diets were provided in pellet form, prepared by Ssniff spezialdiäten gmbh (Soest, Germany). The composition of the diets is shown in Tables 1, 2 and 3.

**Body composition determined by nuclear magnetic resonance (NMR)**

Fat mass and lean mass were determined in weeks 1, 5 and 9 using quantitative magnetic resonance (Minispec mq 7.5, NMR analyser, Bruker, Germany). Body composition used for grouping in the indirect calorimetry experiment was determined using the EchoMRI quantitative magnetic resonance whole-body composition analyser (Echo Medical Systems).

**Analyses of gross energy, fat and nitrogen in diets and faeces**

Gross energy in the diets was measured using a bomb calorimeter, following the manufacturer’s instructions (Parr calorimeter 6300, Parr Instruments, Molinc, IL, USA). The nitrogen content was analysed with the Dumas method (Vario MACRO Cube, Elementar Analysensysteme GmbH, Germany) and a conversion factor of 6.25 was used to calculate crude protein content. Fat in diet and faeces was determined gravimetrically after organic extraction as described in detail elsewhere [19].

**Apparent fat and nitrogen digestibility**

During the last 3–5 days of the experiments, faeces were collected and quantified. The apparent digestibility of nitrogen and fat were calculated using this formula: apparent digestibility (%) = \((I - F)/I \times 100\), where I is dietary nitrogen, or fat intake, and F is faecal nitrogen, or fat output.

**Glucose and insulin tolerance tests**

In week 10 and 11 an oral glucose tolerance (OGTT) and an insulin tolerance test (ITT), respectively, were performed on conscious mice fasted for 6 hours. For the OGTT, mice were given 3 mg glucose/g lean mass orally through a gavage tube. Blood samples were collected from tail vein before (0) and at 15, 30, 60 and 120 minutes after glucose administration and analysed with a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway).
the ITT, mice were given 0.75 units insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) per kg lean mass, intraperitoneally. Blood samples were collected from the tail vein before (0) and at 15 and 30 minutes after insulin injection and analysed with a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway). Incremental area under the curve (IAUC) for the GTT and decremental area under the curve for the ITT were calculated using the following formulas: IAUC= AUC-(Basal glucose*120min). DAUC= (Basal glucose*30min)-AUC.

**Plasma analyses**

MaxMat PL II analyser (MAXMAT S.A., Montpellier, France) and conventional kits were used to measure plasma levels of HDL-cholesterol, glycerol, FFA (Dialab, Austria), LDL-cholesterol, triglyceride, total cholesterol (MaxMat, France) and D-3 Hydroxybutyrate (Randox, United Kingdom) from 6h feed deprived mice.

**Indirect calorimetry and spontaneous locomotor activity**

A separate set of mice was used for the assessment of energy metabolism in young mice at the transition from low-fat to Western diet before the onset of obesity. Based on body mass and composition, mice were divided into either the seafood group (n=8) or the meat group (n=7). Prior to measurements in the CaloCages, mice were placed in training cages for 3 days with free access to the low-fat diet. Following the acclimatisation period, the mice were transferred to CaloCages fitted with infrared light-beam frames (ActiMot2, TSE Systems, Bad Homburg, Germany) where O₂ consumption, CO₂ production and spontaneous locomotor activity were measured using the PhenoMaster open-circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany). In total, the mice were kept in the CaloCages for 6 days. For the first 3 days the mice had free access to a low-fat diet, on day 4 the animals were given free access to the experimental diets for another 3 days. Based on two consecutive light (06.00–17.30h) and dark (18.00–05.30h) phases, the respiratory exchange ratio (RER) was calculated from VO₂ and VCO₂, and spontaneous locomotor activity was defined as total counts of beam breaks. Energy expenditure was calculated using the following equation: 16.3 Kj/L x L VO₂ + 4.6 Kj/L x L VCO₂.
**Meal response test**

After 1 week of acclimatisation, a separate set of 26 mice was divided into two groups based on body mass and composition (n=13/group). Prior to the meal response test, mice were feed-deprived for 16 hours, and a blood sample for basal measurements of glucose and C-peptide was obtained (time point −10). For the meal response test, feed-deprived mice were given free access to the seafood or meat diet for 10 minutes. After removing the food (time point 0), blood was collected at 0, 10, 20 and 30 minutes for determination of plasma C-peptide and blood glucose using a handheld glucometer (Ascensia, Contur, Bayer Healthcare, Oslo, Norway). The blood for plasma C-peptide analyses was collected using EDTA-coated micro tubes (Minivette® POCT, SARSTEDT AG & Co, Nümbrecht, Germany) before transferring to tubes containing a mixture of proteases, esterases and DPP-IV inhibitors optimised for blood (BD P800, Puls, Oslo, Norway). After plasma separation, samples were stored at −80°C. Plasma C-peptide was determined using a commercial ELISA kit in accordance with the manufacturer’s instructions (Mouse C-Peptide ELISA, Crystal Chem, Illinois, USA).

**Diet preference test**

After 1 week of acclimatisation, 30 mice were fasted for 16 hours and subjected to a diet preference test. During the test, the mice were simultaneously offered both the seafood and the meat diet in individual cages. We then observed which diet the mice chose first and quantified the amount eaten during a six-hour period with free access to both diets.

**Statistical analyses**

The data represent group means ± SEM. After homogeneity of variances was checked for by Levene’s test, the data were subjected to ANOVA analyses followed by Tukey’s multiple comparisons, and group means were considered statistically different at $p < 0.05$. Data that were repeatedly measured, i.e. growth, energy intake, OGTT, RER, activity and EE, were analysed by repeated measurements ANOVA followed by Tukey’s post hoc. Data for which the variances were not homogenous were transformed, and after homogeneity of variances was established, the transformed data were subjected to ANOVA followed by Tukey’s post hoc. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software INC., La Jolla, CA, USA) and Statistica (StatSoft Norway AS).
RESULTS

Reduced energy intake and fat mass in mice fed lean seafood compared to mice fed lean meat

To investigate whether intake of lean seafood or lean meat has different effects on weight gain and body composition, Western diets with lean seafood or lean meat were fed to obesity-prone mice.

Mice fed the seafood diet had a lower energy intake than mice fed the meat diet, and this difference was significant from week 3 and throughout the study (Fig.1A). In addition to lower energy consumption, feed efficiency, i.e. body mass gain per energy intake, was significantly lower in mice fed the seafood diet compared to mice fed the meat diet (Fig.1B). There were no difference in apparent digestibility of fat for the experimental diets (Fig.1C).

After 9 weeks of ad libitum feeding, mice receiving the seafood diet had gained less weight than mice receiving the meat diet, and as expected, mice receiving the Western diets had gained more weight during the feeding experiment than mice fed the low-fat reference diet (Fig.1D).

Body composition measurements showed that differences in body mass were due to a higher accumulation of fat mass in mice receiving the meat diet than in mice receiving the seafood diet (Fig.1D). The masses of adipose tissue depots (rWAT, eWAT and iWAT) at week 12 confirmed this difference (Fig.1E). Total lean mass (Fig.1D) and tissue masses of liver, kidney and heart were similar in meat- and seafood-fed mice (Fig.1F). Kidney mass was higher in mice fed the Western diets than in mice fed the reference diet (Fig.1F).

To examine if the increased accumulation of fat mass seen in meat-fed mice was accompanied by reduced glucose tolerance, we performed an oral glucose tolerance test in week 10. Mice fed the Western diet containing meat had the highest 6-hour fasting blood glucose levels (Fig.2A). In addition, mice fed the meat diet had significantly higher glucose levels 15 minutes after receiving the glucose compared to mice fed the seafood diet (Fig.2B). Furthermore, compared to the low-fat reference group, the meat group had higher glucose levels for all time points except at 120 minutes, whereas mice receiving the seafood diet had higher glucose levels only at 15 and 30 minutes (Fig.2B). However, there were no differences in incremental area under the curve (IAUC) (Fig. 2C). To investigate if variations in glucose levels during the OGTT were due to reduced insulin sensitivity, an insulin tolerance test was performed during week 11. Again, 6-hour fasting blood glucose seemed to be higher in the meat-fed mice, even though the difference did not reach statistical significance (Fig.2D). The mice fed the meat diet had
significantly higher blood glucose levels 15 minutes after the injection of insulin compared with the mice fed the seafood diet (Fig.2E). To compensate for differences in basal glucose levels, the decremental area under the curve (DAUC) was calculated (Fig.2F). No differences in DAUC were observed, suggesting that insulin sensitivity was not decreased.

**Pair feeding prevented the differences in body fat mass gain**

To investigate if the reduced weight gain seen in mice fed the seafood diet during the *ad libitum* feeding experiment was due to lower energy intake, we conducted a pair-feeding experiment. In this experiment, the energy intake in the meat group was restricted to the energy intake for the mice receiving seafood *ad libitum*.

During the pair-feeding regime, mice receiving the Western diets had significantly higher feed efficiency compared with the low-fat diet fed mice, but they did not differ from each other (Fig.3B). Furthermore, analyses for fat and nitrogen digestibility showed that the seafood-fed mice had a significantly higher fat digestibility compared to the meat-fed mice, and that mice fed the meat diet had higher nitrogen digestibility compared mice fed the low-fat reference diet (Fig.3C).

No difference was found in body fat or lean mass accretion between meat- and seafood-fed mice. However, both experimental groups accumulated more body fat compared to the low-fat group (Fig.3D). The masses of adipose and lean tissues at week 12 confirmed the body composition measurements and showed that the experimental groups accumulated more eWAT and rWAT than the low-fat group (Fig.3E). The mass of kidneys was also higher in meat- and seafood-fed mice than low-fat-fed mice (Fig.3F).

**Pair feeding mice Western diets containing either lean meat or lean seafood have similar effects on glucose and insulin tolerance**

To explore if there was any alteration in glucose tolerance despite no difference in fat mass, we also here performed an oral glucose tolerance test. There was no difference in 6-hour fasting glucose between the groups (Fig.4A). Measurements of blood glucose levels after receiving the glucose bolus revealed no difference between the Western diets (Fig.4B). Furthermore, there was no difference in IAUC (Fig.4C). Again, we found no difference in 6-hour fasting glucose before the ITT (Fig.4D). The ITT revealed no differences between the groups for any of the
time points (Fig.4E). DAUC confirmed that seafood- and meat-fed mice had no difference in insulin sensitivity (Fig.4F).

The feeding regime and/or body composition are strong drivers of plasma parameters

To examine if the meat and seafood diets modulated plasma parameters associated with fat and carbohydrate metabolism differently, blood was collected after 6 hours of food deprivation, at the end of the study. Plasma levels of LDL, HDL and total cholesterol were higher in meat-fed mice than seafood-fed mice when the mice were fed ad libitum (Fig. 5A) However, when the mice were pair fed, these parameters were similar in seafood- and meat-fed mice (Fig.5C). Measurements of free fatty acids (FFA), triacylglycerol (TAG), hydroxybutyrate (OH-but) and glycerol (gly) showed no differences between seafood- and meat-fed mice when fed ad libitum (Fig.5B). When pair fed, mice fed seafood had higher levels of TAG than both meat- and low-fat-fed mice (Fig.5D).

Mice fed a Western diet with lean seafood have increased spontaneous locomotor activity

The fact that meat-fed mice gained more weight than seafood-fed mice when given free access to the diets, but not when pair fed, indicated that a difference in energy intake was the primary driving factor for the observed differences in body composition during the ad libitum study. Based on this, we decided to investigate the metabolic effects of lean seafood and meat consumption using indirect calorimetry combined with measurements of spontaneous locomotor activity. As expected, mice given the meat diet ate significantly more than mice given the seafood diet (Fig. 6A). Interestingly, during the dark phase, the mice that switched from the low-fat diet to the seafood diet increased spontaneous locomotor activity, whereas those switching to the meat diet decreased spontaneous locomotor activity (Fig. 6B). In addition, meat-fed mice had a larger reduction in RER compared to mice fed seafood during the light phase (Fig.6C). Despite the difference in spontaneous locomotor activity, no difference was found in energy expenditure (Fig. 6D).

Lean seafood and lean meat diets have similar acute effects on blood glucose and plasma C-peptide concentrations

To elucidate whether the diets had acute distinct effects on postprandial glucose concentration and insulin secretion in the mice, we performed a meal response test. After 16 hours of feed
deprivation, the mice were fed 0.15g of their respective diets and allowed to eat for 10 minutes. Blood was collected prior to feeding and after 10, 20 and 30 minutes. Blood glucose levels were similar all time points (Fig. 7A). C-peptide levels seemed to be higher in meat-fed mice, but the difference did not reach statistical significance (Fig. 7B).

**A Western diet with lean meat as the protein source might be more palpable than a Western diet containing lean seafood**

To investigate the possibility that the difference in feed intake was due to different smell and/or taste of the diets, we performed a diet preference test. Mice were fasted for 16 hours before the experimental diets were introduced. We found no difference in first choice between the diets (Fig. 8A), but mice ate significantly more of the meat diet during the following 6 hours (Fig. 8B).
DISCUSSION

A large number of studies has been conducted to elucidate the effects of dietary protein in relation to satiety, thermogenesis and body weight management. However, the knowledge regarding how different protein sources, consumed at normal dietary levels, influence energy balance is scarce. Furthermore, several studies investigating the effects of different protein sources on satiety and energy expenditure have been of short duration [15]. In the present study, we have compared the effects in mice of intake for 12 weeks of a mixture of lean seafood with that of a mixture of lean meat on energy intake and diet-induced obesity.

When given free access to the diets, meat-fed mice ate more and gained significantly more weight compared to seafood-fed mice. No difference was found in the amount of lean mass, so the difference in weight gain was due to increased fat accretion in meat-fed mice. Calculation of feed efficiency revealed that meat-fed mice gained more weight per calorie consumed compared to mice fed seafood. This suggests that the meat diet is more obesogenic independent of energy intake. However, this apparent conclusion could not be sustained as we did not detect any difference between the two diets in relation to weight gain or fat accumulation during pair feeding. This implies that the feeding regime might influence feed efficiency. Restricting feed availability has been shown to reduce feed efficiency compared to a situation with unlimited access [20]. A possible explanation for this phenomenon is differences in digestibility of the diets. However, calculation of apparent digestibility of fat showed that apparent fat absorption was highest in seafood-fed mice.

Interestingly, results from the meal response test showed that consumption of the meat diet tended to stimulate a greater release of C-peptide, indicating increased insulin secretion following consumption of the meat diet. This is intriguing because insulin is a strong anabolic peptide capable of repressing gluconeogenesis, facilitating peripheral nutrient uptake, and inducing lipogenesis [21, 22]. Thus, insulin has been identified as a key component in development of diet-induced obesity [23-25]. Differences in insulin secretion might be involved in driving the observed differences in feed efficiency between seafood- and meat-fed mice during the ad libitum experiment. Moreover, one might speculate that restricting the meat-fed mice blunted differences in insulin secretion, thereby attenuating insulin signalling and differences in fat accretion. However, the fact that insulin functions as a satiety hormone and that we observed differences in feed intake contradicts this [26].
Protein source is not the only difference between the diets, as there are also variations in fatty acid composition, which also may be involved. Intake of n-3 polyunsaturated fatty acids has been shown to be less obesogenic compared to consumption of linoleic acid in rodent models under iso-energetic conditions [27, 28]. Interestingly, the diet composed of seafood was enriched in n-3 polyunsaturated fatty acids, and the meat diet was enriched in linoleic acid. This might, at least in part, explain why the meat-fed mice had increased feeding efficiency during ad libitum feeding and not during the pair feeding. Restricting feed intake in the meat-fed mice decreased the intake of linoleic acid, and this might have blunted the effect of linoleic acid on fat accumulation.

Using indirect calorimetry, we found no difference in energy expenditure between the diets at the transition from low-fat to the experimental diets. We did, however, find that switching to the seafood diet increased spontaneous locomotor activity compared to meat diet. This was surprising, as switching from a low-fat to a high-fat diet has previously been reported to decrease activity in mice [29]. Differences in activity are likely to reflect changes in energy expenditure [30]. However, we have previously observed an inverse correlation between activity level and obesity without detecting a difference in energy expenditure [31]. Furthermore, during measurements using indirect calorimetry, the mice were given free access to the diets, resulting in increased intake in the meat-fed mice.

The mechanisms underlying reduced energy intake in the seafood-fed mice were not established in this study. Differences in amino acid composition could possibly drive differences in energy intake. The seafood diet was enriched in taurine, an amino acid associated with decreased energy intake and satiety in both mice and human studies [19, 32, 33]. However, it has been a matter of dispute as to the ability of taurine to cross the blood-brain barrier and thereby influence satiety when ingested orally [34]. The potential role of taurine in decreased energy intake needs to be investigated more closely before any conclusion can be reached. Glycine is another amino acid of interest as glycine has been associated with decreased accumulation of fat mass in previous rodent studies [19, 35]. However, as mentioned earlier, we observed no difference in fat accretion during the pair feeding, which weakens the association between consumption of glycine and decreased accumulation of fat mass in the present study. Intake of leucine has also been associated with increased satiety [36]; however, there was no difference in leucine content in the diets.
In an *ad libitum* setting mice fed lean seafood consumed less energy and gained less body fat compared to mice fed lean meat suggesting that intake of lean seafood increased satiety. In the *ad libitum* setting feed efficiency was higher in the lean meat fed mice than in the lean seafood fed mice. By contrast, in the pair-feeding experiment we observed no differences in feed efficiency between lean seafood fed and lean meat fed mice. However, we observed a striking increase in spontaneous locomotor activity upon intake of the lean seafood Western diet, which together with the decrease feed intake would contribute to the diminished weight gain in lean seafood fed mice compared to mice fed the lean meat Western diet. The mechanisms behind the observed difference in energy intake and spontaneous locomotor activity remain to be established, and will require further studies on regulation of appetite and physical activity in response to intake of lean seafood.
ACKNOWLEDGMENTS

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GRANTS

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CONFLICT OF INTEREST

The authors have no conflicting interest, financial or otherwise.
LITERATURE CITED


Tables:

Table 1 Diet composition and analysis of the experimental mouse diets

<table>
<thead>
<tr>
<th>Components added (g/kg)</th>
<th>Low-Fat</th>
<th>Seafood</th>
<th>Meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein powder</td>
<td>162</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Seafood powder</td>
<td>-</td>
<td>214</td>
<td>-</td>
</tr>
<tr>
<td>Meatmix powder</td>
<td>-</td>
<td>-</td>
<td>194</td>
</tr>
<tr>
<td>Corn starch</td>
<td>420</td>
<td>302</td>
<td>309</td>
</tr>
<tr>
<td>Dextrine</td>
<td>175</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Crisco</td>
<td>15</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Lard</td>
<td>14</td>
<td>31</td>
<td>43</td>
</tr>
<tr>
<td>Milk fat</td>
<td>15</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Soy oil</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1</td>
<td>5</td>
<td>5</td>
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</table>

Analysed values

<table>
<thead>
<tr>
<th></th>
<th>Low-Fat</th>
<th>Seafood</th>
<th>Meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (g/kg)</td>
<td>45</td>
<td>156</td>
<td>158</td>
</tr>
<tr>
<td>(N*6.25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (g/kg)</td>
<td>45</td>
<td>174</td>
<td>185</td>
</tr>
<tr>
<td>Energy (J/g)</td>
<td>17900</td>
<td>20000</td>
<td>20400</td>
</tr>
</tbody>
</table>

Analysed values represent the mean of duplicate measurements.
Table 2 Fatty acid composition of the diets

<table>
<thead>
<tr>
<th>mg/g</th>
<th>Low-Fat</th>
<th>Seafood</th>
<th>Meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>∑ SFA</td>
<td>12.1</td>
<td>60.3</td>
<td>70.0</td>
</tr>
<tr>
<td>∑ MUFA</td>
<td>9.0</td>
<td>42.0</td>
<td>47.6</td>
</tr>
<tr>
<td>LA. 18:2n-6</td>
<td>6.9</td>
<td>32.8</td>
<td>35.4</td>
</tr>
<tr>
<td>AA. 20:4n-6</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>∑ n-6</td>
<td>6.9</td>
<td>33.3</td>
<td>35.7</td>
</tr>
<tr>
<td>ALA 18:3n-3</td>
<td>0.8</td>
<td>3.9</td>
<td>4.1</td>
</tr>
<tr>
<td>EPA 20:5n-3</td>
<td>&lt;0.1</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>DHA 22:6-n3</td>
<td>&lt;0.1</td>
<td>2.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>∑ n-3</td>
<td>0.9</td>
<td>10.1</td>
<td>4.4</td>
</tr>
<tr>
<td>n-3/n-6 ratio</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Abbreviations: SFA – saturated fatty acids; MUFA – monosaturated fatty acids; LA – linoleic acid; AA – arachidonic acid; ALA – α-linoleic acid; EPA – eicosapentaenoic acid; DHA – docosahexaenoic acid.
Table 3 Amino acid composition of the diets

<table>
<thead>
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<th>mg/g</th>
<th>Low-Fat</th>
<th>Seafood</th>
<th>Meat</th>
</tr>
</thead>
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<tr>
<td>Alanine</td>
<td>4.7</td>
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<td>9.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.5</td>
<td>9.2</td>
<td>8.4</td>
</tr>
<tr>
<td>∑ Aspartate+Aspargine</td>
<td>11.2</td>
<td>16.9</td>
<td>15.6</td>
</tr>
<tr>
<td>∑ Glutamate+Glutamine</td>
<td>34.7</td>
<td>24.3</td>
<td>24.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.8</td>
<td>9.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.1</td>
<td>3.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>-</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.5</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>14.2</td>
<td>12.3</td>
<td>12.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>13.1</td>
<td>15.7</td>
<td>15.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.8</td>
<td>4.4</td>
<td>3.8</td>
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<tr>
<td>Phenylalanine</td>
<td>7.4</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Proline</td>
<td>16.4</td>
<td>5.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Serine</td>
<td>8.9</td>
<td>6.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Taurine</td>
<td>-</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.2</td>
<td>6.7</td>
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<tr>
<td>Tryptophan</td>
<td>1.6</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.5</td>
<td>3.7</td>
<td>3.6</td>
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<tr>
<td>Valine</td>
<td>9.7</td>
<td>7.3</td>
<td>7.5</td>
</tr>
<tr>
<td>∑ BCAA</td>
<td>31.3</td>
<td>26.6</td>
<td>26.9</td>
</tr>
<tr>
<td>∑ DAA</td>
<td>70.6</td>
<td>71.4</td>
<td>71.8</td>
</tr>
<tr>
<td>∑ IAA</td>
<td>84.1</td>
<td>76.0</td>
<td>70.1</td>
</tr>
</tbody>
</table>

IAA – indispensable amino acids (HIS, ARG, LEU, LYS, MET, PHE, THR, VAL, ILE);
DAA – dispensable amino acids (ALA, ASP, GLU, GLY, PRO, SER, TYR);
BCAA – branched-chain amino acids (VAL, ILE, LEU).
Figure legends:

**Figure 1** Male C57BL/6 mice with free access to a diet composed of meat consume more energy and gain more body fat compared to mice with free access to a seafood diet. (A) Cumulative energy intake. (B) Feed efficiency. (C) Apparent digestibility based on the last 3–5 days of the experiment. (D) Body composition at week 0 and 9; noted differences are for fat mass. (E) Adipose tissue mass after 12 weeks of feeding, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), retroperitoneal white adipose tissue (rWAT). (F) Lean tissue dissected out in week 12. The data represent group means (n=10/group) ± SEM. Different letters denote statistical significance (P<0.05).

**Figure 2** Glucose- and insulin tolerance tests performed on male C57BL/6J mice fasted for 6 hours after 10 and 11 weeks with free access to the experimental diets, respectively. (A) Fasting (6hrs) blood glucose levels before starting the oral glucose tolerance test (n=10/group). (B) Glucose response curve after administering 3mg glucose/g by gavage (n=10/group). (C) Incremental area under the curve (IAUC) (n=10/group). (D) Fasting (6hrs) glucose levels before starting the insulin tolerance test (n=10/group). (E) Glucose response curve after administering 0.75 units insulin/kg lean mass, n=10/per group except in LF n=6, 4 mice taken out due to dangerously low glucose levels (F) Decremental area under the curve, n=10 for the experimental groups and n=6 in LF. The data represent group means ± SEM. Different letters denote statistical significance (p<0.05).

**Figure 3** Equalising the energy intake (pair feeding) neutralised/counteracted the differences in body fat mass gain. (A) Cumulative energy intake. (B) Feed efficiency. (C) Apparent digestibility based on the last 3–5 days of the experiment. (D) Body composition at week 0 and 9; noted differences are for fat mass. (E) Adipose tissue mass after 12 weeks of feeding, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), retroperitoneal white adipose tissue (rWAT). (F) Lean tissue dissected out in week 12. The data represent group means (n=10/group) ± SEM. Different letters denote statistical significance (p<0.05).
Figure 4 Glucose and insulin tolerance tests performed on male C57BL/6J mice fasted for 6 hours after 10 and 11 weeks of pair-feeding experimental diets, respectively. (A) Fasting (6-hour) blood glucose levels before starting the oral glucose tolerance test (n=10/group). (B) Glucose response curve after administering 3mg glucose/g by gavage (n=10/group). (C) Incremental area under the curve (IAUC) (n=10/group). (D) Fasting (6-hour) glucose levels before starting the insulin tolerance test (n=10/group). (E) Glucose response curve after administering 0.75 units insulin/kg lean mass, n=10/per group except in LF n=8, where 2 mice were taken out due to dangerously low glucose levels. (F) Decremental area under the curve, (n=10) for the experimental groups and (n=8) in LF. The data represent group means ± SEM. Different letters denote statistical significance (p<0.05).

Figure 5 Plasma metabolite concentrations in male C57BL/6J mice fasted for 6 hours after 12 weeks on experimental diets (ad libitum and pair feeding). (A and C) High-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol (TC). (B and D) Free fatty acids (FFA), triacylglycerides (TG), D-3 hydroxybutyrate (OH-but), glycerol (Gly). The data represent group means ± SEM. Different letters denote statistical significance (p<0.05).

Figure 6 Indirect calorimetry and spontaneous locomotor activity measured on a separate set of male C57BL/6J mice (seafood n=8) (meat n=7) before onset of obesity. (A) Ad libitum energy intake for the 3 days on experimental diet. (B-D) Change between the last 2 days on LF and the last 2 days on experimental diets for: (B) Spontaneous locomotor activity. (C) RER. (D) Energy expenditure (EE). The data represent group means ± SEM. Different letters denote statistical significance (p<0.05).

Figure 7 Diet preference test on a separate set of male C57BL/6J mice (n=30). (A) Choice of diet represents which diet the mice take the first bite of when presented both diets at the same time. (B) Amount eaten represents amount of diet eaten during the course of 6 hours with access to both diets (n=30). The data represent group means ± SEM. Different letters denote statistical significance (p<0.05).
Figure 8 Meal response test on a separate set of male C57BL/6J mice (n=26). (A) Glucose response curve after consumption of 0.15g of experimental diets. (B) C-peptide response curve after consumption of 0.15g of experimental diets. The data represent group means ± SEM. Different letters denote statistical significance ($p<0.05$).
Figure 1

A. Cumulative feed intake

B. Feed efficiency

C. Apparent digestibility

D. Body composition

E. Adipose tissue mass

F. Lean tissue mass
Figure 2

**Glucose before OGTT**

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<tr>
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<th>Low fat</th>
<th>Seafoodmix</th>
<th>Meatmix</th>
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<tr>
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**OGTT**

- Mean glucose concentrations over time.
- Significant differences indicated by asterisks.

**OGTT incremental AUC**

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**Glucose before ITT**

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**ITT**

- Mean glucose concentrations over time.
- Significant differences indicated by asterisks.

**ITT decremental AUC**

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Figure 3

Cumulative feed intake

- Low fat
- Seafood mix
- Meat mix

Feed efficiency

Apparent digestibility

Adipose tissue mass

Lean tissue mass

Body composition

Nitrogen

Fat

Week 0

Week 9

Liver

Kidneys

Heart
Low fat  Seafood  Meat

A  Glucose before OGTT
B  OGTT
C  OGTT incremental AUC
D  Glucose before ITT
E  ITT
F  Decremental AUC

Figure 4
Figure 5

**Ad libitum**

**Pair feeding**

**Low fat**

**Seafood**

**Meat**

<table>
<thead>
<tr>
<th>Cholesterol (mmol/l)</th>
<th>HDL</th>
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**Ad libitum**

**Pair feeding**

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Figure 6

A. Energy intake

B. Activity change

C. RER change

D. EE change
Figure 7

A. Blood glucose

B. Plasma C-peptid

- --- Seafood mix
- --- Meat mix
Figure 8

A. First choice of diet

- Black: Seafood
- Gray: Meat

B. 6hrs feed intake

- Black: Seafood
- Gray: Meat

Bars and error bars indicate statistical significance (a, b).