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Dietary protein in the prevention of diet-induced obesity and co-morbidities

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Preface

The work presented in this PhD thesis was carried out at the Laboratory of Genomics and Molecular Biomedicine, Department of Biology, University of Copenhagen and at National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway during the years 2011-2014, under the supervision of Professor Karsten Kristiansen, associate professor Lise Madsen and Dr. Bjørn Liaset.

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Abstract

Background: Obesity and related co-morbidities are increasing problems worldwide and nutritional approaches to prevent and alleviate these diseases are thus of great interest. High-protein diets have been shown to prevent and alleviate obesity and co-morbidities in rodents and humans through increased energy expenditure and satiety. Similarly, protein from different sources and in different forms has been shown to modulate obesity and co-morbidities. However, the impact of protein from different sources consumed at normal dietary levels remains to be further elucidated. Obesity-prone C57BL/6J mice were fed obesity-promoting diets with protein from different sources, in different forms and at different levels to evaluate the affect on development of obesity, glucose intolerance and dyslipidemia.

Results: In the present study the dietary level of protein, 16 versus 32 percent energy from protein, was found to be negligible in development of obesity and co-morbidities in mice. Seafood protein with high endogenous taurine and glycine contents was found to prevent diet-induced adiposity and dyslipidemia, both in ad libitum and pair-fed settings. The ability of seafood proteins to prevent these metabolic disturbances was found to associate with the high endogenous taurine and glycine concentrations and to concur with increased energy expenditure and a tendency towards increased voluntary locomotor activity. Consumption of a seafood protein-mixture prevented diet-induced development of obesity as compared to intake of chicken filet and preserved glucose tolerance compared to casein intake. Hydrolyzed casein was shown to prevent obesity compared to intact casein, which was associated with increased spontaneous locomotor activity in hydrolyzed casein-fed mice. No increase in energy expenditure, as assessed by indirect calorimetry, was observed after four weeks, but after eight weeks adaptations towards increased energy expenditure-capacity was present concurrent with altered energy substrate utilization in hydrolyzed casein-fed mice compared to mice fed the intact protein.

Conclusions: We found that the source and form of protein has great impact on development and prevention of diet-induced adiposity, dyslipidemia, hyperinsulinemia and impairment of glucose tolerance through modulations of voluntary locomotor activity, energy expenditure and energy substrate metabolism in mice.
**Resumé**

**Baggrund:** Fedme og relaterede sygdomme er et stigende problem på verdensplan og ernæringsmæssige tiltag der kan forebygge og afhjælpe disse sygdoms-tilstande er derfor af stor interesse. Et højt protein indtag har vist sig effektiv i forebyggelse af fedme og relaterede sygdomme, både i gnawere og mennesker, gennem forøget energiforbrug og mæthedsfølelse. Ligeledes har protein fra forskellige kilder vist sig at kunne påvirke fedme og relaterede sygdomme. Dog er det endnu ikke klarlagt hvilke effekter protein fra forskellige kilder har, når disse indtages på normalt niveau. C57BL/6J mus, som har tilbøjelighed til at udvikle fedme, blev fodret med fedme-fremmende foder med protein fra forskellige kilder, i forskellig form og i forskellig mængde, for at vurdere indflydelse på udviklingen af fedme, glukose intolerance og dyslipidæmi.

**Resultater:** Mængden af protein i kosten, 16 versus 32 procent energi fra protein, viste sig ikke at påvirke udviklingen af fedme og følgesygdomme hos mus i dette forsøg. Foder med protein fra fisk og skaldyr forebyggede kost-induceret fedme og dyslipidæmi, både under ad libitum og pair-fed forhold. Forebyggelsen af disse metaboliske forstyrrelser var korreleret med det høje indhold af taurin og glycin i protein fra fisk og skaldyr og derudover var energiforbruget forøget imens aktivitetsniveauet tenderede til at være forøget hos mus der fik protein fra fisk og skaldyr. Indtag af protein fra fisk og skaldyr forhindrede kost-induceret udvikling af fedme i forhold til indtag af kylling filet og bevarede glukose-tolerancen i forhold til indtag af kasein. Hydrolyseret kasein forebyggede kost-induceret fedme i forhold til intakt kasein hvilket var forbundet med øget aktivitetsniveau, men her sås ingen stigning i energiforbrug, målt via indirekte kalorimetri, efter fire uger. Imidlertid øgede hydrolyseret kasein kapaciteten for energiomsætning efter otte uger, set i forhold til de mus der blev fodret med intakt kasein.

**Konklusioner:** Opsummeret viser resultaterne at kilden til og formen af proteiner i kosten har stor betydning for udvikling og forebyggelse af kost-induceret fedme, dyslipidemia, hyperinsulinæmi og glukose intolerance, gennem regulering af aktivitetsniveau og energiforbrug i mus.
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## Abbreviations

- **BA**: Bile acids
- **BCAA**: Branched-chain amino acids
- **BMI**: Body mass index
- **CCK**: Cholecystokinin
- **CVD**: Cardiovascular disease
- **DIO**: Diet-induced obesity
- **DIT**: Diet-induced thermogenesis
- **EE**: Energy expenditure
- **E%**: Energy percent
- **GLP-1**: Glucagon-like peptide 1
- **GTT**: Glucose tolerance test
- **HDL**: High-density lipoprotein
- **HF**: High fat
- **HFHS**: High fat and high sucrose
- **HOMA-IR**: Homeostasis model assessment of insulin resistance
- **iaVAT**: Intra-abdominal visceral adipose tissue
- **iWAT**: Inguinal white adipose tissue
- **LDL**: Low-density lipoprotein
- **MS**: Metabolic syndrome
- **PI 3-kinase**: Phosphatidylinositol 3-kinase
- **RER**: Respiratory exchange ratio
- **SAT**: Subcutaneous adipose tissue
- **TAG**: Triacylglycerides
- **T2DM**: Type 2 diabetes mellitus
1 - Introduction

1.1 - A world spanning health issue

Over the last few decades diseases related to metabolic disturbances, e.g. diet-induced obesity, type II diabetes mellitus (T2DM) and non-alcoholic fatty liver disease have increased dramatically. Worldwide obesity has nearly doubled during the last thirty years [1]. Obesity is defined as a Body Mass Index (BMI [kg/m²]) score greater than 30 and overweight is defined as a score between 25 and 30. For simplicity, obesity will be used as a collective term for unhealthy overweight and obesity for the remainder of this discussion.

Prevalence of obesity differs immensely geographically, constituting an epidemic in certain regions, most pronounced in the Western world but also a problem in certain developing regions, e.g. in Asia [2]. Obesity is associated with a range of co-morbidities, such as T2DM, cardiovascular disease (CVD), depression, dementia and certain cancers [3], and thus negatively affects quality of life and life expectancy in affected individuals, emphasizing the need for action to circumvent this alarming and preventable development.

Despite wide utilization of the BMI score, waist circumference may be a better indicator of unhealthy adiposity as intra-abdominal visceral adipose tissue (iaVAT) is a greater risk factor for T2DM and CVD than fat accumulation in peripheral subcutaneous adipose tissue (SAT) [4-7]. Abdominal fat is a defining factor of the metabolic syndrome (MS), which is a clustering of risk factors that independently and together increase the risk of CVD and T2DM. MS is defined as the concurrent presence of centrally located adiposity and at least two of the following risk factors; high blood pressure, dysglycemia (high fasting plasma glucose), dyslipidemia (high plasma triglyceride and/or low high-density lipoprotein (HDL) cholesterol levels), a pro-thrombotic state, a pro-inflammatory state and insulin resistance [8, 9]. The reasons for iaVAT being more harmful than SAT are multifaceted and reflect differences in physiology and function between different adipose tissues [4].

1.2 - The development of obesity and T2DM

The succession and mechanisms of development of obesity and T2DM and the association between the two remain to be fully resolved. Obesity may be a symptom of underlying metabolic disturbances rather than the cause of associated co-morbidities per se [10], as indicated by the fact that a person may be lean and suffer from T2DM, as well as an obese person may be metabolically healthy [11-13]. T2DM has traditionally
been considered to develop as a consequence of diet-induced obesity when systemic tissues become insulin resistant which in turn causes hyperglycemia followed by hyperinsulinemia as the pancreas secretes compensatory insulin, eventually leading to β-cell exhaustion and consequentially T2DM [14]. However, more recently this view has been challenged as hyperinsulinemia has been proposed to precede insulin resistance and to be a requirement for diet-induced obesity (DIO) [14-16].

During energy surplus SAT expands through hypertrophy and hyperplasia [17], thus acting as a metabolic sink storing surplus energy as triacylglycerides (TAG), potentially protecting non-adipose tissues against lipotoxic lipid spillover [10, 18]. Insulin stimulates uptake of glucose and NEFA, inhibits lipolysis in adipocytes [18-20], is involved in differentiation of pre-adipocytes [20, 21] and may hence increase adipocyte hypertrophy and hyperplasia. Hyperinsulinemia may thus constitute a compensatory function facilitating adipose tissue expansion to prevent lipotoxicity. However, hyperinsulinemia may turn detrimental if energy surplus and adipose tissues expansion persists. Adipocytes are not mere fat-storing depots, but have endocrine functions through secretion of hormones and adipokines, e.g., leptin, adiponectin, TNF-α and IL-6 amongst others [22] and as adipose tissues expand, the secretion of adipokines increases, causing macrophage infiltration which further increases the secretion of adipokines [23].

Adipocyte death increases markedly with increasing adipocyte hypertrophy and macrophage infiltration of expanding adipose tissue may serve an adipocyte debris-clearance function as the macrophages selectively locate surrounding dead adipocytes [24]. During the development from lean to obese, a shift occurs in the macrophages present in adipose tissue, from M2 macrophages, secreting primarily anti-inflammatory cytokines, towards M1 macrophages which mainly secrete pro-inflammatory cytokines [25]. This results in chronic, low-grade inflammation, which has been associated with insulin resistance in adipose tissue [25, 26] as well as in muscle tissue [27].

If SAT fails to accommodate the increasing demand for lipid storage, ectopic fat may accumulate in other insulin sensitive tissues, e.g., liver, iaVAT, heart and skeletal muscle [4]. iaVAT expansion is thus an indication of dysfunctional adipose tissue [28]. iaVAT differs from SAT in multiple ways, as reviewed in [4]. Fatty acids uptake from circulation and insulin-induced inhibition of lipolysis is decreased while lipolytic rate is increased in iaVAT. iaVAT expansion thus leads to increased circulating NEFA levels, which has been shown to lead to insulin resistance in liver and skeletal muscle [29, 30], possibly through attenuated insulin signaling and insulin-stimulated glucose transport activity [31, 32]. Furthermore, iaVAT typically has larger adipocytes, increased adipocyte death rate and an increase in inflammatory cells and immune cells which
results in increased adipokine secretion and inflammation. NEFA and adipokines released from iaVAT drains directly through the portal vein to the liver [33], leading to hepatic inflammation and fat deposition and in turn insulin resistance and hyperinsulinemia [5]. Increased NEFAs in the portal venous system furthermore causes increased hepatic synthesis and secretion of small VLDL particles [5] and iaVAT has been shown to correlate with cholesterol synthesis and reduced HDL cholesterol levels [34]. In summary, SAT expansion may protect against lipotoxicity while iaVAT fat accumulation may lead to dyslipidemia, a pro-inflammatory and pro-thrombotic state and insulin resistance, i.e., features of MS.

1.3 - Nutrition in relation to obesity and T2DM

Severe financial, social and public health consequences related to obesity and associated co-morbidities have initiated considerable research into alleviation and prevention of these metabolic disturbances. Increased availability of high energy density food items and concurrently decreased physical activity are considered the primary causes behind the obesity epidemic. However, also a change in dietary macronutrient composition may be at cause.

1.3.1 - Macronutrients – is a calorie simply a calorie?

Obesity is simply put the consequence of an imbalance where energy intake surpasses energy expenditure. However, as different dietary strategies seem to affect weight gain and loss differently at equal energy intake [16, 35-37], questions are raised whether a calorie is simply a calorie [38] or whether the composition of dietary macronutrients is also important.

In the history of modern humans, agriculture is the single factor affecting dietary macronutrient composition the most. Pre-agricultural humans were limited to hunter-gatherer diets, i.e., wild plants and animal prey [39]. With cultivation of cereals and starch bearing crops, dietary carbohydrate content increased vastly at the expense of protein [40], a development further promoted by industrialization [40], most profoundly in the last few decades; Intake of energy from carbohydrates increased significantly in the United States of America between 1971 and 2000 [41]. Interestingly, the acceleration in prevalence of obesity and diabetes occurred concurrently with the profound increase in carbohydrate intake during the last few decades [42]. It is plausible that these changes in macronutrient proportions are part of the causes behind increased prevalence of obesity. However, correlation and causality is still disputed. No correlation between daily carbohydrate intake and BMI was found in an observational study analyzing seven-day
dietary recall of healthy adults [43], and randomized weight loss trials reported equal weight loss by energy restriction irrespective of macronutrient composition [44, 45].

Nevertheless, low-carbohydrate diets have proven efficient for weight loss in humans [36, 46-50] and partially replacing dietary carbohydrate with protein resulted in significant weight loss in obese subjects at three and six months in randomized trials [49, 50]. Moreover, in rodents fed high fat-diets carbohydrates have been shown to increase weight gain compared to protein [16, 35, 37, 51]. Furthermore, increased protein to carbohydrate ratio was associated with improved glucose homeostasis in mice [51] and improved cardiovascular risk factors in obese human subjects [47, 48]. It is evident from a substantial amount of scientific data that dietary proportions of macronutrients are of great importance in preventing and alleviating features of MS [52]. The remainder of this thesis will focus on the effect of dietary protein on obesity and co-morbidities.

1.4 - Dietary protein

1.4.1 - Satiety and energy expenditure

Increased proportion of dietary protein at the expense of fat or carbohydrate has been shown to prevent diet-induced weight gain in mice [16, 35, 37, 51] and has proven efficient in obese human subjects for both weight loss [53, 54] and subsequent weight maintenance [50, 55], at least short-term [56]. Compared to iso-energetic consumption of fat and carbohydrate, protein reduces appetite in rats [57] and induces greater satiety in human subjects [53, 58] and has furthermore been shown to increase diet-induced thermogenesis (DIT) in both rats [59] and humans [60-62]. DIT is defined as increase in energy expenditure (EE) above basal metabolic rate induced by diet [63]. DIT is thought to have evolved as an adaptive mechanism allowing for regulating nutrient balance in case of unbalanced diet availability [64]. In this way feed intake could be increased to secure sufficient intake of a limiting nutrient while the increase in EE would expend excess energy and macronutrients. In rats DIT was found to be high with low-protein diets and to decrease continuously with increasing protein energy percent (E%) from four to 18 E%, between 18 and 30 E% DIT was at a constant low and then increased continuously again with increasing E% from protein [59]. The higher EE associated with high protein intake is likely derived on energy-expending pathways of protein metabolism, i.e., protein synthesis, ureagenesis and gluconeogenesis [65].

In rats EE was found to be increased by hyperphagia, expending almost 90% of the excess consumed energy [66]. A similar effect was seen in lean mice which increased EE in response to increased energy intake, thus
expending the excess energy ingested [67]. However, in obese mice hyperphagia did not induce EE enough to expend the excess consumed energy, and when comparing lean and obese mice ingesting equal amount of energy the obese mice had lower EE and nearly three times increased body weight accretion compared to the lean mice [67]. This demonstrate that hyperphagia is not a requirement for DIO and highlights the importance of metabolic efficiency and DIT in development of obesity [64]. Indeed low relative EE, i.e. EE adjusted for body mass, has been identified as a risk factor for developing obesity [68] and postprandial thermogenesis has shown to be 40-50% lower on average in obese compared to lean subjects [69, 70] with an inverse correlation between body fat percentage and DIT.

DIT has been found to correlate positively with satiety [71] and it is thought that DIT increases satiety [72, 73], which might explain the greater satiety associated with higher protein intake. An increase in dietary protein may thus likely contribute to weight loss by increasing DIT and satiety and in turn decreasing energy intake. A randomized, three-way crossover trial where healthy, overweight male subjects ingested baseline (normal balanced diet), low-fat, high-carbohydrate or low-fat, high-protein diets with either pork or soy for four days and spend the fourth day inside a respiratory chamber, demonstrated that DIT was higher with both high protein-diets than with the high carbohydrate-diet. Moreover, 24h EE was higher with high-pork than high-soy protein, high-carbohydrate and baseline diets. EE was also higher in high-soy than in high-carbohydrate, but only high-pork significantly increased EE above baseline level. It was calculated that substituting fat with protein increased 24h EE by 3% more than carbohydrate and inclusion of pork instead of soy increased 24h EE by additionally 2% [62].

Our research group have previously shown that increased dietary protein, at the expense of carbohydrates, cause sustained long-term prevention against DIO in mice [16, 35, 37], however it is not fully elucidated whether the beneficial effects of increased dietary protein are maintained over time in human subjects [56]. The satiating effect of protein was found to be inversely associated with habitual protein intake in human subjects [74], which may influence long-term effects of high protein-diets. Furthermore, concerns about the safety of high habitual protein intake have been raised in relation to calcium homeostasis and bone density, renal function, CVD and cancer risk [75]. A meta-analysis of fifteen randomized controlled trials on long-term effects of low-protein versus high-protein diets found neither beneficial nor detrimental effects of high-protein diets on obesity, CVD or glucose metabolism [76]. However, another study found that a five percent iso-energetic replacement of either fat or carbohydrates with protein was associated with weight gain at five year follow up and protein E% above 22 was associated with 23–24% higher risk of becoming overweight or obese [77]. Furthermore, a recent study reports that higher protein intake of
animal origin, especially meats, was positively associated with inflammation markers in obese adults, however this was not the case with proteins from vegetables and fish [78].

Another disadvantageous aspect of high protein diets is the climate impact of dietary protein sources [79]. Animal protein sources come with a higher cost of resources than vegetal protein sources [79], especially red meat sources which happen to be the protein sources that affect EE the most. Animal husbandry is associated with pollution, emission of greenhouse gasses and deforestation to facilitate livestock and feed production for the animals [80]. As is, 70% of all agricultural land, 30% of total terrestrial surface not covered by ice, is used for live stock grazing and feed production [81]. These numbers would increase considerably if just a fraction of the obese individuals in the world were to increase their intake of animal protein and resources would be at higher risk of depletion. Fortunately, the abovementioned differences observed with protein from different sources suggest that not only the quantity but also the quality of dietary protein may be of importance in the prevention of obesity and associated disease states. However, while a lot of research has focused on high proportions of protein, little is still known about the effect of protein from different sources at normal dietary levels, i.e., 10-20 E%, in relation to satiety, EE and prevention of obesity and co-morbidities.

1.4.2 - Protein from different sources

Proteins differ primarily with regards to amino acid (AA) composition and the bioavailability of these, which influence gastric absorption kinetics and postprandial increase in blood AAs [82], which in turn affect satiety [83], possibly in part through differences in brain monoamine neurotransmitters [84].

Casein and whey, two well-studied proteins constituting the majority of proteins in bovine milk, are often compared as examples of proteins with different absorption kinetics. Casein is insoluble and coagulates in the acidic gastric environment upon ingestion leading to delayed gastric emptying [85], whereas whey is soluble and readily absorbed [82]. Whey was shown to increase postprandial plasma total AAs and induce greater satiety compared to casein [83, 86]. In high fat (HF)-fed C57BL/6J mice four independent studies demonstrate that whey protein reduced diet-induced weight gain compared to casein [87-90]. Furthermore, HF-whey feeding was associated with improved glucose tolerance [87] and decreased hepatic lipid accumulation [88-90]. In none of these four studies did HF-whey fed mice ingest less energy than the HF-casein fed mice, suggesting that additional features besides increased satiety are contributing to the beneficial effects of whey protein. Indeed, whey protein has also been shown to increase postprandial EE
more than casein and soy [91] and have greater insulinotropic effect than whole milk and cheese (naturally high in casein and low in whey) [92]. Moreover was postprandial lipemia regulation improved after a very high fat-meal when the protein source was whey instead of casein in diabetic subjects [93]. In a recent study from our group whey was shown to reduce DIO compared to casein due to increased urinary excretion of intermediates of the tricarboxylic acid cycle [89], leading to a loss of energy substrates. These pronounced differences between casein and whey demonstrate the importance as well as the potential of protein sources in prevention of obesity and co-morbidities.

Protein increases DIT more than fat and carbohydrate but as described above also different sources of protein may influence DIT, as pork was shown to increase 24h EE more than soy protein [62] and whey was shown to increase postprandial EE more than casein and soy [91]. However, little is still known about the effect of protein from different sources on DIT. Given the prevalence of obesity and co-morbidities and the findings that DIT is decreased by 40-50% in obese subjects [69, 70] the potential of increasing DIT by dietary interventions is of great interest.

Something that has been better studied is the varying effect of satiety between protein from different sources. Indeed, a range of studies have demonstrated differences in acute satiating effect of different protein sources [83, 84, 94-96], but the interpretation of data is confounded by differences in dietary macronutrient composition and is thus inconclusive. Furthermore, these acute postprandial effects on satiety do not say anything about long term effects on energy intake and whether this ultimately translates into prevention of obesity. Interestingly, in the studies including fish satiety was consistently higher after fish consumption compared to beef and chicken [84], beef [94] and turkey and egg [95].

1.4.3 - Seafood protein

Observational studies have associated consumption of fish with decreased risk of T2DM [97, 98] and CVD [99-104] and a dose-dependent decrease of blood inflammatory markers [105] in humans, while other prospective studies found no such associations [106-108]. However, while the conclusions of prospective and observational studies may be impeded by immense demographic differences in habitual seafood intake, the beneficial and protective effects of seafood consumption have been confirmed in randomized controlled intervention studies as described in the following.

Overweight men and women were given placebo or cod protein capsules (three g/d for the first four weeks and six g/d for the remaining four weeks) for eight weeks. Without causing any differences in
macronutrient and energy intake between groups or any other changes in diet and lifestyle the cod protein supplementation resulted in decreased body weight and improved body composition after four weeks. At the completion of the study cod-supplemented subjects had improved blood cholesterol profile as well as decreased fasting and 2h postprandial glucose levels [109]. Energy-restricted dieting in overweight young adults has been shown to be more effective, i.e. leading to greater weight loss, when fish is included in the diet compared to isoenergetic control diets without seafood [110, 111]. Also, incorporation of a daily fish meal into a weight-loss regimen was more effective than either fish consumption or weight loss alone at improving glucose-insulin metabolism and dyslipidemia [112]. In a crossover design study providing diets that differed only with regards to the protein sources, four weeks of cod-diet improved insulin sensitivity as evaluated by hyperinsulinemic-euglycemic clamp [113] and reduced plasma C-reactive protein [114] compared to a diet containing a mixture of protein from beef, pork, veal, eggs and dairy. A reduction of inflammatory markers was also seen in obese subjects consuming cod or salmon three times per week for eight weeks compared to subjects eating a diet without fish [115].

Beneficial effects of seafood protein consumption in relation to DIO, T2DM and co-morbidities have also been demonstrated in rodent models. In male Wistar rats fed isoenergetic HF diets with protein from either casein, soy or cod for 28 days, casein-feeding was associated with increased fasted plasma glucose, increased fasted and postprandial (1-2h) plasma insulin, decreased glucose tolerance and glucose disposal rate during hyperinsulinemic-euglycemic clamp compared to rats fed soy or cod [116]. Subsequent studies revealed whole body and skeletal muscle insulin resistance in casein and soy-fed compared to cod-fed rats [117]. Cod-protein prevented HF diet-induced insulin resistance through improved insulin-mediated glucose uptake and disposal [117, 118], by acting as an insulin sensitizing agent restoring peripheral insulin signaling by normalizing PI 3-kinase signaling and improving GLUT4 translocation in cod-fed compared to casein and soy-fed mice [118].

In a comparison of other fish species, salmon reduced weight gain and visceral adiposity and improved insulin sensitivity in rats compared to casein, bonito, herring and mackerel while all the fish species reduced visceral adipose inflammation, i.e. TNF-α and IL-6, compared to casein-fed rats [119]. Also sardine protein was found to reduce diet-induced weight gain, adiposity, insulin resistance and inflammation compared to casein [120]. Furthermore, seafood proteins were also shown to improve lipid status [120-122].

Saithe protein hydrolysate reduced diet-induced weight gain, adiposity and plasma triacylglycerides and at the same time increased plasma bile acids (BA) and expression of genes involved in EE energy expenditure.
and fatty acid oxidation compared to casein and soy protein in rats at equal energy intake [123]. Similarly, salmon protein hydrolysate reduced weight gain, body fat, energy efficiency, plasma and liver TAG, fed state plasma glucose and insulin, while increasing plasma BA and induction of genes involved in EE and uncoupling and increased whole body EE as assessed by indirect calorimetry [124]. The beneficial effects of salmon protein hydrolysate were attenuated by pharmacologically lowering plasma BA with the BA-binding resin cholestyramine, demonstrating that salmon hydrolysates prevent DIO and co-morbidities in rats, at least in part, by increased EE through increased BA concentration [124]. BA concentration was most likely increased by the seafood proteins due to higher taurine and glycine concentration found in seafood protein compared to casein.

The positive effects of seafood consumption have traditionally been ascribed primarily to marine n-3 long-chain polyunsaturated fatty acids. However, other factors seem to contribute to the benefits associated with seafood consumption e.g. v iodine, vitamin D, selenium and the taurine [125].

1.4.4 – Taurine

Seafood protein sources differ distinctively from terrestrial protein sources by having a higher concentration of taurine (2-amino ethane sulfonic acid) [126]. Taurine is one of the most abundant molecules in mammalian cells and resides freely since the sulfonic acid group renders the molecule metabolically inert, i.e. neither oxidized nor incorporated into protein. Due to its abundance and participation in a variety of fundamental physiological functions, i.e. regulation of volume and osmolarity, protection against oxidative stress, neuromodulation, stabilization of membranes as well as modulation of channels and cell signaling, taurine has been extensively researched and found to be involved in a myriad of processes, including obesity and glucose and insulin metabolism.

The higher satiating effect of fish protein compared to beef and chicken found by Uhe et al, as described above (1.4.2), was accompanied by increased postprandial plasma taurine concentration [84]. Another study, evaluating the effect of 10 and 25 E% soy on satiety and energy intake, confirms the association between plasma taurine and satiety, as greater satiety following the 25 E% soy meal was shown to correlate significant with postprandial plasma taurine concentration [127]. Supplementation of taurine in high fat, normal protein-diet reduced DIO and improved glucose tolerance and moreover reduced ad libitum energy intake, which was claimed as a consequence of observed increased hypothalamic insulin signaling in mice [128]. Oral taurine supplementation, either in diet or drinking water, was similarly shown to prevent diet-induced weight gain and adiposity in rats [129] and mice [130] which in mice were shown to
associate with a concurrent increase in resting $O_2$-consumption, without differences in activity-related $O_2$-consumption, and increased expression of EE-related genes, indicating that stimulation of resting EE contributed to the decreased weight gain [130]. Taurine supplementation has furthermore been shown to improve blood lipid profiles in rodents [129, 131-134] as well as overweight human subjects [135].

Seafood proteins, i.e. saithe and salmon with endogenous high glycine and taurine, was shown to increase plasma BA level in rats as described above (1.4.3), which was associated with prevention of DIO and improvement of plasma lipids [123, 124]. Other studies have shown plasma lipid improvement by dietary taurine and glycine supplementation through increased fecal excretion of BA and cholesterol in rats [133, 134]. However, in the rats fed the seafood protein with endogenous taurine and glycine the improvements in body composition and plasma lipids was not associated with increased fecal BA excretion, but rather with increased EE as assessed by indirect calorimetry and increased induction of genes involved in EE [123, 124]. It is well-established that BA, apart from their emulsifying properties which aid in absorption of fats and fat soluble vitamins, modulate EE through Farnesoid X receptor and TGRS [136].

The affinity of the bile salt export pump is greater for conjugated than for non-conjugated BA [137]. Conjugation of BA therefore increases biliary secretion of BA [138] and may thus in turn enhance BA synthesis leading to the increase in BA pool seen with increased dietary taurine and glycine in rats [123, 124]. The conjugation of bile acids is highly species specific [139, 140]; rats conjugate primarily with taurine but also to some extend with glycine, rabbits conjugate exclusively with glycine, mice conjugate almost exclusively with taurine, while human subjects conjugate with both glycine and taurine, usually in the proportions 3:1 [141]. It has been demonstrated that taurine and glycine supplementation increases BA pool in hamsters [142] and as described above that seafood protein rich in glycine and taurine increases plasma BA in rats [123, 124].

Taurine-supplementation studies are numerous and have demonstrated several beneficial effects of taurine. However, they often utilize supraphysiological taurine concentrations and thus present a somewhat unnatural setting and taurine concentrations that are not attainable through a healthy, balanced diet. To the best of our knowledge, no previous studies have evaluated the effect of protein sources with varying endogenous taurine concentrations with regard to DIO, T2DM and co-morbidities. From the knowledge obtained through taurine-supplementation, it seems plausible to hypothesize that feeding mice seafood protein with endogenous high taurine concentration may affect satiety, DIO, glucose tolerance, dyslipidemia and EE.
1.4.5 - Hydrolyzed versus intact proteins

Gastric kinetics and absorption differs between casein and whey as described above (1.4.1). While whey (a fast protein) empties rapidly and readily into the intestines from the stomach as intact protein, casein (a slow protein) clots and precipitates in the acidic environment of the stomach and thus remains in the stomach for longer and is subjected to a higher degree of gastric peptic hydrolysis until finally being available to emptying into the intestines as degraded peptides [85]. Interestingly, hydrolyzation of casein has been shown to decrease hydrophobicity [143] and increase the rate of absorption compared to intact casein [144, 145], which was accompanied by increased postprandial plasma insulin and AA concentrations [145]. When comparing intestinal absorption rates of AAs after ingestion of either intact whey, hydrolyzed whey, intact casein or hydrolyzed casein, equal rates were found for all proteins except intact casein which was characterized by slower absorption [146], i.e. hydrolyzation of casein resulted in faster absorption rate. It thus seems plausible to hypothesize that hydrolyzation of casein could confer some of the beneficial effects associated with whey compared to casein, i.e., increased satiety, reduced weight gain and hepatic lipid accumulation, increased EE, increased insulinoortropic effect and improved glucose tolerance.

Infusion of peptides has been shown to increase release of cholecystokinin (CCK) from isolated vascularly perfused rat duodenojejunum [147] and increase release of glucagon-like peptide (GLP-1) from isolated vascularly perfused rat proximal and transverse colon [148] more than infusion of either intact protein or a mixture of free AAs. GLP-1 is important for maintaining normal glucose homeostasis as it induces glucose stimulated insulin secretion, while CCK stimulates digestion by inducing release of pancreatic digestive enzymes and bile from the gallbladder and both hormones furthermore reduces appetite and energy intake. These stimulatory effects of peptides on hormone release, compared to intact protein and free AA, suggest a way in which hydrolyzed proteins may affect DIO, impaired glucose and insulin metabolism and other morbidities.

Indeed, fish protein (blue whiting) hydrolysate but not intact albumin or free AA mixture, was shown to dose-dependently increase release of CCK and active GLP-1 from STC-1 endocrine cell line [149]. Blue whiting hydrolysate furthermore dose-dependently increased plasma CCK and active GLP-1 and dose-dependently decreased energy intake in HF-fed rats. At the termination of the study, after 12 days, weight gain was only significantly decreased compared to control group in the rats fed the intermediate concentration of fish hydrolysate though, not in rats fed the high concentration [149]. Also other studies have shown improvements associated with hydrolyzed proteins [123, 124, 150, 151]. However, since these
studies do not compare the hydrolyzed proteins with the corresponding intact proteins it is not possible to fully derive the effect of hydrolyzation and the effect of using differing protein sources in these studies.
1.5 - Working hypotheses

These working hypotheses laid the ground for the work conducted as part of this PhD Project:

1. Protein sources with varying endogenous taurine concentration affect diet-induced weight gain, adiposity, dyslipidemia, metabolism of glucose and insulin, DIT and EE

2. Protein level and form, i.e. hydrolyzed versus intact, affect diet-induced weight gain, adiposity, dyslipidemia, metabolism of glucose and insulin, DIT and EE

1.6 - Animal model

These hypotheses were tested in male C57BL/6J mice fed obesity-promoting diets with protein from different sources, at different levels and in different forms. Mice (Mus musculus) are widely used in research and numerous commercial, inbred mouse strains differing tremendously with regard to propensity of DIO [152] are available. C57BL/6J mice are one of the most commonly used strains in research on obesity and metabolism as this strain is prone to obesity [152], dyslipidemia, hyperinsulinemia, insulin resistance, and glucose intolerance [153] compared to SV129, which is aggravated by HF feeding [152, 154, 155].

A deletion affecting the Nicotinamide nucleotide transhydrogenase (Nnt) gene, encoding an integral inner mitochondrial membrane protein catalyzing the interconversion of NADH and NADPH, has been identified in C57BL/6J mice [156], which was proposed to be linked to the glucose intolerance seen in these mice, presumably through reduced insulin secretion from β-cells [157]. However, the link between truncated Nnt and glucose intolerance was later disputed [158]. Fortunately, C57BL/6JBomTac used for these studies has been shown not to carry the Nnt mutation [159].

C57BL/6J mice was shown to excrete approximately 10 times more taurine in the urine (hypertaurinuria) through reduced tubular taurine reabsorption [160], than other (normal taurine excretor) inbred strains [160, 161]. It has unfortunately not been possible to obtain information on whether the C57BL/6JBomTac mice used for these studies are hypertaurinuric. However, of the two cited studies on hypertaurinuria in C57BL/6J, the first paper was published in 1953 from a group in the United Kingdom and the second paper over twenty years later in 1976 from a group in Canada, implying that this trait dates long back and is preserved over time and at different suppliers. This should be kept in mind when analyzing the results of these studies.
2 - Objectives and aims

The objectives and aims of this PhD project were to evaluate how protein from different sources, in different forms and at different levels affect the development of DIO and co-morbidities, more specifically:

1. To evaluate whether protein from different sources, i.e., casein, chicken and lean seafood, with varying endogenous taurine concentrations, affect diet-induced adiposity as well as disturbances in plasma lipid levels and glucose and insulin metabolism, possibly by altering energy expenditure.

2. To evaluate whether the form of a protein, i.e., intact vs. hydrolyzed casein, impacts diet induced adiposity, dyslipidemia as well as impaired glucose and insulin metabolism, possibly by altering energy expenditure.

3 - List of manuscripts

The papers included in this PhD thesis are:

1. Tastesen HS, Keenan AH, Madsen L, Kristiansen K, Liaset B. 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 Epub ahead of print, DOI 10.1007/s00726-014-1715-1.


4 - Results and discussion

4.1 - Dietary protein sources in the ad libitum setting

Previous studies have shown that protein sources can modulate DIO and co-morbidities. In rats seafood protein with high endogenous taurine and glycine contents was found to reduce DIO, increase plasma BA, increase energy expenditure and induce genes involved in energy metabolism compared to casein and soy [123, 124]. In Paper 1 feeding male C57BL/6J mice various protein sources with increasing taurine and glycine concentrations ad libitum in a high-fat, high-sucrose (HFHS) background-diet revealed that the protein sources with the highest taurine and glycine contents, i.e. scallop, prevented diet-induced weight gain, adiposity and dyslipidemia (Paper 1, Fig. 1-3). Significant negative correlations were found between taurine and glycine intake and adiposity and significant positive correlations were found between taurine and glycine intake and improved plasma cholesterol profile (Paper1: Table 3). Crab-feeding, i.e. the protein with second highest taurine and glycine concentrations, also led to reduced body mass gain compared to cod-fed mice, but these mice were not protected from diet-induced increase in adiposity and dyslipidemia (Paper 1: Fig. 1-3).

Cod protein-fed mice were found to gain the most weight and fat of all groups, significantly more than crab- and scallop-fed mice (Paper 1: Fig. 1A-B and Fig. 2A). DIO is associated with metabolic disturbances, but despite significant body mass gain, the cod-fed mice presented no signs of impaired glucose or insulin metabolism (Paper 1: Supplemental Table 2). This is in accordance with previous studies where cod-protein, when fed as the sole protein, was shown to prevent diet-induced glucose intolerance and insulin insensitivity in rats compared to casein and soy [116-118], by maintaining peripheral insulin signaling [118].

The increased DIO in cod-fed mice is in part explained by the higher energy intake in the cod and chicken-fed compared to the crab and scallop-fed mice (Paper 1: Fig. 1C) and equally the reduced body mass gain in crab and scallop-fed mice may, at least in part, be explained by the lower energy intake compared to cod and chicken-fed mice. However, even though energy intake was lower in both crab and scallop-fed mice, only scallop-fed mice presented with reduced adiposity and improved plasma lipid profile suggesting that the beneficial effect of scallop-consumption is not only caused by the decreased energy intake. Indeed, feed efficiency relating weight gain to energy intake, was higher in cod and chicken-fed than in scallop-fed mice (Paper 1: Fig. 1D), indicating that the differences in energy intake do not fully explain the differences in weight gain between these groups. Chicken and cod-fed mice simply have a higher body mass and adipose accretion per joule ingested, suggestive of increased EE in scallop-fed mice.
Significant correlations were found between dietary taurine and glycine intake and decreased adiposity and improved plasma lipid profile in the present study. However, it should be taken into account that taurine and glycine coincidentally had the same distribution pattern in the protein sources used, with the lowest concentration in chicken and the highest in scallop. It is a possibility that only one of these AA is actually imparting the beneficial effects and that the other is found to correlate with these beneficial effects simply due to having similar distribution pattern in the protein sources as the first one. This study does not allow deciphering of the independent contribution of taurine and glycine. Furthermore, dietary constituents other than taurine and glycine, that we did not measure, may have affected the outcome.

4.2 - Dietary protein sources in the pair-fed setting

To be able to compare the effect of the different protein sources on DIO and EE without interference from possible differences in palatability and satiety and thus energy intake associated with the different diets, mice were pair-fed and subjected to indirect calorimetry measurements in a subsequent study. The diets in the pair-feeding experiment were similar to those from the ad libitum experiment with minor differences. Instead of having different seafood-fed groups it was decided to combine the groups. However, since crab-fed mice presented with unexplained indications of hyperlipidemia despite a lean phenotype, it was decided not to include this protein source in the subsequent study. Furthermore, to have a HFHS-control for the indirect calorimetry measurements it was decided to include a HFHS diet with casein as the sole protein source.

At equal energy intake chicken-fed mice gained significantly more weight and adipose tissue than casein and cod/scallop-fed mice (Paper 2: Fig. 1A-C), reflected in a higher feed efficiency in chicken-fed mice (Paper 2: Fig. 1D) suggestive of underlying reduced EE. The adiposity in the chicken-fed mice was associated with a tendency ($P = 0.09$) towards hyperinsulinemia (Paper 2: Fig. 3G). Traditionally hyperinsulinemia has been regarded as a compensatory response to systemic insulin resistance, but has more recently been shown to be a driving force in DIO [14]. This is in accordance with the findings of the present study where chicken-fed mice are hyperinsulinemic and have increased adiposity but remains glucose tolerant (Paper 2: Fig. 2A). Hyperinsulinemia may have contributed to the elevated hepatic TAG accumulation [162] in the chicken-fed mice (Paper 2: Table 3). Interestingly, the discrepancy between the tendency towards increased plasma insulin and concomitant increased plasma glucose and lactate (Paper 2: Fig. 2 E-F) in chicken-fed compared to casein-fed mice is indicative of incipient peripheral insulin insensitivity, but further experiments would be needed to elucidate this.
The decreased feed efficiency in the casein- and cod/scallop-fed mice was suggestive of increased EE or in some other way less efficient utilization of ingested energy. Indeed, cod/scallop-feeding was associated with increased EE in the dark phases and a tendency of increased EE in the light phases compared to the casein-fed mice (Paper 2: Fig. 3G) concomitantly with a tendency of increased spontaneous locomotor activity (Paper 2: Fig 3E). Moreover, apparent fat digestibility was decreased by approximately three percent in the casein-fed compared to the chicken- and cod/scallop-fed mice (Paper 2: Fig. 1F). Although seemingly a minor difference, this might amount to significant reduced feed efficiency over time. HF casein-feeding in mice have previously been shown to reduce body mass gain and increase fecal fat excretion compared to mice fed a HF salmon diet [163]. It is thus plausible that the reduced apparent fat digestibility contributed to the reduced fat accretion in casein-fed mice in the present study.

On a related note, it has recently been shown that whey consumption was associated with increased urinary excretion of tricarboxylic acid cycle substrates, i.e. loss of energy, leading to reduced body mass accretion compared to casein consumption in mice [89]. Interestingly, excretion of energy substrates may thus be modulated by dietary protein source and may in turn affect energy efficiency and DIO. This is an important aspect to consider, especially in instances where EE and DIT fail to account for observed differences in feed efficiency. In relation to the observation that dietary protein source modulate urinary excretion of energy substrates, the finding that taurine supplementation increases glomerular filtration in rats [164] is interesting, since the combination of certain protein sources and supplementation of taurine could possibly lead to considerable loss of energy through urinary excretion. However, it remains to be evaluated whether these observations in rodents applies to human subjects.

Our finding that casein-feeding decreased fat absorption in mice is in accordance with a previous study demonstrating that dietary casein dose-dependently reduced fat absorption in the chick, mainly due to inhibition of absorption in ileum [165]. Casein constitutes the majority of protein found in milk (80%) along with whey (20%). High dairy consumption has previously been shown to increase fecal fat excretion, however this effect was ascribed to higher calcium-intake associated with high dairy intake, in rats [166] and in human subjects [167-169]. The sodium caseinate used in the present study does not contain calcium, excluding diary calcium as the underlying cause behind the decreased apparent fat digestibility in casein-fed mice in this study and at the same time allowing the possibility that other factors, attributable to casein, than high calcium content may contribute to the increased fecal fat excretion associated with high diary consumption.
The casein-diet was found to be high in branched-chain amino acids (BCAA) especially compared to the cod/scallop-diet where BCAA content was approximately 39% lower than in the casein-diet (Paper 2: Table 2). The finding that the casein-fed mice had impaired glucose tolerance, at 30 and 60 minutes after a 2g/kg glucose bolus, despite very lean phenotype (Paper 2: Fig. 1A-B and Fig. 2A+D), is in accordance with a study where rats fed a HF-diet supplemented with BCAA developed glucose intolerance despite lean phenotype and decreased energy intake compared to non-supplemented HF and LF-fed rats [170]. The impaired glucose tolerance was found to be associated with chronic phosphorylation of mammalian target of rapamycin, Jun N-terminal kinase and insulin receptor substrate-1, negatively regulating insulin signal transduction and leading to insulin resistance [170].

The increased energy expenditure and prevention of DIO associated with cod-feeding in rats was, at least in part, due to increased plasma BA level [123, 124]. On the contrary to what was observed in rats, no increase in plasma BA (Paper 1: Supplemental Table 2 and Paper 2: Table 3) was associated with seafood consumption in the present studies. The prevention against DIO was thus most likely independent of increased BA synthesis and signaling in C57BL/6J mice. The lack of increase in BA in seafood-fed mice in the present studies is probably due to the species and strain-dependent characteristics inherent to C57BL/6J mice. Renal taurine handling is altered in C57BL/6J mice which are hypertaurenuric and excrete ten times more taurine in the urine than non-hypertaurenuric mice strains, due to impaired tubular taurine reabsorption [160, 161] as previously described (1.6). To add insult, chronic taurine supplementation has been shown to cause renal hyperfiltration in rats [164]. The seafood-feeding may essentially be regarded as chronic taurine supplementation compared to casein and chicken-feeding, and may thus potentially cause renal hyperfiltration. A combination of impaired taurine reabsorption in C57BL/6J mice and possible hyperfiltration brought on by high dietary taurine would further increase taurine excretion. However, as filtration rate of the kidneys was not evaluated this remains speculative. What can be concluded is that the increased dietary taurine intake did not translate into increased plasma BA concentration. If the hypertaurinuria renders C57BL/6J mice incapable of increasing the plasma or body pool of taurine sufficiently to impact BA metabolism, no effect on BA will arise from feeding protein endogenously high in taurine and glycine since mice are inherently incapable of utilizing glycine for BA conjugation [139, 140].

Based on the observation that chronic taurine supplementation caused renal hyperfiltration in rats [164], the increased kidney mass observed in cod/scallop-fed compared to casein-fed mice (Paper 2: Fig. 1) might be considered a consequence of taurine-induced renal hyperfiltration in mice in the present study. Hyperfiltration and hypertrophy is highly studied in type I diabetes mellitus where both manifests early
after onset [171, 172]. It is hypothesized that hyperfiltration occurs as a consequence of impaired vascular control leading to increased intraglomerular pressure and that hypertrophy follows as a compensatory mechanism, serving to increase reabsorption to circumvent excessive urinary loss. It could be speculated that similar mechanisms may be at play in the seafood-fed mice. Here hypertrophy similarly is a compensatory measure, serving to prevent excess urinary loss, brought on by hyperfiltration. However, here hyperfiltration is caused by the chronic presence of taurine instead of poor vascular control. The findings that C57BL/6J mice have impaired renal taurine reabsorption [160, 161] and that chronic taurine supplementation causes renal hyperfiltration in rats [164] supports this hypothesis. In Paper 1 no differences in absolute kidney mass were reported between the mice fed the different protein sources (Paper 1: Fig. 2B). However, the scallop-fed mice had considerably lower body mass than the chicken and cod-fed mice, and the scallop-fed mice had indeed significantly increased kidney mass, as compared to chicken, cod and crab-fed mice ($P < 0.001$, unpublished data), when reported as a percentage of body mass.

Ad libitum energy intake differed between the groups in Paper 1 which may be caused by differences in satiety or palatability associated with the diets. By extending into a pair-fed setting we removed these aspects and demonstrated that the benefits of seafood protein were maintained at equal energy intake in Paper 2. Findings from these two studies confirms the claim from the introduction (1.2) that obesity may be an adaptation to prevent lipotoxicity caused by disturbances in metabolism rather than the cause of these disturbances. Despite gaining significant weight and fat mass, cod-fed mice did not present with alterations in plasma glucose, plasma insulin, QUICKI or HOMA-IR scores, whereas crab-fed mice that gained less weight than the cod-fed mice tended ($P = 0.10$) to be hyperinsulinemic. Furthermore, casein-fed mice were glucose-intolerant despite lean phenotype, while chicken-fed mice remained glucose tolerant despite increased adiposity. The chicken-fed mice tended ($P = 0.09$) to be hyperinsulinemic though, which may have contributed to the weight gain, adiposity and hepatosteatosis in this group. The prevention of DIO and metabolic disturbances associated with seafood protein was seen both in ad libitum and pair-feed setting, highlighting that protein from different sources modulate metabolism and may prevent DIO irrespective of energy intake.

4.4 – Dietary proteins at different levels and forms

Surprisingly, we found no difference in weight gain between mice fed 16 or 32 E% protein for eight weeks (Paper 3: Fig. 1A-B). Previous studies have otherwise shown that higher protein intake leads to reduced weight gain in mice [16, 35, 37, 51]. The pair-feeding in the present study was performed between diets
with equal protein concentrations, i.e. the groups receiving 16 E% hydrolyzed casein and 16 E% intact casein were pair-fed and the groups receiving 32 E% hydrolyzed casein and 32 E% intact casein were pair-fed. However, still no differences in energy intake were seen between any of the groups, demonstrating that protein E% did not affect energy intake in this study. This is in line with a previous study showing that whereas 48 E% protein reduced ad libitum energy intake in mice, 31 E% did not, indicating that protein E% higher than 31 is needed to effectively reduce energy intake [173]. On a different note, based on the notion that DIT induces satiety [72, 73] it may be speculated that the equal energy intake between 16 and 32 E% protein-diets in the present study is due to the absence of differences in EE.

Hydrolyzed casein significantly reduced diet-induced weight gain and adiposity compared to intact casein irrespective of protein level (Paper 3: Fig. 1A-B, Fig. 2 and Table 3). The hydrolyzed casein-fed mice were more voluntarily active in the cages during indirect calorimetry measurements (Paper 3: Fig. 1E), however no increase in EE was detected after four weeks on the different diets. Interestingly, after eight weeks hydrolyzed casein-fed mice was found to have greater capacity for β-oxidation as well as increased expression of genes involved in EE in iWAT (Paper 3: Fig. 3), i.e. a greater capacity for EE. This together with the reduced weight gain and adiposity certainly suggests that EE was indeed increased in hydrolyzed casein-fed mice after eight weeks. It may thus be speculated that the adaptations towards increased EE, e.g. induction and expression of genes and proteins, was not fully implemented after four weeks at the time of indirect calorimetry. In support of this notion is the course of the body mass curve (Paper 3: Fig. 1A), as it is not until weeks five-six the body mass development really starts to differ between diet groups. Whereas body masses of intact casein as well as LF-fed mice continue to increase past week five, the increase in body mass levels off in the hydrolyzed casein-fed mice, which could be indicative of increasing EE in these groups, as suggested by the increased capacity for EE in iWAT after eight weeks.

It is counterintuitive that we find increases in activity without corresponding increases in EE. Increased locomotor activity would, all things being equal, result in increased expenditure of energy. However, this discrepancy may depend on the sensitivity and resolution of the indirect calorimetry set-up. Minor differences in O₂ consumption and CO₂ production, as little as two to five percent, is sufficient to translate into significant modulation of EE [174]. However, measurements of these miniscule changes require set-up and devices of very high sensitive and resolution to be detected efficiently.

While no differences in EE were detected by indirect calorimetry after four weeks, differences in RER were demonstrated (Paper 3: Fig. 1F). In the dark phases RER was decreased by 32 E% protein compared to 16
E%, irrespective of protein form, indicating less carbohydrate and higher fat or protein use as energy substrate. In the light phase RER tended to by decreased in hydrolyzed casein-fed mice. This together with the increased plasma OH-butyrate in the non-fasted state (Paper 3: Table 2) suggests increased fat utilization and AA deamination and decreased utilization of carbohydrate in hydrolyzed casein-fed mice, which is further supported by the decreased non-fasted plasma glucose and lactate levels. In accordance with the lower plasma glucose, plasma insulin concentrations were found to be lower in hydrolyzed casein-fed mice compared to intact casein-fed mice in the non-fasted state. This may play an important part in the prevention against DIO in the hydrolyzed casein-fed mice as hyperinsulinemia has been demonstrated to be a requisite for developing obesity [14-16].

**In summary**

In summary we found that the source and form of protein has great impact on development and prevention of diet-induced adiposity, dyslipidemia, hyperinsulinemia and impairment of glucose tolerance in mice. The ability of seafood proteins to prevent these metabolic disturbances was found to associate with endogenous taurine and glycine concentrations and to concur with increased EE and a tendency towards increased voluntary locomotor activity. The prevention against diet-induced obesity by hydrolyzed casein was also associated with increased spontaneous locomotor activity but no increase in EE, as assessed by indirect calorimetry, was observed after four weeks. However, after eight weeks adaptations towards increased EE-capacity was present in iWAT concurrent with altered energy substrate utilization in hydrolyzed casein-fed mice compared to mice fed the intact protein.
5 - Conclusions

5.1 - Protein from different sources

5.1.1 - Ad libitum fed;

- Scallop protein prevents ad libitum diet-induced adiposity and dyslipidemia, which however could be caused in part by decreased energy intake
- Chicken and cod protein caused diet-induced weight gain, adiposity and impaired plasma lipid profile
- Crab protein prevented weight gain compared to cod possibly through decreased energy intake. However, despite leaner phenotype, crab protein-feeding was associated with sharply increased fasted plasma insulin concentration suggesting impairment of glucose and insulin metabolism
- Taurine and glycine intake correlated with decreased adiposity and improved plasma lipid profile

5.1.2 - At equal energy intake (pair-fed);

- Casein and cod/scallop protein prevented diet-induced weight gain and adiposity
- Prevention of DIO with casein-feeding was, at least in part, caused by decreased fat digestibility. However casein-feeding was despite lean phenotype associated with impaired glucose tolerance
- Chicken protein caused greatly increased weight gain and adiposity which was associated with dyslipidemia, hepatosteatosis and a tendency towards hyperglycemia and hyperinsulinemia
- The prevention of DIO and co-morbidities of cod/scallop was shown to associate with increased EE and a tendency towards increased spontaneous locomotor activity

5.2 - Hydrolyzed versus intact protein

- The level of dietary protein, 16 versus 32 E%, was found to be negligible is this study
- Hydrolyzed casein prevented diet-induced weight gain and adiposity compared to intact casein
- Hydrolyzed casein tended to decrease RER during the light phases and decrease plasma glucose and glucose metabolites, suggesting decreased use of carbohydrates as energy substrate
- Hydrolyzed casein increased spontaneous locomotor activity, but no difference in energy expenditure was demonstrated after four weeks. However, after eight weeks β-oxidation capacity as well as expression of genes involved EE was increased in iWAT
6 - Perspectives

The effect of macronutrient proportions, i.e. quantity, has been thoroughly researched. Also the quality of dietary carbohydrates, e.g. with regard to glycemic index and load, has been evaluated in detail, whereas the impact of protein from different sources is less studied and less well-understood. However, identifying dietary approaches to prevent and alleviate obesity and co-morbidities is of great importance in light of the current epidemic rise in obesity and T2DM. In the present studies the source and form of protein was found to significantly affect the development of diet-induced adiposity, dyslipidemia and disturbances in glucose and insulin metabolism, while the level of dietary protein was found to be negligible. This demonstrates the importance of protein quality in prevention of DIO and co-morbidities in mice. However as often is the case in science, answers leads to new exciting questions and further research is needed to fully elucidate the impact of protein source in DIO and co-morbidities.

Consumption of taurine and glycine was found to correlate negatively with total fat mass and positively with improved plasma cholesterol profile (Paper 1: Table 3). However, from the present study it was not possible to decipher the independent effect of either taurine or glycine. And furthermore, we cannot fully rule out the possibility that one or more dietary constituents, that we did not measure, may to some extent have affected the outcome of these experiments. Therefore, further studies evaluating the effects of taurine and glycine would be instrumental. It would be interesting to test whether the weight gain, adiposity, dyslipidemia and hyperlipidemia associated with chicken-feeding is alleviated by supplementing the chicken-diet with taurine and glycine to the concentrations found in scallop or cod/scallop-diets. Furthermore, supplementing with only taurine or glycine would help unravel the independent effect of taurine and glycine and would further help demonstrate whether high dietary taurine and glycine concentrations do in fact affect development of DIO, dyslipidemia and disturbances of glucose and insulin metabolism.

Given the altered taurine renal handling in C57BL/6J mice it would be interesting to feed diets varying in endogenous taurine concentration to C57BL/6J mice and a mouse strain with normal renal taurine handling and measure taurine concentration in plasma and urine as well as plasma BA, to see whether plasma and total body levels of taurine are affected by increased dietary taurine in C57BL/6J mice compared to non-hypertaurinuric mice and furthermore to see whether dietary taurine increases plasma BA in mice with normal renal taurine handling.
In Paper 2 the chicken-fed mice tended to be hyperinsulinemic in the fasted state and had increased ‘Quantitative Insulin Sensitivity Check Index’- and ‘Homeostatic Model Assessment of Insulin Resistance’-scores but remained glucose tolerant, as assessed by 6h fasted oral-GTT. However, despite increased plasma insulin concentration, plasma glucose was increased compared to casein-fed mice, which may imply incipient hepatic or peripheral insulin sensitivity. To further evaluate glucose tolerance and insulin sensitivity in the chicken-fed mice the hyperinsulinemic euglycemic clamp technique, which is considered the gold standard for assessing insulin resistance, could beneficially be used. The hyperinsulinemic euglycemic clamp is more advanced than GTT and insulin tolerance test and may thus register the minute changes in incipient insulin resistance more efficiently than the GTT used in the present study.

Application of the hyperinsulinemic euglycemic clamp might also have revealed differences between mice fed hydrolyzed and intact casein in Paper 3. In this study no differences were seen in blood glucose clearance rates, as assessed by intraperitoneal-GTT (Paper 3: Supplemental Figure 1), despite reduced diet-induced weight gain and adiposity in hydrolyzed casein-fed fed compared to intact casein-fed mice. However, whereas plasma insulin level was highly increased in the fed compared to the fasted state in the intact casein-fed mice, no fed-state increase of plasma insulin was seen in the hydrolyzed casein-fed mice. The equal blood glucose clearance rates despite markedly higher fed-state plasma insulin in intact casein-fed mice is indicative of increased insulin sensitivity in mice fed hydrolyzed casein, but further studies employing more sensitive methods, e.g. hyperinsulinemic euglycemic clamp, is needed to establish this.

Sensitivity and resolution of the method used may also have affected the results of the indirect calorimetry measurements performed in Paper 2 and Paper 3. As described minute changes in EE are sufficient to translate into alterations of long term energy balance. It is counterintuitive that the observed differences in spontaneous activity between mice fed hydrolyzed and intact casein were not reflected in EE. Too low sensitivity of the measurements of O2-consumption and VO2-production in the indirect calorimetry set-up may explain why no differences in EE were observed between groups. Thus it would be interesting to perform the measurements again in a set-up with higher sensitivity.

As the ultimate goal would be to prevent further rise in the global public health problems, it would be interesting to test whether the beneficial effects of different protein sources observed in mice would translate to prevention and alleviation of obesity and co-morbidities in humans. Simple dietary adjustments with the potential of preventing obesity and co-morbidities through increased EE and improved metabolism would be of great importance in the fight against the current obesity and T2DM-pandemics.
7. Cited literature


159. Seymour J.L., M.A.Y., G.W.M. Bothe, A.V. Perez. *Taconic C57Bl/6J/BomTac, C57Bl/6NTac and C57Bl/10SgSnAiTac mice do not carry the Nnt mutation*. in 59th National Meeting of the American Association for Laboratory Animal Science (AALAS). 2008. Indianapolis, IN, USA.


The following manuscript and published articles are enclosed:

1. **Tastesen HS**, Keenan AH, Madsen L, Kristiansen K, Liaset B. 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 Epub ahead of print, DOI 10.1007/s00726-014-1715-1


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
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

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Abstract High-protein diets induce alterations in metabolism that may prevent diet-induced obesity. However, little is known as to whether different protein sources consumed at normal levels may affect diet-induced obesity and associated co-morbidities. We fed obesity-prone male C57BL/6J mice high-fat, high-sucrose diets with protein sources of increasing endogenous taurine content, i.e., chicken, cod, crab and scallop, for 6 weeks. The energy intake was lower in crab and scallop-fed mice than in chicken and cod-fed mice, but only scallop-fed mice gained less body and fat mass. Liver mass was reduced in scallop-fed mice, but otherwise no changes in lean body mass were observed between the groups. Feed efficiency and apparent nitrogen digestibility were reduced in scallop-fed mice suggesting alterations in energy utilization and metabolism. Overnight fasted plasma triacylglyceride, non-esterified fatty acids, glycerol and hydroxy-butyrate levels were significantly reduced, indicating reduced lipid mobilization in scallop-fed mice. The plasma HDL-to-total-cholesterol ratio was higher, suggesting increased reverse cholesterol transport or cholesterol clearance in scallop-fed mice in both fasted and non-fasted states. Dietary intake of taurine and glycine correlated negatively with body mass gain and total fat mass, while intake of all other amino acids correlated positively. Furthermore taurine and glycine intake correlated positively with improved plasma lipid profile, i.e., lower levels of plasma lipids and higher HDL-to-total-cholesterol ratio. In conclusion, dietary scallop protein completely prevents high-fat, high-sucrose-induced obesity whilst maintaining lean body mass and improving the plasma lipid profile in male C57BL/6J mice.

Keywords Cholesterol · Diet-induced obesity · Plasma lipid profile · Protein · Seafood · Taurine

Introduction

As obesity and a range of co-morbidities, such as type 2 diabetes mellitus and cardiovascular disease, have become major public health challenges worldwide, research into prevention and alleviation of obesity has intensified. Proteins induce greater satiety than carbohydrates and fat in human subjects (Rolls et al. 1988; Weigle et al. 2005) and rats (Bensaïd et al. 2002). Consequently, increasing the proportion of protein in the diet potentially decreases energy consumption and ultimately may contribute to weight loss. Indeed, in human subjects an increase in dietary protein level, at the expense of fat or carbohydrate, has been shown to promote weight loss (Weigle et al. 2005) and to improve body composition and short-term weight maintenance (Westerterp-Plantenga et al. 2004). It is still uncertain whether the beneficial effects of increasing protein in the diet are maintained over time in humans (Due et al. 2004), whereas in
mice we have previously shown that increasing dietary protein (casein) at the expense of carbohydrates (sucrose) reduces diet-induced obesity over time (Hao et al. 2012; Ma et al. 2011; Madsen et al. 2008). Apart from quantity, the quality of dietary proteins is of significance in the prevention of obesity. Prospective cohort studies have demonstrated that consumption of fish as a part of healthy diet is associated with lower body weight (Schulze et al. 2006; Shubair et al. 2005) and randomized controlled studies show that the inclusion of fish in energy-restricted diets resulted in greater weight loss compared to control diets without seafood (Thorsdottir et al. 2007; Ramel et al. 2009). In addition, incorporation of a daily fish meal into a weight-loss regimen was more effective than either fish consumption or weight loss alone at improving glucose-insulin metabolism and dyslipidemia (Mori et al. 1999). The beneficial properties of seafood have mainly been ascribed to the marine n-3 long-chain PUFAs, but other factors, e.g., vitamin D, selenium, iodine and taurine, have been reported to also contribute to the benefits associated with consumption of seafood (Lund 2013). Insulin resistant subjects showed improved insulin sensitivity after 4 weeks on a cod-based diet compared with subjects ingesting a meat-based diet (Ouellet et al. 2007); an effect possibly associated with the cod-based diet’s ability to reduce C-reactive protein levels (Ouellet et al. 2008). Also, a daily supplement of cod protein resulted in improved body composition and decreased blood LDL-cholesterol in overweight adults (Vikøren et al. 2013). Seafood protein is characterized by high levels of taurine compared to terrestrial protein sources (Spitze et al. 2003). Supplementation of taurine in the diet or drinking water has been shown to prevent diet-induced weight gain and adiposity in rodents (Camargo et al. 2013; Nardelli et al. 2011) and to improve blood lipid profiles in rodents (Fukuda et al. 2011; Murakami et al. 1998; Nardelli et al. 2011; Yokogoshi et al. 1999) and human subjects (Zhang et al. 2004). To the best of our knowledge no studies have been published evaluating the effects of dietary protein sources with endogenously varying contents of taurine on the development of diet-induced obesity and related diseases. We hypothesize that an increase in dietary taurine through consumption of seafood protein might prevent diet-induced fat accretion and improve plasma lipid profile in a dose-dependent way. To test this hypothesis we fed male C57BL/6J mice high-fat, high-sucrose diets with protein containing increasing endogenous levels of taurine, i.e., chicken fillet, cod fillet, white crab meat and scallop muscle, as the sole protein source for 6 weeks. C57BL/6J mice are prone to diet-induced obesity (West et al. 1992) and are thus widely used in research on obesity, glucose intolerance, insulin resistance, dyslipidemia and related disease states. A high-fat, high-sucrose diet was used in this study as it has been shown to be more obesogenic than high fat (Surwit et al. 1995) and high-fat, high-protein feeding (Ma et al. 2011). We found that the scallop diet prevented diet-induced obesity without affecting lean body mass. Furthermore, the plasma lipid profile was improved in scallop-fed mice compared to chicken, cod and crab-fed mice.

**Materials and methods**

**Ethical statement**

The animal experiments were approved by the National Animal Health Authorities (Norwegian approval identification number 2497). Care and handling were performed in accordance with local institutional rules and the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. No adverse events were observed.

**Animal studies**

Male C57BL/6J BomTac mice, weighing approximately 25 g, 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 ± 1 °C). To obtain both fasted and non-fasted (randomly fed) blood plasma at the termination, two identical studies were carried out in parallel 3 weeks apart, i.e., experiment 1 (Expt. 1) and experiment 2 (Expt. 2), respectively. Upon arrival, the mice were let to acclimatize for 5 days and were then assigned to the different experimental diet groups (n = 8 mice per group) by body mass. The mice were fed ad libitum, and the feed intake as well as body mass was measured throughout the feeding period. After 4 weeks on the experimental diets, the mice were kept in cages with paper lining, instead of the standard wood-chip bedding, for 48 h with the purpose of collecting feces (Expt. 1). Following 6 weeks of feeding, the mice were terminated by inhalation of isoflurane (4 %, Baxter, IL, USA) followed by cardiac puncture exsanguination either in the overnight fasted (Expt. 1) or in the non-fasted (Expt. 2) state. At termination selected tissues were dissected and weighed. Total fat mass was compared between the groups by comparing the combined mass of dissected fat depots; inguinal white adipose tissue (iWAT), interscapular brown adipose tissue (iBAT), mesenteric white adipose tissue (MeWAT), epididymal white adipose tissue (eWAT) and perirenal and retroperitoneal white adipose tissue (p/rWAT). Lean mass was compared between the groups by comparing the mass of dissected heart, tibialis anterior and soleus. Unless otherwise stated data shown in this paper is from Expt. 2.
Experimental diets

Based on a high-fat, high-sucrose background diet of Lavigne et al. (2001), four different isonitrogenous and isoenergetic experimental diets were made containing protein from four different sources with varying taurine concentrations; chicken breast fillets (chicken, taurine in diet 0.3 g/kg), wild caught cod fillets (cod, taurine in diet 1.7 g/kg), white crab meat (crab, taurine in diet 2.4 g/kg) and Canadian scallop muscles (scallop, taurine in diet 12.9 g/kg). A fifth group of mice fed a low-fat diet (LF, OpenSource Diet no. D12450B, Research Diets Inc., NJ, USA) with casein as the sole protein source was included as a reference group only and was not included in the statistical analyses. The protein sources were the sole source of AAs and no supplementary AAs were added to the diets. The protein sources had differing endogenous ash content, i.e., the residue left after combustion reflecting mineral content, which may influence energy density (J/g). To balance dietary ash content, varying amounts of potassium chloride (KCl) were added to the chicken (13.4 g/kg), cod (10.8 g/kg) and scallop (5.9 g/kg) diets (Table 1). Potassium chloride was used to avoid elevations in blood pressure. The final compositions of the diets are listed in Tables 1, 2 and Supplemental Table 1 (Online Resource 1). Feed efficiency was calculated as body mass gain per energy intake as follows (Eq. 1):

\[
\text{Feed efficiency} = \frac{\text{body mass gain (g)}}{\text{energy intake (MJ)}}
\]

Analyses of diet compositions

Energy contents were determined by bomb calorimetry following the manufacturer’s instruction (Parr Instruments, Moline, IL, USA). Fatty acids were extracted from the samples with 2:1 chloroform: methanol (v/v) and internal standard 19:0 methyl ester was added. The samples were filtered, saponified and esterified in 12% BF3 in methanol (v/v). Fatty acid methyl esters were separated on a gas chromatograph (GLC Trace GC 2000, Thermo Scientific, USA) and detected with a flame ionization detector (Thermo Scientific) (Lie and Lambertsen 1991). The fatty acids were identified by retention time using standard

### Table 1 Composition of the experimental diets

<table>
<thead>
<tr>
<th>Composition (g/kg)</th>
<th>LFa</th>
<th>Chickenb</th>
<th>Codc</th>
<th>Crabd</th>
<th>Scallope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chicken</td>
<td>–</td>
<td>231</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cod</td>
<td>–</td>
<td>–</td>
<td>228</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Crab</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>253</td>
<td>–</td>
</tr>
<tr>
<td>Scallop</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>248</td>
</tr>
<tr>
<td>KCl</td>
<td>13.4</td>
<td>10.8</td>
<td>25</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>Sucrose</td>
<td>350</td>
<td>226</td>
<td>232</td>
<td>218</td>
<td>217</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Lard</td>
<td>20</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>198</td>
</tr>
<tr>
<td>Vegetable oilf</td>
<td>25</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>198</td>
</tr>
<tr>
<td>Mineral mixg</td>
<td>10</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Vitamin mixh</td>
<td>10</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Choline biturrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Analyzed (g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein (N×6.25)</td>
<td>167</td>
<td>199</td>
<td>206</td>
<td>199</td>
<td>207</td>
</tr>
<tr>
<td>Fat</td>
<td>46</td>
<td>407</td>
<td>400</td>
<td>405</td>
<td>402</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.06</td>
<td>0.83</td>
<td>0.84</td>
<td>1.10</td>
<td>0.51</td>
</tr>
<tr>
<td>Ash</td>
<td>30</td>
<td>71</td>
<td>74</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Gross energy (kJ/g)</td>
<td>17.7</td>
<td>25.8</td>
<td>25.6</td>
<td>25.8</td>
<td>25.4</td>
</tr>
</tbody>
</table>

a OpenSource diet no. D12450B (Research Diets, Inc., NJ, USA)
b Chicken breast fillets (Ytterøykylling AS, Ytterøy, Norway)
c Cod fillets (Wild caught in the Northeastern Atlantic)
d White crab meat (HitraMat AS, Ansnes, Norway)
e Canadian scallops (Placopecten magellanicus)
f LF: soybean oil. Chicken, cod, crab and scallop: corn oil
g LF: Mineral Mix S10026, HFHS: AIN76 mineral mix
h LF: Vitamin Mix V100001, HFHS: AIN76 vitamin mix

### Table 2 Composition of amino acids in the diets

<table>
<thead>
<tr>
<th>g/kg</th>
<th>LF</th>
<th>Chicken</th>
<th>Cod</th>
<th>Crab</th>
<th>Scallop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>5.3</td>
<td>12.1</td>
<td>12.2</td>
<td>10.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Arg</td>
<td>5.6</td>
<td>11.4</td>
<td>10.8</td>
<td>14.6</td>
<td>14.6</td>
</tr>
<tr>
<td>Asx</td>
<td>13.4</td>
<td>20.6</td>
<td>22.0</td>
<td>22.1</td>
<td>17.7</td>
</tr>
<tr>
<td>Cys</td>
<td>3.2</td>
<td>2.4</td>
<td>2.8</td>
<td>3.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Glx</td>
<td>40.2</td>
<td>30.0</td>
<td>29.1</td>
<td>30.7</td>
<td>25.3</td>
</tr>
<tr>
<td>Gly</td>
<td>3.1</td>
<td>7.8</td>
<td>8.6</td>
<td>9.3</td>
<td>19.3</td>
</tr>
<tr>
<td>His</td>
<td>4.3</td>
<td>6.3</td>
<td>4.0</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Ile</td>
<td>8.6</td>
<td>9.6</td>
<td>8.8</td>
<td>9.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Leu</td>
<td>16.0</td>
<td>16.1</td>
<td>15.7</td>
<td>15.5</td>
<td>12.3</td>
</tr>
<tr>
<td>Lys</td>
<td>14.6</td>
<td>19.5</td>
<td>19.1</td>
<td>17.1</td>
<td>14.2</td>
</tr>
<tr>
<td>Met</td>
<td>4.5</td>
<td>5.3</td>
<td>6.0</td>
<td>5.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Phe</td>
<td>8.3</td>
<td>7.6</td>
<td>7.9</td>
<td>8.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Pro</td>
<td>18.1</td>
<td>7.0</td>
<td>6.5</td>
<td>8.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Ser</td>
<td>9.9</td>
<td>7.9</td>
<td>8.8</td>
<td>8.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Thr</td>
<td>7.3</td>
<td>8.9</td>
<td>8.6</td>
<td>9.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Trp</td>
<td>1.8</td>
<td>2.1</td>
<td>2.0</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>7.8</td>
<td>5.7</td>
<td>5.9</td>
<td>7.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Val</td>
<td>10.8</td>
<td>10.1</td>
<td>9.8</td>
<td>9.8</td>
<td>6.4</td>
</tr>
<tr>
<td>OH-Pro</td>
<td>0.0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Tau</td>
<td>0.0</td>
<td>0.3</td>
<td>1.7</td>
<td>2.4</td>
<td>12.9</td>
</tr>
<tr>
<td>EAA</td>
<td>81.6</td>
<td>96.9</td>
<td>92.7</td>
<td>97.2</td>
<td>75.1</td>
</tr>
<tr>
<td>BCAA</td>
<td>35.3</td>
<td>35.8</td>
<td>34.3</td>
<td>34.8</td>
<td>25.7</td>
</tr>
<tr>
<td>Total</td>
<td>191.6</td>
<td>201.6</td>
<td>200.0</td>
<td>209.4</td>
<td>184.6</td>
</tr>
</tbody>
</table>

EAA sum of essential amino acids, BCAA sum of branched-chain amino acids (Leu, Ile and Val), Total sum of total amino acids

a Essential amino acids
mixtures of methyl esters (Nu-Chek-Prep, Elyian, MN, USA) and quantified by the internal standard method. For total amino acid analysis norvaline was added as an internal standard before samples were hydrolyzed in 6 M HCl at 110 ± 2 °C for 22 h. Thereafter, HCl was removed and samples were derivatized with the AccQ-Tag Ultra Derivatization Kit (Waters, USA). Amino acids (AAs) were separated and detected on the ACQUITY UPLC System (Waters, USA). AAs were identified using an amino acid (AA) standard (Pierce Amino Acid Standard H, Thermo Fisher Scientific Inc., IL, USA) to which norvaline, taurine and hydroxy-proline were added. AAs were quantified by internal and external standard regression. Tryptophan was quantified in the samples after basic hydrolysis with Ba(OH)₂ for 20 h at 110 °C. The samples were pH adjusted to 6.2, separated on a HPLC column (4.6 mm × 15 cm) and detected in a UV-spectrophotometer (Shimadzu SPD 6A) at 280 nm. Tryptophan was analyzed in the samples after basic hydrolysis with Ba(OH)₂ for 20 h at 110 °C. The samples were pH adjusted to 6.2, separated on a HPLC column (4.6 mm × 15 cm) and detected in a UV-spectrophotometer (Shimadzu SPD 6A) at 280 nm. Tryptophan was quantified using a standard curve of L-Tryptophan (Sigma T-0254) based on 0.05 and 0.1 mg L-tryptophan mL⁻¹. Total cysteine in the samples was determined after oxidation of cysteine/cystine with 9:1 performic acid (88 %): H₂O₂ (30 %) (v/v) to yield cysteic acid. Total cysteine analysis was performed by the Norwegian Institute of Food, Fishery and Aquaculture.

Total fat in diets and feces

Total fat content was determined gravimetrically after extracting samples with organic solutions. First, samples were mixed with n-heptane, mixed for 10 min and centrifuged. The organic phase was collected. Thereafter, n-heptane and 4 M HCl was added to the sample remnant before the mixture was heated to 90 °C for 2 h. After cooling and centrifugation, the organic top fraction was collected. The aqueous bottom fraction including the sample remnant was quantitatively transferred to liquid–liquid extraction column (Chem Elut CE1010, Varian) mixed with Hydromatrix to remove water, and extracted twice with petroleum ether. The eluates were collected and all organic fractions combined, evaporated under vacuum (Evaporator Syncore Analyst, Büchi), dried at 103 °C for 30 min and weighed.

Nitrogen in diets and feces

The nitrogen content was determined by the Dumas method in a Leco FP-528 nitrogen analyzer (Leco Corp, MI, USA). The crude protein content in the diets was calculated as nitrogen content multiplied by 6.25 (Greenfield and Southgate 2003). The 6.25 conversion factor operates on the underlying assumption that proteins on average contain 16 % nitrogen (100/16 = 6.25).

Apparent fat and nitrogen digestibility

Feces from the 48 h collection was weighed and analyzed for nitrogen and total fat content. Apparent digestibility was calculated as follows (Eq. 2):

$$\text{Apparent digestibility} = \frac{100 \times (\text{intake (mg)} - \text{fecal output (mg)})}{\text{intake (mg)}}.$$  

Plasma metabolite measurements

Heparinized plasma was prepared from blood collected by cardiac puncture at the termination and stored at −80 °C prior to analysis. Insulin concentrations were analyzed by DRG Ultrasensitive Mouse Insulin ELISA kit (DRG Diagnostics, Germany). Plasma alanine aminotransferase, total cholesterol, LDL-cholesterol, triacylglycerides (TAG), glucose (MaxMat, France), non-esterified fatty acids (NEFA), HDL-cholesterol, total bile acids (DiaLab, Austria), hydroxy-butyrate (OH-butyrate), glycerol (Randox, UK) and lactate (Sentinel Diagnostics, Italy) concentrations were analyzed by conventional kits using a MaxMat PL II analyzer (MAXMAT S.A., Montpellier, France).

HOMA-IR and QUICKI

The homeostatic model assessment of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI) scores were calculated based on overnight fasting plasma glucose and overnight fasting plasma insulin as follows, respectively;

$$\text{HOMA-IR} = \frac{\text{Glucose (mmol/l)} \times \text{insulin (\mu U/ml)}}{22.5}$$  \hspace{1cm} (3)

$$(\text{Matthews et al. 1985})$$

$$1/(\log(\text{insulin [mU/l]}) + \log(\text{glucose [mg/dl]}))$$  \hspace{1cm} (4)

$$(\text{Katz et al. 2000}).$$

Correlation analyses

Data were visualized in a correlation matrix to facilitate variable selection using imDEV (Grapov and Newman 2012). Variables that are included in this manuscript were those that were statistically significant ($P < 0.05$) and biologically relevant based on available literature. While there were many significant correlations observed within the data set between amino acids and other measured variables, for simplicity we only present here those that helped to guide an explanation of the observed biological phenotype in our study (Table 3). Correlations were calculated by linear regression. Amino acid and fatty acid intake was calculated by multiplying the feed intake of each animal by the quantity of amino acid and fatty acid, respectively, in the diet. Because of differences in feed
intake, data that were regressed against amino acid intake (i.e., body fat mass and plasma lipids) were normalized to energy intake prior to regression analysis. Significance was noted at \( P < 0.05 \) using critical values for Pearson’s correlation coefficient (\( df = 28 \)).

Statistical analysis

All data are presented as group mean and standard error of the mean. The data were subjected to Analysis of Variance after homogeneity of the variances was confirmed by Levene’s test. Differences between group means were considered statistically significant at \( P < 0.05 \). Statistical analyses were performed using MiniTab version 16.1 (Minitab Ltd., Coventry, UK) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data from mice fed the low-fat reference diet were not included in the statistical analyses.

Results

Reduced body mass gain and energy intake in crab and scallop-fed mice

During 6 weeks on the experimental diets scallop-fed mice gained significantly less body mass than chicken and cod-fed mice, whereas cod-fed mice gained significantly more body mass than crab and scallop-fed mice (Fig. 1a, b, \( P < 0.001 \)). Chicken and cod-fed mice ingested significantly more energy than crab and scallop-fed mice (Fig. 1c, \( P < 0.001 \)). The feed efficiency, as defined in Eq. 1, was significantly higher in chicken and cod-fed mice than in scallop-fed mice (Fig. 1d, \( P < 0.001 \)). Similar results with regards to body mass gain, energy intake and feed efficiency were obtained during the parallel study (Expt. 1, data not shown) indicating that the results are reproducible.

Lower apparent nitrogen digestibility in scallop-fed mice

The dietary intake of fat and nitrogen reflected the differences in feed intake and was thus lower in scallop-fed compared to chicken and cod-fed mice (\( P = 0.001 \) and \( P = 0.004 \), respectively). No differences were detected in the excretion of nitrogen between groups, while the excretion of fat was significantly lower in scallop-fed mice (\( P = 0.038 \)) compared to chicken-fed mice. Based on intake and excretion, as defined in Eq. 2, no differences in apparent fat digestibility were observed between groups, whereas the apparent nitrogen digestibility was significantly lower in scallop-fed mice than in chicken, cod and crab-fed mice (Fig. 1e, \( P = 0.003 \), Expt. 1).

Reduced body mass gain in scallop-fed mice caused by reduced fat accretion

Consistent with the reduced body mass gain, scallop-fed mice had reduced visceral and subcutaneous fat depots; MeWAT (\( P < 0.001 \)), eWAT (\( P < 0.001 \)), p/rWAT (\( P < 0.001 \)), iWAT (\( P < 0.001 \)) and iBAT (\( P = 0.001 \)) compared to chicken, cod and crab-fed mice (Fig. 2a). No significant differences were observed in lean tissue mass, i.e., tibialis, soleus and heart or in the mass of pancreas and kidneys between the groups, but the liver weighed less in scallop-fed mice (Fig. 2b, \( P = 0.028 \)) than in chicken, cod and crab-fed mice.

Improved plasma metabolite profiles in scallop-fed mice

Fasted (Expt. 1) and non-fasted (Expt. 2) levels of plasma metabolites are shown in Fig. 3 and Supplemental Table 2 (Online Resource 2). Plasma TAG (Fig. 3a, \( P = 0.004 \)), NEFA (Fig. 3b, \( P = 0.001 \)) and OH-butyrate (Fig. 3c, \( P = 0.001 \)) were significantly lower in the fasted state in scallop-fed mice than in chicken and cod-fed mice. Furthermore, in the fasted state plasma glycerol was lower in scallop-fed mice than in chicken, cod and crab-fed mice (Fig. 3d, \( P < 0.001 \)). In the non-fasted state, no differences in NEFA, glycerol and OH-butyrate levels were seen.

Table 3 The strongest and most biologically relevant correlations found by linear regression

<table>
<thead>
<tr>
<th>Equation</th>
<th>( R^2 )</th>
<th>( P ) value</th>
</tr>
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<tbody>
<tr>
<td>Correlations between AA intake and total fat mass (normalized to energy intake)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>( y = -1.100x + 1.816 )</td>
<td>0.4296</td>
</tr>
<tr>
<td>Gly</td>
<td>( y = -1.256x + 2.587 )</td>
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</tr>
<tr>
<td>Met</td>
<td>( y = 5.952x - 1.196 )</td>
<td>0.4864</td>
</tr>
<tr>
<td>Trp</td>
<td>( y = 12.07x - 0.399 )</td>
<td>0.4048</td>
</tr>
<tr>
<td>EAA</td>
<td>( y = 0.3505x - 0.749 )</td>
<td>0.4157</td>
</tr>
<tr>
<td>BCAA</td>
<td>( y = 0.8298x - 0.791 )</td>
<td>0.4141</td>
</tr>
<tr>
<td>Total AA</td>
<td>( y = 0.1905x - 1.603 )</td>
<td>0.3903</td>
</tr>
<tr>
<td>Correlations between AA intake and HDL/total cholesterol (norm. to energy intake)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>( y = 0.1044x + 0.2535 )</td>
<td>0.4694</td>
</tr>
<tr>
<td>Gly</td>
<td>( y = 0.1057x + 0.1925 )</td>
<td>0.3564</td>
</tr>
<tr>
<td>Total AA</td>
<td>( y = -0.0242x + 0.6754 )</td>
<td>0.7676</td>
</tr>
<tr>
<td>BCAA</td>
<td>( y = -0.1001x + 0.5585 )</td>
<td>0.7364</td>
</tr>
<tr>
<td>EAA</td>
<td>( y = -0.0422x + 0.5528 )</td>
<td>0.7358</td>
</tr>
<tr>
<td>Trp</td>
<td>( y = -1.439x + 0.5085 )</td>
<td>0.7027</td>
</tr>
<tr>
<td>Met</td>
<td>( y = -0.6357x + 0.5707 )</td>
<td>0.6781</td>
</tr>
</tbody>
</table>

Total fat mass and HDL-to-total-cholesterol ratio (HDL/total cholesterol) were normalized to energy intake

EAA sum of essential amino acids, BCAA sum of branched-chain amino acids (Leu, Ile and Val), total AA total sum of amino acids
between the groups, but TAG tended to be lower in scallop-fed mice ($P = 0.057$). Plasma total cholesterol (Fig. 3e) was significantly lower in scallop-fed mice than in chicken and cod-fed mice in the non-fasted state ($P = 0.015$) and tended to be lower in scallop-fed mice in the fasted state ($P = 0.066$). The ratio of HDL-to-total-cholesterol (Fig. 3f) was higher in scallop-fed mice than in crab-fed mice in the fasted state ($P = 0.044$) and higher in scallop-
fed mice than in chicken and cod-fed mice in the non-fasted state ($P = 0.001$). No significant differences between groups were observed for plasma levels of HDL-cholesterol, LDL-cholesterol, glucose, lactate, total bile acids and alanine aminotransferase, neither in the fasted nor in the non-fasted state, but lactate tended to be lower in scallop-fed mice in the fasted state ($P = 0.062$) and LDL-cholesterol tended to be lower in scallop-fed mice in the non-fasted state ($P = 0.086$) (Supplemental Table 2). No differences were seen in fasted plasma insulin levels between groups. Based on overnight fasted glucose and insulin HOMA-IR and QUICKI scores were calculated, as defined in Eqs. 3 and 4, respectively, but no significant differences were found between the groups (Supplemental Table 2).

Negative correlations between dietary taurine and glycine intake and total fat mass

To identify factors that might contribute to the observed diet-induced differences in body mass, fat accretion, feed efficiency, apparent nitrogen digestibility and plasma lipid profiles data were visualized in a correlation matrix. The strongest and most biologically relevant correlations are shown in Table 3. The analyses showed highly significant, strong positive correlations between total AA intake and total fat mass normalized to energy intake ($R^2 = 0.39$, $P < 0.0001$). Positive correlations of comparable magnitudes were found between fat mass and branched-chain amino acids (BCAA, $R^2 = 0.41$, $P < 0.0001$) and essential amino acids (EAA, $R^2 = 0.41$, $P < 0.0001$).
observations were expected as AA ingestion reflects feed intake and thereby energy intake; however, after normalizing for energy intake, these correlations were still significant. Of all the AAs intake of methionine was found to positively correlate the strongest with total fat mass ($R^2 = 0.48$, $P < 0.0001$), whereas the correlation with tryptophan stands out by having a remarkably high regression coefficient ($12.07x$, $R^2 = 0.40$, $P < 0.0001$). While intake of all other AAs correlated positively to total fat mass, strong negative correlations between the consumption of taurine ($R^2 = 0.42$, $P < 0.0001$) and glycine ($R^2 = 0.41$, $P < 0.0001$) and total fat mass were observed.

Positive correlations between taurine and glycine intake and improved plasma lipid profile

While dietary intake of most AAs did not correlate significantly with plasma TAG and NEFA, dietary taurine and...
Scallop protein with endogenous high taurine and glycine

glycine intake correlated negatively with plasma TAG ($R^2 = 0.16$, $P = 0.032$ and $R^2 = 0.21$, $P = 0.011$, respectively) and NEFA ($R^2 = 0.18$, $P = 0.019$ and $R^2 = 0.17$, $P = 0.019$, respectively) in the fasted state. No significant correlations were found between intake of any AAs and total plasma cholesterol. However, we observed negative correlations between intake of all AAs and the ratio of HDL-to-total-cholesterol, except taurine ($R^2 = 0.46$, $P < 0.0001$) and glycine ($R^2 = 0.35$, $P < 0.0003$) which were positively correlated with HDL-to-total-cholesterol ratios (Table 3).

**Discussion**

Reduced energy intake in crab and scallop-fed mice

It is likely that the reduced energy intake seen in crab and scallop-fed mice, compared to chicken and cod-fed mice, contributed to the altered phenotypes. The underlying mechanism for the reduced energy intake is not established, but the taurine content was high in these diets, particularly in the scallop diet. Interestingly, a single intracerebroventricular injection of taurine was shown to enhance the anorexigenic effect of insulin in hypothalamus and to reduce energy intake in rats (Solon et al. 2012). It has been reported that taurine enters the brain after oral administration (Urquhart et al. 1974) and intraperitoneal injection (Peck Jr and Awapara 1967) in the rat, but it is still debated whether and to what extent orally and peripherally administered taurine is able to traverse the blood brain barrier (Ripps and Shen 2012). Therefore, it is also debatable whether the effects of taurine seen after an intracerebroventricular injection are relevant in the physiological setting. However, it was recently reported that in male C57BL/6J mice taurine supplementation in the drinking water enhanced hypothalamic insulin action which in turn decreased energy intake and prevented high-fat diet-induced obesity (Camargo et al. 2013). Furthermore, in humans a positive correlation between satiety and elevated postprandial blood taurine concentrations after consumption of test meals was reported (Veldhorst et al. 2009). Thus, the high taurine content in the crab diet and especially in the scallop diet might have contributed to the reduced energy intake but further analyses are needed to establish whether taurine affected satiety and thereby decreased energy intake in crab and scallop-fed mice in this experiment. Even though the energy intake was lower in both crab and scallop-fed mice, the body and fat mass gain was significantly reduced in scallop-fed mice only. Together with the reduced feed efficiency and apparent nitrogen digestibility this suggests that metabolism was affected in scallop-fed mice.

Unlike all other amino acids, the intake of taurine and glycine correlated negatively with fat mass

Although the experimental diets were isonitrogenous, the scallop diet had slightly lower total AA content, including EAA and BCAA, than the chicken, cod and crab diets. Importantly, except slightly decreased liver mass in scallop-fed mice no differences were seen in lean body mass between groups, effectively ruling out impaired protein synthesis as the explanation of the reduced body mass gain in scallop-fed mice. We hypothesize that the reduced liver mass in scallop-fed mice may be associated with the observed reduced level of circulating lipids, i.e., fasted plasma TAG and NEFA and non-fasted plasma total cholesterol. Decreased storage of hepatic lipids may have contributed to the reduced liver mass in scallop-fed mice, but further studies are needed to confirm this. Our correlation analyses showed highly significant, strong positive correlations between dietary intake of all AAs, except taurine and glycine, and total fat mass, even after correcting for differences in energy intake. Dietary methionine intake was found to positively correlate the strongest with fat mass. Interestingly, methionine restriction has been shown to prevent diet-induced obesity in rodents (Malloy et al. 2006; Hasek et al. 2010; Perrone et al. 2013; Ables et al. 2012). Therefore, even though the mice’s dietary sulfur-amino acid requirements (Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995) were fulfilled by the experimental diets, the dietary methionine load might have influenced the growth and adiposity in the present study. Highly significant negative correlations were found between the consumption of taurine as well as glycine and fat mass in male C57BL/6J mice. This is consistent with other studies showing that taurine supplementation reduced body weight in human subjects (Zhang et al. 2004) and fat mass in rodents (Nardelli et al. 2011; Tsuboyama-Kasaoka et al. 2006), and that glycine supplementation reduced adipocyte size and visceral fat mass in rats (El Hafidi et al. 2004). Scallop-fed mice were found to have decreased fasting plasma TAG, NEFA, glycerol and OH-butyrate and a tendency ($P = 0.062$) towards increased fasting plasma lactate suggesting a shift towards decreased lipid metabolism and increased glucose metabolism. Taurine supplementation has previously been associated with changes in fat metabolism in rodents (Murakami et al. 1998; Tsuboyama-Kasaoka et al. 2006) and with upregulating signaling cascades that increase nutrient utilization and energy expenditure in humans (Yeh et al. 2011) and mice (Tsuboyama-Kasaoka et al. 2006) while decreasing fed state energy expenditure and glucose oxidation rate in diabetic rats (Harada et al. 2004). Similarly, glycine supplementation has been shown to increase energy expenditure in mice (Alarcon-Aguilar et al. 2008;
Almanza-Perez et al. 2010). We have previously shown that a diet with fish protein hydrolysate, rich in taurine and glycine, elevated plasma bile acid concentration and reduced adiposity in rats (Liaset et al. 2009, 2011). It is suggested that bile acids increase energy expenditure, possibly through activation of farnesoid-X-receptor and TGR5, which both affect metabolism and energy expenditure (Prawitt et al. 2011). However, despite the large differences in taurine and glycine intake, no differences in fasted or non-fasted plasma total bile acid concentration were observed in mice from the present study. Thus, it is unlikely that the alteration of body and fat mass gain was affected by bile acids in the present study. Taken together, intake of taurine and glycine correlated negatively with body and fat mass gain possibly due to changes in metabolism and energy substrate utilization, but further experiments are needed to identify the mechanisms behind the observed differences.

Improved plasma lipid profile correlate with taurine and glycine intake

The scallop-fed mice had improved plasma lipid profiles with lower plasma TAG, NEFA and total cholesterol and increased HDL-to-total-cholesterol ratio, compared to mice fed the other diets. Due to a small contribution from the protein sources the seafood diets contained low levels of the marine n-3 long-chain PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), Supplemental Table 1 (Online Resource 1), that hypothetically could contribute to the reduced body mass gain and improved plasma lipid profiles (Alvheim et al. 2012; Frøyland et al. 1997; Janovska et al. 2013; Madsen et al. 1998) seen in scallop-fed mice. However, our analyses revealed no significant correlation between EPA or DHA intake and body mass gain or plasma lipid profile and consequently, the low doses of n-3 PUFAs are likely to be negligible in the present study. Our correlation analyses not only showed highly significant, strong correlations between especially taurine but also glycine intake and plasma HDL-to-total-cholesterol ratio. These findings are in line with previous studies showing that taurine supplementation improved serum lipid profile in overweight subjects (Zhang et al. 2004) and improved cholesterol profile in rodents (Murakami et al. 1998; Nardelli et al. 2011; Yokogoshi et al. 1999), specifically by decreasing non-HDL cholesterols (Chen et al. 2012; Murakami et al. 2002; Sugiyama et al. 1989) and that glycine supplementation reduced plasma lipids in sucrose-fed rats (El Hafidi et al. 2004). The increased HDL-to-total-cholesterol ratio suggests increased reverse cholesterol transport or clearance, and taurine has in fact been shown to upregulate key genes involved in reverse cholesterol transport in cultured cells in vitro (Hoang et al. 2012), and to upregulate LDL receptor binding and activity in vitro (Stephan et al. 1987) and in the liver of hamsters (Murakami et al. 2002). The reduced plasma total cholesterol level in scallop-fed mice may be associated with the lower cholesterol content in the scallop diet compared to the chicken, cod and crab diets (Table 1). However, this does not explain the increased plasma HDL-to-total-cholesterol ratio in the scallop-fed mice. Moreover, the LF diet had very low cholesterol content (0.06 g/kg). If LF-fed mice are included in the statistical analyses no statistically significant differences were found in plasma total cholesterol between any of the groups in the fasted state. In the non-fasted state LF-fed mice had decreased plasma total cholesterol compared to cod and chicken-fed mice ($P = 0.001$), while cod-fed mice had higher plasma total cholesterol levels than LF and scallop-fed mice ($P = 0.001$). No differences were found in HDL-to-total-cholesterol ratio between LF-fed mice and chicken, cod or crab-fed mice neither in the fasted nor the non-fasted state, while scallop-fed mice maintained an increased HDL-to-total-cholesterol ratio compared to chicken and cod-fed mice in the non-fasted state ($P = 0.001$) and tended to have increased HDL-to-total-cholesterol ratio compared to crab-fed mice in the fasted state ($P = 0.059$). Therefore, our data indicated that the lower dietary cholesterol concentration did not by itself explain the higher HDL-to-total-cholesterol ratio observed in scallop-fed mice. Taken together, the improved HDL-to-total-cholesterol ratio in scallop-fed mice did not correlate with the differences in lipid composition between the diets but did correlate significantly with dietary taurine and glycine intake.

Apart from the described highly significant linear correlations with intake of taurine and glycine, we cannot exclude that other nutritional factors in the scallop protein might also have contributed to the reduced body mass gain and improved plasma lipid profile, but further analyses are needed to identify other contributing nutrients. Furthermore, non-linear correlations that we are unable to identify in the present study may exist between consumed nutrients and body mass gain and plasma lipid profile.

In conclusion, intake of scallop muscle as the sole dietary protein source completely prevented high-fat, high-sucrose-induced body mass gain and fat accretion without affecting lean body mass. Furthermore, scallop feeding improved plasma lipid profile in C57BL/6J mice compared to mice fed diets with protein from chicken, cod or crab. Correlation analyses revealed strong, highly significant inverse correlations between intake of taurine and glycine and body fat mass, as well as strong, highly significant correlations between glycine and especially taurine intake and improved plasma lipid profiles. Changes in satiety, energy expenditure, energy substrate utilization and cholesterol metabolism cannot be ruled out as significant.
Scallop protein with endogenous high taurine and glycine contributing factors in the present study, but further experiments are needed to explore these variables fully.

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Conflict of interest The authors declare that they have no conflict of interest.

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References

Ables GP, Perrone CE, Orentreich D, Orentreich N (2012) Methionine-restricted C57BL/6J mice are resistant to diet-induced obesity and insulin resistance but have low bone density. PloS ONE 7(12):e51357. doi:10.1371/journal.pone.0051357


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Scallop protein with endogenous high taurine and glycine


**Supplemental Table 1** Composition of fatty acids in the experimental diets

<table>
<thead>
<tr>
<th>(g/kg)</th>
<th>LF</th>
<th>Chicken</th>
<th>Cod</th>
<th>Crab</th>
<th>Scallop</th>
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<tr>
<td>10:0</td>
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<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
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<tr>
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<td>2.75</td>
<td>2.75</td>
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<tr>
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<td>&lt; 0.01</td>
<td>1.85</td>
<td>0.95</td>
<td>0.90</td>
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^a eicosapentaenoic acid

^b docosahexaenoic acid
## Supplemental Table 2
Measured plasma metabolites and calculated HOMA-IR and QUICKI scores

<table>
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<tr>
<th>Metabolite</th>
<th>State</th>
<th>LF</th>
<th>Chicken</th>
<th>Cod</th>
<th>Crab</th>
<th>Scallop</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>TAG (mmol/l)</td>
<td>Fasted</td>
<td>1.58 ± 0.11</td>
<td>^A1.34 ± 0.08</td>
<td>1.33 ± 0.08</td>
<td>^AB1.19 ± 0.09</td>
<td>^0.89 ± 0.10</td>
<td>0.004</td>
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<tr>
<td></td>
<td>Non-fasted</td>
<td>0.91 ± 0.13</td>
<td>1.27 ± 0.13</td>
<td>1.58 ± 0.20</td>
<td>1.22 ± 0.19</td>
<td>0.91 ± 0.11</td>
<td>0.057</td>
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<tr>
<td>NEFA (mmol/l)</td>
<td>Fasted</td>
<td>0.78 ± 0.04</td>
<td>^A0.82 ± 0.05</td>
<td>0.74 ± 0.03</td>
<td>^AB0.69 ± 0.04</td>
<td>^0.55 ± 0.05</td>
<td>0.001</td>
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<td>0.26 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>0.29 ± 0.03</td>
<td>0.32 ± 0.04</td>
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<tr>
<td>Glycerol (mmol/l)</td>
<td>Fasted</td>
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<td>^A0.48 ± 0.02</td>
<td>0.46 ± 0.03</td>
<td>^A0.43 ± 0.02</td>
<td>^0.31 ± 0.01</td>
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<tr>
<td></td>
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<td>0.30 ± 0.02</td>
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<td>0.323</td>
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<tr>
<td>OH-butyrate (mmol/l)</td>
<td>Fasted</td>
<td>3.57 ± 0.29</td>
<td>^A3.76 ± 0.21</td>
<td>3.78 ± 0.26</td>
<td>^AB3.27 ± 0.18</td>
<td>^2.50 ± 0.19</td>
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</tr>
<tr>
<td></td>
<td>Non-fasted</td>
<td>0.13 ± 0.02</td>
<td>0.27 ± 0.05</td>
<td>0.29 ± 0.16</td>
<td>0.28 ± 0.09</td>
<td>0.30 ± 0.05</td>
<td>0.260</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>Fasted</td>
<td>3.09 ± 0.16</td>
<td>3.06 ± 0.09</td>
<td>3.07 ± 0.10</td>
<td>3.07 ± 0.16</td>
<td>2.70 ± 0.06</td>
<td>0.066</td>
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<tr>
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<td>Non-fasted</td>
<td>3.54 ± 0.16</td>
<td>^A4.43 ± 0.23</td>
<td>^A4.59 ± 0.27</td>
<td>^AB4.18 ± 0.19</td>
<td>^3.72 ± 0.09</td>
<td>0.015</td>
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<td>LDL-cholesterol (mmol/l)</td>
<td>Fasted</td>
<td>0.57 ± 0.04</td>
<td>0.56 ± 0.04</td>
<td>0.53 ± 0.02</td>
<td>0.60 ± 0.07</td>
<td>0.45 ± 0.02</td>
<td>0.119</td>
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<tr>
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<td>Non-fasted</td>
<td>0.63 ± 0.04</td>
<td>0.81 ± 0.06</td>
<td>0.78 ± 0.06</td>
<td>0.72 ± 0.05</td>
<td>0.66 ± 0.03</td>
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<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>Fasted</td>
<td>2.25 ± 0.10</td>
<td>2.22 ± 0.06</td>
<td>2.20 ± 0.09</td>
<td>2.13 ± 0.11</td>
<td>2.16 ± 0.04</td>
<td>0.880</td>
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<tr>
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<td>2.50 ± 0.06</td>
<td>2.55 ± 0.06</td>
<td>0.944</td>
</tr>
<tr>
<td>HDL/total cholesterol</td>
<td>Fasted</td>
<td>0.73 ± 0.02</td>
<td>^AB0.73 ± 0.02</td>
<td>^AB0.72 ± 0.02</td>
<td>^0.70 ± 0.04</td>
<td>^0.80 ± 0.02</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Non-fasted</td>
<td>0.64 ± 0.02</td>
<td>^b0.56 ± 0.02</td>
<td>^b0.56 ± 0.03</td>
<td>^AB0.60 ± 0.02</td>
<td>^0.69 ± 0.01</td>
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<tr>
<td>ALAT (U/l)</td>
<td>Fasted</td>
<td>23.33 ± 2.87</td>
<td>31.86 ± 4.86</td>
<td>29.00 ± 3.25</td>
<td>28.00 ± 3.64</td>
<td>20.71 ± 1.11</td>
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<tr>
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<td>27.75 ± 3.60</td>
<td>21.50 ± 1.05</td>
<td>25.00 ± 2.67</td>
<td>18.75 ± 1.26</td>
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</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>Fasted</td>
<td>1.53 ± 0.03</td>
<td>1.94 ± 0.11</td>
<td>1.70 ± 0.10</td>
<td>2.25 ± 0.23</td>
<td>2.13 ± 0.11</td>
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<tr>
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<td>4.27 ± 0.42</td>
<td>3.80 ± 0.44</td>
<td>4.01 ± 0.40</td>
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<td>3.47 ± 0.29</td>
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<tr>
<td>Total bile acids (µmol/l)</td>
<td>Fasted</td>
<td>2.20 ± 0.52</td>
<td>1.77 ± 0.42</td>
<td>1.45 ± 0.22</td>
<td>1.60 ± 0.39</td>
<td>2.73 ± 0.68</td>
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<td>3.86 ± 0.48</td>
<td>1.30 ± 0.44</td>
<td>1.71 ± 0.35</td>
<td>1.49 ± 0.40</td>
<td>2.16 ± 0.33</td>
<td>0.381</td>
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<td>Glucose (mmol/l)</td>
<td>Fasted</td>
<td>6.52 ± 0.51</td>
<td>9.63 ± 0.52</td>
<td>8.96 ± 0.53</td>
<td>9.07 ± 0.72</td>
<td>10.18 ± 0.91</td>
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<tr>
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<td>Non-fasted</td>
<td>16.32 ± 0.78</td>
<td>16.17 ± 0.44</td>
<td>16.46 ± 0.90</td>
<td>14.80 ± 0.61</td>
<td>16.55 ± 0.40</td>
<td>0.214</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>Fasted</td>
<td>15.12 ± 6.54</td>
<td>19.95 ± 4.80</td>
<td>23.75 ± 4.34</td>
<td>37.47 ± 6.60</td>
<td>20.78 ± 5.35</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>Non-fasted</td>
<td>0.91 ± 0.23</td>
<td>1.46 ± 0.29</td>
<td>1.61 ± 0.26</td>
<td>2.19 ± 0.40</td>
<td>1.41 ± 0.42</td>
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</tr>
<tr>
<td>QUICKI</td>
<td>Fasted</td>
<td>0.43 ± 0.02</td>
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<td>0.37 ± 0.01</td>
<td>0.36 ± 0.02</td>
<td>0.39 ± 0.02</td>
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Data represent group means (n=6-8) ± standard error. Data was analysed by one-way analysis of variance followed by Tukey's pairwise comparisons. Means that do not share a letter are significantly different (P<0.05). HOMA-IR: Homeostasis Model of Assessment-Insulin Resistance, QUICKI: Quantitative Insulin Sensitivity Check Index.
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
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

1This 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.


3Supplemental 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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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/6J BomTac mice (Taconic, Ejby, Denmark) weighing approximately 25 g at arrival were housed individually at thermoneutrality (28 ± 1°) 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 \((n = 8/\text{group})\) 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 \((n = 10/\text{group})\). 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 \((6 \text{ M HCl, } 110±2^\circ\text{C, 22h})\) 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 \((\text{Ba(OH)}_2, 110±2^\circ\text{C, 20h})\), 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: \(1/(\log(\text{insulin} \ [\mu U/l]) + \log(\text{glucose} \ [mg/dl]))\) [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 mice 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, β-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-metylglutaryl-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.
References Cited


27. Tastesen HS, Keenan AH, Madsen L, Kristiansen K, Liaset B. 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.


49. Randle PJ. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes/Metabolism Reviews 1998;14:263-83.


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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 experimental diets

<table>
<thead>
<tr>
<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/scallopd&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>215</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chicken</td>
<td>-</td>
<td>-</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td>Cod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>114</td>
</tr>
<tr>
<td>Scallop</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>133</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>KCl</td>
<td>-</td>
<td>10.2</td>
<td>5.4</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>350</td>
<td>242</td>
<td>223</td>
<td>221</td>
</tr>
<tr>
<td>Lard</td>
<td>20</td>
<td>198</td>
<td>198</td>
<td>198</td>
</tr>
<tr>
<td>Vegetable oil&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>198</td>
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<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>AIN-76 mineral mix</td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>AIN-76 vitamin mix</td>
<td>10&lt;sup&gt;g&lt;/sup&gt;</td>
<td>14</td>
<td>14</td>
<td>14</td>
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<tr>
<td>Choline bitartrate</td>
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<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BHT</td>
<td>-</td>
<td>0.4</td>
<td>0.4</td>
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</table>

**Analyzed (g/kg)**

<table>
<thead>
<tr>
<th></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/scallopd&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Crude protein&lt;sup&gt;i&lt;/sup&gt;</td>
<td>170</td>
<td>190</td>
<td>190</td>
<td>200</td>
</tr>
<tr>
<td>Ash</td>
<td>31</td>
<td>48</td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>Fat</td>
<td>44</td>
<td>390</td>
<td>400</td>
<td>390</td>
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<tr>
<td>Gross energy kJ/g</td>
<td>17.4</td>
<td>26.2</td>
<td>26.0</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>i</sup> Crude protein: N x 6.15 for casein; N x 5.6 for chicken and cod/scallop
Table 2: Amino acid composition of experimental diets

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>Casein</th>
<th>Chicken</th>
<th>Cod/scallop</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
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<tr>
<td>Asx</td>
<td>100</td>
<td>109</td>
<td>154</td>
<td>149</td>
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<tr>
<td>Cys</td>
<td>28</td>
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<td>48</td>
<td>42</td>
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<tr>
<td>Glx</td>
<td>274</td>
<td>307</td>
<td>212</td>
<td>191</td>
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<tr>
<td>Gly</td>
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<td>His*</td>
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<td>Ile*</td>
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<tr>
<td>Leu*</td>
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<td>126</td>
<td>107</td>
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<tr>
<td>Lys*</td>
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<td>112</td>
<td>136</td>
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<tr>
<td>Met*</td>
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<td>Phe*</td>
<td>51</td>
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<td>Pro</td>
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<td>47</td>
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<tr>
<td>Ser</td>
<td>94</td>
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<td>78</td>
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<td>Thr*</td>
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<td>Tyr</td>
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<td>Val*</td>
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<td>88</td>
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<tr>
<td>Hyp</td>
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<td>&lt;0.1</td>
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<td>3</td>
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<tr>
<td>Tau</td>
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<td>&lt;0.1</td>
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<tr>
<td>EAA</td>
<td>551</td>
<td>640</td>
<td>630</td>
<td>512</td>
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<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 ± 0.18</td>
<td>4.53 ± 0.19</td>
<td>3.88 ± 0.15</td>
<td>0.003</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>2.68 ± 0.13</td>
<td>3.14 ± 0.13</td>
<td>3.72 ± 0.17</td>
<td>3.23 ± 0.13</td>
<td>0.022</td>
</tr>
<tr>
<td>HDL:total cholesterol ratio</td>
<td>0.27 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>0.82 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.87 ± 0.05</td>
<td>0.97 ± 0.08</td>
<td>1.42 ± 0.08</td>
<td>1.16 ± 0.07</td>
<td>0.002</td>
</tr>
<tr>
<td>Total bile acids (mmol/L)</td>
<td>3.0 ± 0.29</td>
<td>2.7 ± 0.23</td>
<td>1.9 ± 0.17</td>
<td>2.4 ± 0.19</td>
<td>0.027</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.75 ± 0.07</td>
<td>0.40 ± 0.03</td>
<td>0.65 ± 0.05</td>
<td>0.50 ± 0.06</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 ± 2.61</td>
<td>48.6 ± 8.56</td>
<td>24.7 ± 2.82</td>
<td>0.009</td>
</tr>
<tr>
<td>Total neutral lipids (mg/g)</td>
<td>35.2 ± 6.90</td>
<td>30.0 ± 2.62</td>
<td>52.9 ± 8.72</td>
<td>28.7 ± 2.84</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>
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<tr>
<td>Diacylglycerides (mg/g)</td>
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<td>0.2 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>0.1 ± 0.02</td>
<td>0.43</td>
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<tr>
<td><strong>Liver relative mRNA expression</strong></td>
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<tr>
<td>Pck-1</td>
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<td>0.24 ± 0.06</td>
<td>0.40 ± 0.09</td>
<td>1.06 ± 0.36</td>
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<tr>
<td>Scd-1</td>
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<td>0.02 ± 0.01</td>
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<td>Srebf1</td>
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<tr>
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<td>Dgat-1</td>
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<tr>
<td>Fasn</td>
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<tr>
<td>Hmgcr</td>
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<td>2.89 ± 0.61</td>
<td>3.77 ± 1.06</td>
<td>0.36</td>
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</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 2
Figure 3

A. Respiratory Exchange Ratio (VCO₂/VO₂) (Time of day)

B. RER, average 48h

C. Spontaneous activity, beam breaks (Time of day)

D. Activity, 48h

E. Activity, 48h, total

F. Energy expenditure (kJ/h-kg) (Time of day)

G. EE, average 48h

Legend:
- LF (Casein)
- LF (Chicken)
- LF (Cod/scallop)
- Casein
- Chicken
- Cod/scallop

Significance levels:
- A
- B
- C
- P values
  - P = 0.09
  - P = 0.06
  - P = 0.08
## Online Supporting Material

### Supplemental Table 1 Genes and corresponding primer sequences used for qRT-PCR

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<tr>
<th>Abbreviation</th>
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<th>5'prime</th>
<th>3'prime</th>
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<td>Pck-1</td>
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<td>CATATTCTTCAGCTTGCGG</td>
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<td>Scd-1</td>
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<td>ATGAAGCACATCAGCAGGAGG</td>
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<td>Srebfi</td>
<td>Sterol regulatory element-binding transcription factor-1</td>
<td>GGAGCCATGGATTGCACATT</td>
<td>GCTTCCAGAGGAGGCCAG</td>
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<td>Acaca</td>
<td>Acetyl-Coenzyme A carboxylase alpha</td>
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<td>CCAACAAGATGCCACTTTGA</td>
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<td>TATA-box binding protein</td>
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Hydrolyzed casein reduces diet-induced obesity in male C57BL/6J mice
Hydrolyzed Casein Reduces Diet-Induced Obesity in Male C57BL/6J Mice

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Abstract

The digestion rate of dietary protein is a regulating factor for postprandial metabolism both in humans and animal models. However, few data exist about the habitual consumption of proteins with different digestion rates with regard to the development of body mass and diet-induced obesity. Here, we used a factorial ANOVA design to investigate the effects of protein form (intact vs. hydrolyzed casein) and protein level (16 vs. 32 energy percent protein) on body mass gain and adiposity in obesity-prone male C57BL/6J mice fed Western diets with 35 energy percent fat. Mice fed the hydrolyzed casein diets had higher spontaneous locomotor activity than mice fed intact casein. During the light phase, mice fed hydrolyzed casein tended (P = 0.08) to have a lower respiratory exchange ratio, indicating lower utilization of carbohydrates as energy substrate relative to those fed intact casein. In further support of less carbohydrate oxidation, plasma concentrations of glucose and those of the glucose metabolite lactate were lower in fed mice that consumed the hydrolyzed compared with the intact casein diet. Concomitantly, the plasma insulin concentration was strongly reduced in fed mice given hydrolyzed casein relative to those given intact casein. The mice fed hydrolyzed casein had greater ex vivo inguinal white adipose tissue non-CO2 β-oxidation capacity along with induced expression of genes involved in mitochondrial fatty acid oxidation and mitochondrial uncoupling. The physiological changes induced by hydrolyzed casein ingestion translated into decreased body and adipose tissue masses. We conclude that chronic consumption of extensively hydrolyzed casein reduces body mass gain and diet-induced obesity in male C57BL/6J mice. J. Nutr. 143: 1367–1375, 2013.

Introduction

The worldwide increment in body weight is of an alarming character. Between 1980 and 2008, the mean BMI worldwide was estimated to have increased by 0.4 and 0.5 kg/m2 per decade for men and women, respectively (1). The increase in BMI is caused by a positive energy balance over time. Therefore, elucidating dietary impact on energy metabolism is crucial. Dietary protein has been shown to increase postprandial thermogenesis, i.e., convert more of the ingested food energy to heat, relative to carbohydrates in humans (2). The reduced energy efficiency, or the reduced ability to use ingested energy for growth, by intake of dietary proteins has been verified in humans in a cross-sectional epidemiological study (3), in a controlled intervention study (4), in studies with caloric restriction (5–7), in studies with overeating participants (8,9), and in a weight-maintenance study following weight reduction (10). Therefore, intake of dietary protein might be beneficial to prevent or reduce body weight gain.

Data on the ability of different protein sources to reduce the metabolic efficiency is limited. However, postprandial digestion rate is an independent factor modulating protein retention (11–13). Intake of soluble milk proteins with a high intestinal absorption rate increases the deamination of the ingested amino acids, leading to a higher urinary urea excretion compared with micellar casein or total milk proteins (14). Thus, ingestion of dietary proteins with a rapid amino acid delivery might induce a lower net energy accretion compared with expenditure in the postprandial period (14). Over time, a reduced energy accretion might translate into reduced body weight gain.

In addition to varying intestinal kinetics of amino acid delivery in intact milk proteins (11,12,14), ingestion of extensively hydrolyzed casein induces increased gastric emptying, higher amino acid absorption rates, and elevated plasma glucose-dependent insulinotropic polypeptide compared with ingestion of an equal amount
of intact casein (15). Moreover, in a study with young adults receiving a semi-synthetic meal with 23 g fat, 96 g carbohydrates, and 28.5 g protein either as casein or hydrolyzed casein, the hydrolyzed casein meal favored a higher splanchnic nitrogen utilization but a lower peripheral amino acid anabolism (16). In a crossover design study with elderly men, a single bolus of 35 g hydrolyzed casein induced accelerated protein digestion and absorption from the gut, a transient increase in plasma amino acid concentrations, and a tendency to increase the incorporation rate of amino acids into skeletal muscle protein relative to ingestion of casein (17). Thus, relative to intact casein, ingestion of hydrolyzed casein implies a different nitrogen utilization in human beings that possibly could modulate body weight gain.

All of the above-mentioned studies with casein (11–17) were performed as single meal tests. To the best of our knowledge, no published data exist on longitudinal, controlled intervention studies with ingestion of extensively hydrolyzed casein in adult human participants. However, extensively hydrolyzed casein is used in infant formulas for the prevention of allergy and food intolerance. In fact, 3 independent studies report decreased BMI due to lower body weight gain in the period of exclusive formula feeding with hydrolyzed casein-based formulas relative to those given intact casein-based formulas (18–20). Importantly, there was no decrease in length growth in those studies (18–20). Hence, studies with infants indicate that habitual intake of extensively hydrolyzed casein could be associated with a reduced body mass gain.

Based on the findings that ingestion of extensively hydrolyzed casein alters postprandial metabolism in single meal test studies with adults (15–17) and in feeding studies with rats (21) and pigs (22), as well as the findings that habitual intake of extensively hydrolyzed casein was associated with reduced body mass in infants (18–20), we hypothesized that intake of diets with extensively hydrolyzed casein would prevent or decrease body mass gain. To test this hypothesis, we fed mice diets with intact or extensively hydrolyzed casein.

Materials and Methods

**Ethical statement.** The animal experiments were approved by the National Animal Health Authorities (Norwegian approval identification: 1062, 1840, and 2500). Adverse events were not observed.

**Mice.** Data in this paper are based on the results from 4 animal studies (Expts. 1–4). In Expts. 1–3, one group of mice was fed a low-fat diet (D12450B, Research Diets) (Supplemental Table 1). These mice were used as a reference only and data from these mice are not included in the statistical analyses.

In all studies, male C57BL/6J BomTac mice (Taconic) 8 wk of age were assigned by weight to the experimental diets with hydrolyzed (H) or intact (I) casein at 16 energy percent protein to mimic a contemporary Western diet (23), with 16, 49, and 35 energy percent from protein, carbohydrate, and fat, respectively, or with 32 energy percent protein and 33 and 33 energy percent from carbohydrate and fat, respectively. The experimental diets were designated 16 energy percent protein, intact casein (I-16), 16 energy percent protein, hydrolyzed casein (H-16), 32 energy percent protein, intact casein (I-32), or 32 energy percent protein, hydrolyzed casein (H-32) (Supplemental Table 1). The amino acid content in the diets was balanced. The mice were individually housed and maintained on a 12-h-light/dark cycle at thermo-neutrality, 28 ± 1°C. To ensure equal energy intake, the mice were pair-fed. The mice were fed daily and body weight was recorded twice per week. At the end of the studies, mice were anesthetized with isoflurane (Isoba Vet) and killed by cardiac puncture.

Expt. 1 consisted of 38 mice (n = 8/group, except low-fat diet reference, n = 6). After 3 wk of feeding, the mice spent 48 h in wire-bottom cages for feces collection. After 8 wk of feeding, mice were killed during feed access, blood was collected for plasma separation, and tissues were harvested and weighed. A part of the liver, inguinal white adipose tissue (iWAT), soleus, and interscapular brown adipose tissue (iBAT) tissues were homogenized for non-CO₂ β-oxidation capacity measurements. Samples were taken from the epididymal white adipose tissue (eWAT) and iWAT tissues and fixed for histology. The rest of the liver, eWAT, iWAT, soleus, tibialis anterior, and iBAT tissues were freeze-clamped in liquid nitrogen and stored at −80°C. Samples from this study were used for plasma measurements and tissue qPCR analyses from fed mice.

Expt. 2 consisted of 35 mice (n = 7/group). After 7 wk of feeding, one representative mouse from each group was subjected to MRI.

Expt. 3 consisted of 49 mice (n = 9–10/group). After 7 wk of feeding, a glucose tolerance test was performed. After 8 wk of feeding, the mice were killed. Blood was collected from mice fed deprived overnight and plasma separated. Plasma from this study was used to analyze plasma metabolites and hormones from feed-deprived mice.

Expt. 4 consisted of 16 mice (n = 4/group). Mice were fed for 4 wk, body composition was analyzed at the start and at the end of the feeding period, and indirect calorimetric measurements were started after 3 wk of feeding.

**Body composition by quantitative magnetic resonance (QMR).** In Expt. 4, whole-body fat and lean tissue masses were determined using the EchoMRI quantitative magnetic resonance whole-body composition analyzer (Echo Medical Systems).

**Indirect calorimetry and spontaneous locomotor activity.** In Expt. 4, O₂ consumption, CO₂ production and spontaneous locomotor activity of the mice were measured in the Phenomaster open-circuit indirect calorimetry system (TSE Systems) using CaloCages fitted with infrared light-beam frames (ActiMort2). Indirect calorimetric calculations and spontaneous locomotor activity counts were made based on 3 consecutive light periods (0730–1900 h) and 3 dark periods (1930–0700 h). Energy expenditure [kcal/(h · kg body weight)] was calculated from the equation: 16.3 L O₂ used + 4.6 L CO₂ produced, as described in details elsewhere (24).

**Gross energy in diets.** The energy contents were determined in a bomb calorimeter following the manufacturer’s instruction (Parr Instruments).

**MRI.** In Expt. 2, one representative mouse from each group was anesthetized with isoflurane (Isoba Vet). The adipose tissues in the abdominal region of the mice were visualized on a 7-Tesla Bruker Pharmascan small animal Magnetic Resonance scanner (Bruker Biospin MRI). The MRI was performed at the Molecular Imaging Center, Department of Biomedicine, University of Bergen. Axial and coronal T1 weighted, whole-body scans were acquired with the following scan parameters: repetition time, 1300 ms; echo time, 7.5 ms, 6 acquisitions, 256 × 256 scan matrix; field of view, 4.0 cm; slice thickness, 1.0 mm; interslice distance, 1.0 mm, 12 slices; scan time, 6 min 14 s.

**Histology.** In Expt. 1, sections of adipose tissue were fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer overnight at 4°C, washed in 0.1 mol/L phosphate buffer, dehydrated in ethanol, and embedded in paraffin after clearing with xylene. Then 5-μm-thick sections of the embedded tissue were stained with eosin and hematoxylin.

**Plasma measurements.** In Expts. 1 and 3, hepatic plasma was prepared from blood collected at killing and stored at −80°C prior to analysis. Leptin (BioVendor) and insulin (DRG Diagnostics) concentrations were analyzed by ELISA kits. Glucagon (Millipore) and adiponectin (LINCO Research) were measured by RIA kits.
Plasma β-hydroxybutyrate (OH-butyrate; Randox), TG, FFA, glucose (all DIALAB), and lactate (Sentinel Diagnostics) concentrations were analyzed by conventional enzymatic kits using a MaxMat PL II (MAXMAT).

Non-CO2 β-oxidation capacity measurements. In Expt. 1, one part of tissue from iWAT and iBAT was homogenized in 5 parts (wt:v) of glycyl-glycine homogenization buffer (25). One part of liver tissue was homogenized in 9 parts (wt:v) homogenization buffer (200 mM mannitol, 50 mM/L sucrose, 10 mM/L HEPES-KOH, 1 mM/L EDTA, 0.1% fatty acid-free BSA, pH 7.4) and one part muscle (soleus) was homogenized in 19 parts (wt:v) homogenization buffer (250 mM/L sucrose, 10 mM/L Tris- HCl, 2 mM/L EDTA, 2 mM/L taurine, 50 U/mL heparin, pH 7.4). Non-CO2 palmitate oxidation capacity was measured in the aqueous fraction (iWAT, after centrifugation at 1000 x g for 5 min) or whole homogenates (liver, iBAT, and muscle) by the acid-soluble product method (26). In brief, [1-14C] palmitate oxidation by tissue homogenates was performed at 37°C using a medium containing a final concentrations of 13.3 mM/L HEPES, 83.3 mM/L KCl, 16.7 mM/L MgCl2, 0.7 mM/L EDTA, 1.0 mM/L L-carnitine, 0.4 mM/L CoASH, 2.0 mM/L ATP, 0.5 mM/L L-malate, and 25 mM/L cytochrome c. Then 40 μL of tissue homogenate was added to each vial containing 0.31 mL reaction medium. After 2 min of preincubation, the reaction was initiated by the addition of 0.15 mL [1-14C] palmitate (1.5 Ci/mol, at 100 μL/mL of final concentration) as potassium salt bound to BSA in a 2:1 molar ratio. After incubation for 15 min, the reaction was stopped by the addition of 1.5 mL of 10% perchloric acid. Non-CO2 palmitate oxidation capacity was normalized to the total protein content in the homogenates, measured using the BCA protein Assay Reagent kit (Pierce).

qRT-PCR. In Expt. 1, total RNA was purified from samples with TRIzol Reagent (Invitrogen) and the quality was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies). A 50-μL RT (0.5 μg total RNA) reaction was performed at 48°C for 60 min utilizing a GeneAmp PCR 9700 machine (Applied Biosystems). Individual RT reactions contained 1× TaqMan RT buffer (10×), 5.5 mM/L MgCl2, 500 μmol/L dNTP (of each), oligo(dT) primers (2.5 μmol/L), 0.4 U/μL RNase inhibitor, 1.25 U/μL Multiscribe Reverse Transcriptase (N808–0324, Applied Biosystems), and RNase-free water. qRT-PCR was run in 10-μL reactions on a LightCycler 480 Real-Time PCR System (Roche Applied Sciences) containing 2.0 μL cDNA and using SYBR Green Master Mix (Light Cycler 480 SYBR Green master mix kit, Roche Applied Sciences) and gene-specific primers designed using Primer Express 2.0 (Applied Biosystems) (Supplemental Table 2). GeNorm was used to determine the most suitable normalization gene for each tissue [TATA box binding protein (Tbp); eWAT, liver, and soleus; and β-actin (Actb)]: iBAT and iWAT).

Glucose tolerance test. In Expt. 3, after 7 wk of receiving the experimental diets, mice were feed deprived for 12 h before intraperitoneal injection of 2 g glucose/kg body weight. Blood was collected from the saphenous vein and blood glucose was measured with a Bayer Contour glucometer (Bayer) before and 15, 30, 60, and 120 min after glucose injection.

Statistical analyses. All data represent means ± SEs. All data sets were tested for homogeneity of variances using the Levene’s test. Data for growth curves were analyzed with repeated-measures ANOVA with body mass in weeks 0–8 as the dependent variable and protein level and protein form as categorical predictors. Repeated measures (within-subject effects) were defined as body mass at the 9 different times of weighing (weeks 0–8). The rest of the data were analyzed using a factorial ANOVA test with protein level and protein form as categorical predictors. In the case of an interaction effect between protein level and protein form, a post-hoc Fisher least significant difference test was performed. Data with heterogeneous variances were log-transformed before statistical analyses. P < 0.05 was considered significant. Statistics were performed with STATISTICA 9.0 (StatSoft).

Results

Reduced body mass gain with hydrolyzed casein ingestion (Expt. 1). After 8 wk of feeding, the body mass gain of mice fed hydrolyzed casein was significantly lower than that of the mice fed intact casein (Fig. 1A,B). Because there was no difference in the accumulated energy intake between experimental groups (3.28 ± 0.06 MJ/8 wk), the mice fed intact casein had significantly higher energy efficiency (body weight gain per energy intake) than the mice fed hydrolyzed casein. Yet, no significant difference in tissue weight was found for liver or skeletal muscles, soleus, and tibialis anterior (Table 1). Moreover, no significant difference was found in fecal fat concentration, even though mice fed hydrolyzed casein (42 ± 4 mg fat/g feces) tended (P = 0.09) to have a greater fecal fat concentration compared with those fed intact casein (34 ± 2 mg fat/g feces). Thus, we cannot exclude the possibility that a somewhat higher fecal fat excretion, particularly in the H-32-fed mice, might have contributed to their reduced body mass and lean phenotype.

Increased spontaneous locomotor activity and altered body composition with hydrolyzed casein ingestion. Another set of mice was fed the experimental diets for 4 wk (Expt. 4). Again, at equal energy intake, hydrolyzed casein feeding resulted in less body mass gain relative to intact casein feeding. The body mass gain was less in the mice fed 32 energy percent protein compared with those fed 16 energy percent protein irrespective of casein type (Fig. 1C). The reduction in body mass gain by hydrolyzed casein feeding was due to both reduced fat and lean mass (Fig. 1D). During this shorter feeding trial (4 wk), the H-32 fed mice actually lost body mass, which was caused by a loss of lean mass (Fig. 1C,D). In the previous 8-wk feeding trial (Expt. 1), the mice did not experience reduced body mass loss (Fig. 1A,B) or lower lean tissue masses (Table 1); the negative body mass gain was therefore likely to be a short-term effect. Interestingly, the hydrolyzed casein-fed mice had higher spontaneous locomotor activity (Expt. 4) during both the light and dark periods (Fig. 1E). Despite the lower body mass gain, the altered body composition, and the greater spontaneous locomotor activity induced by hydrolyzed casein ingestion, no difference was found in energy expenditure during either the light [42.6 ± 0.5 kJ/(h · kg body weight)] or dark periods [61.0 ± 0.7 kJ/(h · kg body weight)]. However, the respiratory exchange ratio (RER; CO2 exhaled/O2 inhaled) tended (P = 0.08) to be lower during the light periods in mice fed hydrolyzed casein compared with those fed intact casein. In the dark periods, ingestion of the higher amount of protein caused reduced RER (Fig. 1F). The plasma concentrations of glucose and the glucose metabolite lactate were lower in fed mice (Expt. 1) that consumed hydrolyzed compared with intact casein (Table 2). The plasma concentration of OH-butyrate was higher in fed mice (Expt. 1) given hydrolyzed compared with intact casein (Table 2). No significant difference between dietary treatments (Expt. 1) was found for the plasma concentration of TG or FFAs in fed mice (Table 2). Moreover, no difference in plasma concentrations in any of these variables were found in plasma from mice (Expt. 3) deprived of feed overnight (Table 2).

Reduced adipose tissue masses in mice fed hydrolyzed casein. In agreement with the reduced body fat mass measured after 4 wk of hydrolyzed casein feeding (Fig. 1D), MRI in another set of mice (Expt. 2) revealed that the reduced adiposity by hydrolyzed casein feeding was still present after 7 wk (Fig. 2A). At termination after 8 wk of feeding (Expt. 1), the masses of
eWAT and iWAT (Table 3) and also that of iBAT (Table 2) were all significantly reduced in the mice fed hydrolyzed casein compared with the mice fed intact casein. Lean tissues such as liver and skeletal muscles (soleus and tibialis anterior) did not differ in weight after 8 wk (Expt. 1) of feeding (Table 1). Thus, the small reduction in body lean mass measured after 4 wk (Expt. 4) of hydrolyzed casein feeding (Fig. 1D) was not present in lean tissue masses after 8 wk (Expt. 1) of feeding. Histology of eWAT and iWAT samples (Expt. 1) showed that the mice fed hydrolyzed casein had smaller adipocytes, which was particularly evident in iWAT (Fig. 2B). As expected, both plasma leptin concentrations and white adipose tissue (WAT) leptin (Lep) mRNA levels reflected the WAT masses, with lower levels in the mice fed hydrolyzed casein (Table 3). The plasma adiponectin concentration and WAT adiponectin (Adipoq) mRNA levels, on the other hand, were not affected by WAT accumulation, but the plasma adiponectin concentration was lower in the mice receiving 32 energy percent protein compared with those fed 16 energy percent protein irrespective of the type of casein (Table 3).

**Increased iWAT non-CO2 β-oxidation capacity in mice fed hydrolyzed casein** (Expt. 1). The liver and soleus ex vivo non-CO2 β-oxidation capacity did not differ between dietary treatments (Table 1). Moreover, the hepatic expression of genes involved in fatty acid oxidation, i.e., the mitochondrial carnitine palmitoyltransferase 1a (Cpt1a) and medium-chain acyl-CoA dehydrogenase (Mcad) or the peroxisomal acyl-CoA oxidase 1 (Acox1), did not differ between treatment groups (Table 1). In soleus, the expression of genes encoding mitochondrial fatty acid oxidation enzymes was reduced (Cpt1b), tended (P = 0.053) to be lower (Mcad), or was unchanged [cytochrome c oxidase, subunit VIIIb (Cox8b)] in hydrolyzed casein-fed mice (Table 1).

In iBAT, both ex vivo non-CO2 β-oxidation capacity and uncoupling protein 1 (Ucp1) mRNA level were downregulated by hydrolyzed casein feeding relative to intact casein feeding (Table 1). Moreover, mice that consumed H-32 or I-32 diets had lower iBAT Ucp1 mRNA levels than mice that consumed H-16 or I-16 diets (Table 1). Taken together, our data do not support that hydrolyzed casein feeding induced higher fatty acid oxidation capacity in liver, soleus muscle, or iBAT.

In contrast, mice fed the H-16 and H-32 diets had significantly higher ex vivo non-CO2 β-oxidation capacity in iWAT (Fig. 3A) and also significantly increased mRNA levels of Ucp1, Cox8b, and myelin protein zero-like 2 (Mpzl2), a gene marker of brown adipose tissue that is also known as Eva, in this tissue (Fig. 3B-D). In addition, gene expression of the thyroid activating enzyme deiodinase, iodothyronine, type II (Dio2) tended (P = 0.06) to be induced by hydrolyzed casein feeding (Fig. 3E). We also measured the FFA concentration in the iWAT homogenates that were used to measure ex vivo non-CO2 β-oxidation capacity and found that it was unaltered in homogenates from mice fed the different experimental diets (Fig. 3F). Thus, our data suggest that mice fed hydrolyzed casein had induced fatty acid oxidation capacity and uncoupling in iWAT.

**Reduced plasma insulin concentrations in mice fed hydrolyzed casein**. Mice fed hydrolyzed casein tended (P = 0.08) to exhibit lower RER during light periods (Expt. 4), indicating less use of carbohydrates as an energy source (Fig. 1F). Concomitantly, plasma glucose and lactate concentrations (Expt. 1) were lower in mice fed hydrolyzed casein diets (Table 2). In agreement with the lower plasma glucose, the plasma insulin concentration (Expt. 1) was also reduced in mice fed hydrolyzed casein diets (Table 2). The plasma concentration of glucagon, a hormone that contributes to raise blood glucose, was also lower (Expt. 1) in fed mice hydrolyzed casein diets (Table 2). Despite the low plasma insulin concentration in mice fed hydrolyzed casein diets, these mice showed no signs of reduced glucose tolerance (Expt. 3) (Supplemental Fig. 1).

**Discussion**

We tested and substantiated the hypothesis that chronic consumption of extensively hydrolyzed casein given in a Western-type diet would prevent body mass gain and diet-induced obesity in male C57BL/6J mice. Our data from mice are in agreement
with the reduced body weight gain that was previously observed in infants consuming extensively hydrolyzed casein-based formulas (18–20). The underlying mechanisms for the infants’ reduced body weight gain is not yet fully elucidated, even though some studies indicate that infants fed the hydrolyzed casein-based formula consume less energy due to a greater satiating effect (19,27). In the present study in mice, we observed reduced body weight gain and adiposity at equal energy intake, which is in agreement with data from the infant study reported by Giovannini et al. (18). Therefore, it is likely that another mechanism than satiety could also contribute to the body weight-reducing effect of hydrolyzed casein ingestion.

Mice fed the hydrolyzed casein diets had higher spontaneous locomotor activity after 3 wk of the experimental diets during both the light and dark periods. Despite this, we were unable to detect differences in energy expenditure quantified by indirect calorimetric measurements. However, the mice that consumed hydrolyzed casein diets tended to have a lower RER during the light periods, indicating that these mice used less carbohydrate as an energy substrate. This notion was further supported by lower plasma concentrations of glucose and lactate in mice fed hydrolyzed relative to intact casein diets. During the light periods, the RER approached 0.83 for mice fed the hydrolyzed casein diets. A RER value <1 could be due to higher whole-body long-chain fatty acid oxidation, or it could be due to higher amino acid oxidation, which would drive RER toward 0.8 (24). We did not measure higher non-CO₂ palmitate oxidation capacity in iBAT, skeletal muscle, or liver. Moreover, plasma from mice fed hydrolyzed casein did not have a higher concentration of FFA. Thus, it is likely that the mice fed hydrolyzed casein used more amino acids as their energy substrate. A higher deamination of ketogenic amino acids would also explain the deamination of rapidly absorbed proteins would be in agreement with previous observations in humans (14). A higher deamination of ketogenic amino acids would also explain the higher plasma concentrations of OH-butyrate in mice fed hydrolyzed relative to intact casein diets.

In the present study, the plasma insulin concentrations were lower in mice fed hydrolyzed compared with intact casein. Dietary modulation of insulin signaling is important in the development of obesity (28,29). Insulin concentrations normally rise after feeding, promoting anabolic processes in the body such as protein and glycogen synthesis and lipid storage (30). Inhibition of insulin signaling in adipose tissue by fat-specific knockout of the insulin receptor protects against obesity in mice (31), whereas overexpression of the insulin receptor substrate (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to (IRS) increases adiposity in mice (32). We previously showed in mice that the obesogenic potential of high-fat diets is related to (IRS) increases adiposity in mice (32).
Brown adipose tissue is considered the major adipose tissue for dissipation of chemical energy in the form of heat (37), and the presence of brown-like adipocytes has recently been reported in adult humans (38,39). Importantly, cold exposure induces expression of UCP1 in the brown-like adipocytes (40,41). Also in animal models, the expression of Ucp1 can be induced in WAT under certain conditions, such as elevated intracellular cAMP (34,42–46). In keeping with the fact that insulin may

### TABLE 2 Plasma metabolite and hormone concentrations from mice fed diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk and terminated after overnight feed deprivation (Expt. 3) or during feed access (Expt. 1)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Metabolites, mmol/L</th>
<th>Hormones, pmol/L</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
<td>TG2</td>
</tr>
<tr>
<td>I-16</td>
<td>6.3 ± 0.4</td>
<td>3.2 ± 0.3</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td>I-16</td>
<td>8.2 ± 0.8</td>
<td>4.0 ± 0.3</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>I-32</td>
<td>7.6 ± 0.7</td>
<td>4.0 ± 0.2</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>I-32</td>
<td>7.7 ± 0.6</td>
<td>3.8 ± 0.3</td>
<td>0.89 ± 0.15</td>
</tr>
<tr>
<td>H-16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>0.53</td>
<td>0.50</td>
<td>0.49</td>
</tr>
<tr>
<td>F</td>
<td>0.14</td>
<td>0.44</td>
<td>0.41</td>
</tr>
<tr>
<td>L x F</td>
<td>0.18</td>
<td>0.20</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Note:** Data are means ± SEs, n = 6–10. F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; L, protein level; OH-butyrate, β-hydroxybutyrate.

1 Statistics on log-transformed data.

2 Data are means ± SEs, n = 6–10. F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; L, protein level; OH-butyrate, β-hydroxybutyrate.

### FIGURE 2 Abdominal MRI scan (A, Expt. 2) showing transverse and longitudinal pictures of the abdomen (white areas represent adipose tissue) and histology (B, Expt. 1) of iWAT and eWAT adipose depots from mice fed a low-fat diet or diets containing 16% or 32% of energy as intact or hydrolyzed casein for 7 wk (A) or 8 wk (B). eWAT, epididymal white adipose tissue; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; iWAT, inguinal white adipose tissue; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; LF, low-fat diet.
lower intracellular cAMP (47,48) and that mice fed the hydrolyzed casein diets had lower plasma insulin concentrations in the present study, the gene expression pattern and the increased \( b \)-oxidation capacity in iWAT strongly suggest a higher amount of brown-like adipocytes in iWAT. Brown-like adipocytes in WAT is one of the characteristics of obesity-resistant mouse strains such as the A/J mouse (49). Importantly, hyperinsulinemia was recently shown to drive diet-induced obesity in mice, and prevention of hyperinsulinemia reprogrammed WAT to express Ucp1 and increase energy expenditure (50). Thus, in the present study, the reduced fed plasma insulin concentration was most likely one underlying factor of the alterations observed in iWAT in the hydrolyzed casein-fed mice. Even though the observed differences in iWAT functions are insufficient to explain all the metabolic consequences of intake of hydrolyzed compared with intact casein, it is conceivable that the WAT phenotype contributed to the lower body mass gain in mice consuming hydrolyzed casein.

Whether our data obtained in mice are of relevance for ingestion of hydrolyzed casein in humans still needs to be investigated. However, our data from the mouse study resemble those from studies of infants where those given extensively hydrolyzed casein formula also exhibited reduced body mass gain relative to those given an intact casein formula (18–20).

TABLE 3  eWAT and iWAT masses, plasma leptin and adiponectin concentrations, and relative gene expressions from fed mice consuming diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk (Expt. 1)

<table>
<thead>
<tr>
<th>Diet</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-16</td>
</tr>
<tr>
<td>Tissue mass, g</td>
<td></td>
</tr>
<tr>
<td>eWAT</td>
<td>1.35 ± 0.20</td>
</tr>
<tr>
<td>iWAT</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td>Plasma concentration, ( \mu )mol/L</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>10 ± 0.9</td>
</tr>
<tr>
<td>eWAT, relative mRNA gene expression</td>
<td></td>
</tr>
<tr>
<td>Lep</td>
<td>96 ± 16</td>
</tr>
<tr>
<td>Adipoq</td>
<td>665 ± 97</td>
</tr>
<tr>
<td>iWAT, relative mRNA gene expression</td>
<td></td>
</tr>
<tr>
<td>Lep</td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>Adipoq</td>
<td>80 ± 9</td>
</tr>
</tbody>
</table>

1 Data are means ± SEs, \( n = 5–8 \). eWAT, epididymal white adipose tissue; F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iWAT, inguinal white adipose tissue; L, protein level.

2 Statistics on log-transformed data.

\( \text{FIGURE 3} \)  

iWAT non-\( \text{CO}_2 \) ex vivo palmitate oxidation capacity (A, Expt. 1), iWAT relative gene expressions (B-E, Expt. 1), and FFAs in iWAT homogenates used for the ex vivo palmitate oxidation capacities (F, Expt. 1) in mice fed a low-fat diet or diets containing 16% or 32% of energy as intact or hydrolyzed casein for 8 wk. Values are means ± SEs, \( n = 4–8 \). Statistics in B-E performed on log-transformed data. ASP, acid-soluble products; F, protein form; H-16, 16 energy percent protein, hydrolyzed casein; H-32, 32 energy percent protein, hydrolyzed casein; I-16, 16 energy percent protein, intact casein; I-32, 32 energy percent protein, intact casein; iWAT, inguinal white adipose tissue; L, protein level; LF, low-fat diet.
Data on postprandial hormone concentrations from the infant studies are not available from the literature at this time. In healthy adults, single meal tests (given as solutions) containing either hydrolyzed casein alone (15,17) or in a mixture with carbohydrates and fat (16) are reported to either transiently increase postprandial plasma insulin (16,17) or not alter the plasma insulin concentration (15). The transient increase in plasma insulin is thought to be secondary to a transient increase in plasma amino acids, in particular leucine, one amino acid that can stimulate pancreatic insulin release (51–53). Further, in type 2 diabetic subjects, single meal tests with mixtures of carbohydrate and hydrolyzed casein transiently elevate plasma insulin concentration and decrease the plasma glucose concentration (54). The insulinotropic effect of hydrolyzed casein seems to be dose dependent (55).

In both healthy and type 2 diabetic adults, intake of a bolus of ~30–35 g hydrolyzed casein generally results in a postprandial, transient elevation in plasma insulin concentration. This is in contrast to our findings in the present study with mice consuming hydrolyzed casein as the sole protein source for 8 wk. However, all of the reported human studies were performed with single test meals only, which very well may not reflect the situation with repeated/habitual intake of hydrolyzed casein. Therefore, human intervention studies with intake of hydrolyzed casein over time are needed to elucidate whether the insulino- tropic effects of hydrolyzed casein intake are maintained beyond single meal tests in humans.

Acknowledgments

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Literature Cited


Online supporting material

Supplemental Table 1: Experimental diet composition

<table>
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<tr>
<th>Composition</th>
<th>LF&lt;sup&gt;1&lt;/sup&gt;</th>
<th>I-16&lt;sup&gt;2&lt;/sup&gt;</th>
<th>H-16&lt;sup&gt;3&lt;/sup&gt;</th>
<th>I-32&lt;sup&gt;3&lt;/sup&gt;</th>
<th>I-32&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Casein&lt;sup&gt;4&lt;/sup&gt;</td>
<td>189.6</td>
<td>177.1</td>
<td>-</td>
<td>349.7</td>
<td>-</td>
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<tr>
<td>Hydrolyzed casein&lt;sup&gt;5&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>177.1</td>
<td>-</td>
<td>349.7</td>
</tr>
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<td>Supplemental AA&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>4.7</td>
<td>14.4</td>
<td>9.3</td>
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<td>L-Cystine</td>
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<td>KCI</td>
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<td>-</td>
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<td>Soybean oil</td>
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<td>Lard</td>
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<td>Mineral mix S10026&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>Disodium Phosphate</td>
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<td>14.2</td>
<td>14.2</td>
<td>14.2</td>
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<tr>
<td>Calcium Phosphate</td>
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<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
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<tr>
<td>Potassium Citrate, 1 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>15.6</td>
<td>18.0</td>
<td>18.0</td>
<td>18.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Vitamin Mix V10001&lt;sup&gt;8&lt;/sup&gt;</td>
<td>9.5</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
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<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Analyzed (kJ/g)

| Gross energy        | not analyzed   | 20.5           | 20.3           | 21.5           | 21.2           |

<sup>1</sup>The low fat control diet (LF) was bought commercially: D12450B (Research Diets, USA), and contained 20 % protein, 70 % carbohydrate and 10 % fat (% of energy), gross energy 16.1 kJ/g

<sup>2</sup>Containing 16 % protein, 49 % carbohydrate and 35 % fat (% of energy)

<sup>3</sup>Containing 32 % protein, 33 % carbohydrate and 35 % fat (% of energy)

<sup>4</sup>MP Biomedicals prod# 901293

<sup>5</sup>MP Biomedicals prod# 960138

<sup>6</sup>The concentration of individual amino acids was analyzed and supplemental amino acids were added to balance each amino acid in the final diets.

<sup>7</sup>Containing (g/kg mineral mix): sodium chloride, 259; magnesium oxide, 41.9; magnesium sulfate, 7H<sub>2</sub>O, 257.6; ammonium molybdate, 4H<sub>2</sub>O, 0.3; chromium potassium sulfate, 1.925; copper carbonate, 1.05; ferric citrate, 21; manganese carbonate, H<sub>2</sub>O, 12.25; potassium iodate, 0.035; sodium fluoride, 0.2; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose, 399.105

<sup>8</sup>Containing (g/kg vitamin mix): retinyl palmitate, 0.8; cholecalciferol, 1.0; all-rac-a-tocopheryl acetate, 10; menadione sodium bisulfite, 0.08; biotin, 2; cyanocobalamin, 1; folic acid, 0.2; nicotinic acid, 0.2; calcium panthotenate, 1.6; pyroxidine-HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6, sucrose, 978.42
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<tr>
<th>Abbreviation</th>
<th>Gene name</th>
<th>5'prime</th>
<th>3'prime</th>
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<tr>
<td>Acox1</td>
<td>Acyl-Coenzyme A oxidase 1</td>
<td>GGGTCATGGAACTCATCTTCGA</td>
<td>GAATGAACCTCTTTGGCTTTGGG</td>
</tr>
<tr>
<td>Adipoq</td>
<td>Adiponectin</td>
<td>GATGGC AGA GATGG ACTCC</td>
<td>CTTGCCAGTGCTGCCGTC AT</td>
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<tr>
<td>Actb</td>
<td>Beta Actin</td>
<td>ATGGGTCAGAAGGACTCCTACG</td>
<td>AGTGCTACGACCAGAGGCATAAC</td>
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<tr>
<td>Cox8b</td>
<td>Cytochrome c oxidase, subunit VIIIb</td>
<td>GACACCATGAAGCCAACGACT</td>
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<tr>
<td>Cpt1a</td>
<td>Carnitine Palmitoyltransferase 1a</td>
<td>ACAAAATTATGTGAGTGACTGG</td>
<td>GATCCCCAGAGAGCAATAGG</td>
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<tr>
<td>Cpt1b</td>
<td>Carnitine palmitoyltransferase 1b</td>
<td>CACAAATTCCGGGTACTTTGG</td>
<td>TAATAAGCACAACATCCATGGC</td>
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<tr>
<td>Dio2</td>
<td>Deiodinase, iodothyronine, type II</td>
<td>GCC CAGCAAATGTAGAC</td>
<td>TGG CAA TAA GGA GCT AGA A</td>
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<tr>
<td>Mcad</td>
<td>Acyl-CoA dehydrogenase, medium-chain</td>
<td>AGTATGCCTGGATAGGAACAT</td>
<td>CTTGGTGCTCCACTAGCAGCT</td>
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<td>Mpzi2 (Eva1)</td>
<td>Myelin protein zero-like 2</td>
<td>GTCCCAACCAGACCCTCAAC</td>
<td>CTCCATCTTTCTCGAAGGC</td>
</tr>
<tr>
<td>Leptin</td>
<td>Leptin</td>
<td>ATTTTACACACGACAGGTGAT</td>
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<tr>
<td>Tbp</td>
<td>TATA-Box Binding Protein</td>
<td>ACCCTTACCAATGACTCTATG</td>
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<tr>
<td>Ucp1</td>
<td>Uncoupling protein 1</td>
<td>AGCCGGCTTATGACTGGAG</td>
<td>TCTGTAGGCTGCCAATGAC</td>
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</tbody>
</table>

**Online Supporting Material**

**Supplemental Table 2:** Primer sequences used for real-time qPCR analyses
Online supporting material

Supplemental Figure 1 Blood glucose concentrations during the intraperitoneal glucose tolerance test (A, Expt. 3), calculated blood glucose area under the curve (AUC) (B, Expt. 3) in mice fed a low-fat diet or diets containing 16 or 32 % of energy as intact or hydrolyzed casein for 7 weeks. Values are given as mean + SE, n = 9-10. F, protein form; L, protein level.