Dietary proteins and body weight regulation

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Chapter 1

Introduction

GENERAL

Body weight changes are the result of changes in energy balance, while energy balance is the result of energy intake minus energy expenditure. A positive energy balance results in weight gain, while a negative energy balance results in weight loss. To maintain body weight, a balance between energy intake and energy expenditure should be maintained. Since obesity is a major health concern and the number of people with obesity is still increasing, strategies for weight loss and weight maintenance thereafter are necessary. These strategies should affect both short-term as well as long-term mechanisms involved in body weight regulation.
Recent findings suggest that an elevated protein intake affects both short- and long-term mechanisms by 1) increased satiety, despite similar or lower energy intakes, 2) an increased thermogenesis, 3) lower energy efficiency during overfeeding, and 4) sparing of fat free mass (1-5). An increased satiety may reduce energy intake, while increased thermogenesis, lower energy efficiency and sparing of fat free mass contribute to increase/maintain energy expenditure. These factors may contribute to prevent weight gain (or promote weight loss under conditions of negative energy balance) and in this way prevent the development of overweight. In addition to prevention of weight gain and promotion of weight loss, weight maintenance after weight loss is most crucial. Sparing of fat free mass is a conditional factor in weight maintenance after weight loss. As fat free mass is the main determinant of basal energy expenditure, the weight loss-induced decrease in energy expenditure needs to be counterbalanced, and this is achieved if fat free mass is preserved (6). The following part of this introduction will subsequently present how dietary protein may affect appetite, energy expenditure substrate balance, and body weight and composition.

APPETITE: PROTEIN-INDUCED SATIETY

Proteins are the most satiating of the three macronutrients, while fats are the least satiating (3, 7, 8). The increased satiety (or decreased hunger) with an increased protein intake is observed after acute high-protein meals (1, 3, 7, 9-11), as well as after high-protein diets for one day or longer (3, 8, 12-14). Several mechanisms may be involved in the higher satiating effect of high-protein meals or diets, like (an)orexigenic hormones, plasma amino acid responses, and energy expenditure.
One hypothesized mechanism for protein-induced satiety is a change in the secretion of gut neuropeptides, with increased secretions of GLP-1 and PYY, or decreased secretion of ghrelin (15). The (an)orectic actions of PYY, GLP-1 and ghrelin are thought to be mediated by the vagus nerve, the brainstem and/or the hypothalamus (16). GLP-1 and PYY are hormones that are known to promote satiety (17), and therefore are classified as anorexigenic hormones. Both hormones are predominantly released by the distal gut in response to ingestion of food (17). The extent of PYY release depends on calories ingested and on macronutrient composition of the meal (17). High-protein meals result in higher hunger suppression and higher increases in PYY levels compared with high-carbohydrate or high-fat meals (18). It is suggested that PYY is more important as a satiety factor, i.e. postponing initiation of the next meal, than as meal terminator (16). It is largely unknown which mechanisms underly the synthesis and release of GLP-1 by the distal gut (16). Studies on high protein meals/diets showed that after four days of consumption of a high-protein diet compared with a normal-protein diet satiety was higher and GLP-1 concentrations were higher after dinner (13). However after an acute high-protein lunch compared with a high-carbohydrate, normal-protein lunch satiety was also higher, but GLP-1 concentrations were lower than after the high-carbohydrate lunch (9). In addition to its anorectic effect, GLP-1 is an incretin hormone, which potentiates insulin secretion from the pancreatic islands (17, 19), indicating that GLP-1 concentrations are not satiety specific but nutrient specific (9). Ghrelin, known as an orexigenic hormone, meaning that it stimulates appetite, is released from mucosa cells in the stomach. Plasma ghrelin concentrations increase until the onset of a meal and decrease again rapidly after the meal is started. Therefore, it appears to function as a meal initiator (16). The role of postprandial ghrelin secretion in protein-induced satiety is still controversial (15).
Another mechanism that may contribute to protein-induced satiety is the aminostatic hypothesis by Mellinkoff. This theory suggests that an increase in amino acid concentrations in the blood is correlated with a decrease in appetite. The suggestion is that a satiety center in the brain exists, which is sensitive to serum amino acid levels. Once amino acid concentrations reach a certain point, appetite decreases (1, 20).

A third mechanism that may contribute to protein-induced satiety is an increase in energy expenditure. The measured thermic effect of the 3 separate macronutrients is highest for protein (20–30%), followed by carbohydrate (5–10%) and fat (0–3%) (21). Previous studies observed a positive relationship between high-protein-induced satiety and energy expenditure (8, 13, 22), or, more detailed, a relationship between the difference in diet-induced energy expenditure and the difference in satiety between a high- and normal-protein diet (8). A suggestion for the theoretical basis of this relationship is based on studies that observed higher satiety scores under limited oxygen availability conditions (23-25): increased metabolic rate at rest implies an increased oxygen consumption and body temperature and limits oxygen availability, which is perceived by the subjects as a reduction in the possibility to eat and therefore is rated as an increase in satiety (8). Several processes mediate the observed higher energy expenditure after protein ingestion, like high ATP costs for postprandial protein synthesis and costs for amino acid oxidation, especially when given in excess of protein disposition (26). In addition, costs for urea production and gluconeogenesis are involved (27).
Little research is done on differences in satiating properties between different protein types. The amino acid composition may influence the satiating properties of a dietary protein. In particular, the amino acid tryptophan (TRP) has gained specific interest, as it is a precursor for the neurotransmitter serotonin (28). Brain serotonin has been suggested to be involved in appetite regulation (29), which is supported by the anorexigenic effects of serotonergic drugs in human subjects (30, 31). TRP uptake in the brain not only depends on plasma TRP concentrations, but also on the plasma ratio of TRP to the sum of the other large neutral amino acids (LNAA) valine, leucine, isoleucine, tyrosine and phenylalanine (28, 32). As protein sources dramatically differ in their TRP content, they distinctly influence plasma TRP concentrations or plasma TRP/LNAA ratio, and in this way influence brain serotonin activity. The whey peptide, α-lactalbumin, contains relatively high levels of TRP, and has been shown to increase plasma TRP concentrations and plasma TRP/LNAA ratio when ingested alone (33, 34) or as part of a meal (32). In contrast, gelatin contains very low levels of TRP. To reveal if the differences in TRP content contribute to differences in appetite or energy intake, α-lactalbumin and gelatin need to be compared with a third protein, namely gelatin+TRP, by adding TRP to gelatin and create a similar TRP content in gelatin as in α-lactalbumin. It is unknown whether these three proteins types affect appetite differently.

ENERGY EXPENDITURE

Daily energy expenditure consists of four components: 1) sleeping metabolic rate (SMR), 2) energy costs of arousal, 3) diet-induced energy expenditure (DEE), also known as the thermic effect of food or diet-induced thermogenesis (DIT), and 4) energy costs of physical activity, also known as activity-induced energy expenditure (AEE). The SMR and the energy costs for arousal together are known as basal metabolic rate (BMR), which is the main component of daily energy expenditure (35). Processes that determine BMR are 1) the resting metabolic requirements of splanchnic tissues, brain, and skin, which vary little under normal conditions because of relatively constant tissue mass and protein turnover rates, and 2) energy expenditure related to resting muscle; this is determined by the rate of muscle protein turnover (i.e. synthesis and breakdown of muscle protein) and is the only component that might vary considerably since large variations in muscle mass are possible and the rate of muscle protein may vary as well (36).
Proteins have the highest thermic effect of the 3 macronutrients (21). In this way, increasing the protein content of a diet may increase energy expenditure. Higher 24-h EE, DIT or SMR have been observed after meals or diets with a relatively high protein content compared with meals or diets lower in protein content (8, 13, 22, 27, 37-41). The increased energy expenditure with an increased protein intake is due to the fact that the body has no storage capacity for protein when ingested in high amounts, and therefore it immediately needs to be processed metabolically, which increases thermogenesis (27). Several mechanisms contribute to this increased energy expenditure. A first mechanism is an increased postprandial protein synthesis with high ATP costs for peptide bond synthesis (1, 27, 39). A positive correlation between amino acid-induced energy expenditure and amino acid-induced protein synthesis has been observed (42). Two other mechanisms that contribute to increased energy expenditure with an increased protein intake are the high ATP costs for urea production or gluconeogenesis (1, 27, 39).
The before mentioned mechanisms are possible mechanisms that contribute to protein-induced energy expenditure. However, little research is done on differences in energy expenditure

between different protein types. The amino acid composition of a protein is an important determinant for the metabolic efficiency of protein oxidation. Because amino acid catabolism results in a wide variety of carbon chains and cofactors, large differences exist with respect to the efficiency by which amino acids are oxidized (26). For instance, the number of amino groups that undergo conversion to urea in the urea cycle (at a cost of 4 ATP) differs between amino acids. If the stoichiometry of amino acid catabolism and urea synthesis are taken into account, the calculated energy expenditure to produce ATP ranges from 153 kJ/ATP for cysteine to 99 kJ/ATP for glutamate (26). With respect to the amino acid composition of different protein types and the energy expended for ATP synthesis for each amino acid, the energy expended for ATP synthesis per gram of protein ranges from 15.6 kJ/ATP per gram gelatin to 8.2 kJ/ATP per gram casein. Thus, gelatin oxidation, compared with oxidation of other protein sources, is hypothesized to be more inefficient. This inefficiency contributes, by increasing energy expenditure. In addition to gelatin, casein is of interest as it is a 'slow' protein, indicating that the plasma appearance of dietary amino acids is slower, lower and prolonged after digestion and absorption of casein compared to for example gelatin (43). The most likely reason for the slower appearance of amino acids into the plasma is a slower gastric emptying, as casein clots into the stomach (43). As the plasma appearance of dietary amino acids affects postprandial protein synthesis and amino acid oxidation, it affects energy expenditure in this way, as well as satiety.

SUBSTRATE BALANCE

The difference between macronutrient intake and macronutrient oxidation determines macronutrient balance, also known as substrate balance. The protein, fat and carbohydrates balances together determine energy balance. The macronutrient composition of a diet plays a role in postprandial macronutrient oxidation. The oxidation of the macronutrients can be determined with the respiratory quotient (RQ), which is calculated as the measured carbon dioxide (CO_2) production divided by the measured oxygen (O_2) consumption. The RQ is approximately 0.8 for pure protein oxidation, 0.7 for pure fat oxidation and 1.0 for pure carbohydrate oxidation. The RQ of a mixed diet varies between 0.7 and 1.0. Actual 24-h protein, fat and carbohydrate oxidations (in g/d) can be calculated from oxygen (O_2) consumption, carbon dioxide (CO_2) production, and 24-h urinary nitrogen excretion (44). Substrate balance can be determined by subtracting macronutrient oxidation from macronutrient intake. As proteins are the main building blocks of muscles, protein balance plays an important role in maintaining muscle mass, which is, as mentioned previously, an important determinant of BMR. If protein oxidation is higher than protein intake, proteins will be broken down from muscle mass. To prevent this, the protein content of a diet is important in achieving protein balance or even creating a positive protein balance. Over 24 hours a relatively high-protein diet resulted in a higher positive protein balance compared with an adequate-protein diet, in which protein balance was achieved (13). In addition, although fat content was the same between both diets, a negative fat balance was achieved with a high-protein diet, but not with an adequate-protein diet. In the long-term this negative fat balance may favor fat loss (13). The positive effect of an increased protein intake on substrate balances in the short-term may affect body weight and composition in the long-term, and may play a role during weight loss as well as weight maintenance, as described later on in this introduction.

Although protein content of a diet affects substrate balances differently, little research is done on differences in substrate balances between different protein types. An important factor in protein metabolism is the availability of amino acids. Amino acids can be divided in two groups: nutritionally indispensable (or essential) amino acids and nutritionally dispensable (or non-essential) amino acids. The nutritionally indispensable amino acids cannot be synthesized in the body and have to be derived from the diet: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The other amino acids are the nutritionally dispensable: tyrosine, arginine, proline, cysteine, glycine, glutamate, glutamine, aspartate, asparagine, alanine and serine (45). As protein types differ in their amino acid composition, this affects protein metabolism, and thus may affect substrate balances. Casein and gelatin are two contrasting proteins with respect to their amino acid composition. Casein is a complete protein as it contains all essential amino acids, while gelatin is an incomplete protein as it lacks the essential amino acid tryptophan and contains low amounts of for instance methionine and histidine.

WEIGHT LOSS AND MAINTENANCE

The amount of protein in a diet plays a role in body weight regulation and in body composition in the long-term through the before mentioned effects on appetite, energy expenditure and substrate balances. The World Health Organization recommends a minimum protein intake of 0.83 g/kg per day (45). Protein intake is expressed in grams (absolute protein intake) or as percentage of energy intake (relative protein intake). This distinction is important when considering low-, normal- or high-protein diets. For instance, in neutral energy balance (i.e. energy intake matches energy requirements set by energy expenditure) a relatively normal-protein diet of 10-15% of energy from protein has the same absolute amount of protein as a relatively high-protein diet of 20-30% of energy from protein in a negative energy balance (i.e. energy intake is lower than energy requirements set by energy expenditure) when subjects consume only half of their energy requirements in order to lose body weight (6). Thus, such a weight loss diet is relatively high in protein, but in absolute grams of protein it only contains a sufficient amount of protein. To prevent subjects from being in a negative protein balance during weight loss, as this results in breakdown of the metabolically active fat free mass, the absolute amount of daily protein intake is more important than the relative protein content of the diet (6). In this way, fat free mass is spared, which is conditional for preventing a decrease in BMR. Via these processes protein intake affects body weight and body composition and plays a role during weight loss as well as during weight maintenance afterwards.

In most weight loss studies relatively high protein diets are considered. Studies performed under ad libitum conditions observed higher weight losses with relatively high-protein diets than with relatively normal-protein diets, as a result of lower energy intakes with the relatively high-protein diets (46, 47). However, under iso-energetic conditions or energy-restricted conditions, no differences in body weight change were observed between relatively high- and normal-protein diets (46, 48, 49), while hunger was decreased or satiety increased with the high-protein diets (48, 49). After a 12-months ad libitum dietary intervention, a higher decrease in intra-abdominal adipose tissue was observed with a relatively high-protein diet compared with a relatively normal-protein diet, while weight losses were similar (50). During weight maintenance after weight loss, the 18 En% protein diet showed less weight regain after 3 months, while the regained weight only consisted of fat free mass after the 18 En% protein diet, but also of fat mass after the 15 En% protein diet (14). These results were confirmed in a similar study with a 6 months weight maintenance period after weight loss (12). It seems that a higher relative protein intake induces weight loss under ad libitum conditions as a result of a reduced energy intake, in which protein-induced satiety plays a role. In addition, relatively high protein diets improve body composition during weight loss as well as weight maintenance, in which sparing of fat free mass and/or higher fat losses play a role. The lower weight regain during weight maintenance with relatively higher protein diets results from 1) favoring regain of FFM at the cost of FM at a similar physical activity level, 2) reducing the energy efficiency (kg body mass regain/energy intake) with respect to the body mass regained, and 3) increasing satiety (6).

Most weight loss and weight maintenance studies use relative protein intake to express the intervention, instead of absolute protein intake, which can imply a lower absolute protein intake than required or just meeting protein requirements during negative energy balance with relatively normal- or high-protein diets, respectively. For preservation of fat free mass during weight loss and/or an improved fat free mass/fat mass ratio during weight maintenance, a sustained absolute minimum required protein intake is more important than relative protein intake. Therefore, a protein diet with a sustained absolute minimum required protein amount should be compared with a supra-sustained, i.e. a higher amount than the minimum required protein intake, diet in order to reveal possible differences in changes in body weight and composition during weight loss and weight maintenance. In addition, no research is done on possible differences between protein types in changes in body weight and composition during weight loss and weight maintenance. We observed that, under 10En% as well as under 25En% protein conditions, energy intake after a single-protein breakfast was less with gelatin compared with casein, soy or whey without glycomacropeptide (51). In addition, under 10En% protein conditions, gelatin decreased hunger more than casein after a single-protein breakfast (51) as well as after a single-protein diet for one day (see chapter 5). To study if the beneficial short-term effect of gelatin on hunger and energy intake plays a role in the long-term during weight loss and weight maintenance, gelatin may be used as a protein source in a weight loss/maintenance diet. However, gelatin is an incomplete protein, because it is deficient in certain essential amino acids, and therefore cannot be used as a single-protein source in a

long-term diet. To create a high-protein diet without lacking the essential amino acids, gelatin should be complemented with a complete protein source, for which milk protein is a good candidate.

OUTLINE OF THE THESIS

The research presented in this thesis investigates the effects of different types of protein under normal and higher protein conditions on satiety, energy expenditure, substrate balances, and body weight loss and maintenance.
From literature it is known that TRP is a precursor for brain serotonin, which has been suggested to be involved in appetite regulation. The hypothesis that a protein high in TRP content (α-lactalbumin) leads to a higher satiety respons than a protein low in TRP content (gelatin), due to its higher TRP content, is tested in chapter 2. Therefore the effects of α-lactalbumin, gelatin and gelatin with added tryptophan on appetite and energy intake in the short-term are compared under relatively normal protein (10En%) breakfast conditions.
In chapter 3 and 4 we tested the hypothesis that a single-protein diet relatively high in protein content results in a higher satiety respons and higher energy expenditure than a single-protein diet relatively normal in protein content, for casein (chapter 3) and gelatin (chapter 4) respectively. Therefore we studied single-protein diets with either casein or gelatin as protein source, under 10En% as well as under 25En% protein conditions, with respect to their effects on 24-h energy expenditure, substrate balances and appetite. In chapter 5 these effects were compared between the protein types casein and gelatin, under 10 En% as well as under 25 En% protein conditions, since we hypothesized that the use of an incomplete protein (gelatin), compared with a complete protein (casein), in single-protein diets may stimulate appetite suppression and energy expenditure, and may limit a positive protein balance.
Absolute protein intake may be more important than relative protein intake. Therefore we studied sustained and supra-sustained protein diets during weight loss and weight maintenance. The sustained protein diet provided a sustained absolute minimum required daily protein intake, while the protein intake in the supra-sustained protein diets was even higher than the minimum required daily amount of protein intake. In order to reveal if the beneficial short-term effects of the incomplete protein gelatin on appetite and energy intake may affect weight loss and weight maintenance in the long-term, we added gelatin to a sustained milk protein diet, while creating a supra-sustained gelatin-milk protein diet. The supra-sustained gelatin-milk protein diet is tested in the long-term with respect to effects on body weight loss and maintenance, and compared with the effects of a sustained and supra-sustained milk protein diet. We tested the hypothesis that the addition of gelatin to a milk protein diet will promote weight loss during a weight loss period (chapter 6) and will improve weight maintenance during a 4-months weight maintenance period after weight loss (chapter 7).

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Chapter 2

Acute effects of breakfasts containing alpha-lactalbumin, or gelatin with or without added tryptophan, on hunger, 'satiety' hormones and amino acid profiles

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ABSTRACT

Proteins are the most satiating macronutrients. Tryptophan (TRP) may contribute to the satiating effect, as it serves as a precursor for the anorexigenic neurotransmitter serotonin.
To address the role of TRP in the satiating properties of dietary protein, we compared 3 different breakfasts, containing either α-lactalbumin (high in TRP), gelatin (low in TRP) or gelatin with added TRP (gelatin+TRP, high in TRP), on appetite.
Twenty-four subjects (22-29 kg/m^2,19-37 years) received a subject-specific breakfast at t=0 with 10/55/35En% protein/carbohydrate/fat in a randomized, single-blind design. Hunger, GLP-1, ghrelin, amino acid concentrations, and energy intake during a subsequent lunch were determined.
Suppression of hunger was stronger 240 min after the breakfast with α-lactalbumin compared to gelatin and gelatin+TRP. Total plasma amino acid concentrations were lower with α-lactalbumin compared to gelatin±TRP (from t=180-240 min). TRP concentrations were higher after α-lactalbumin than after gelatin±TRP from t=0-100 min, whereas from t=100-240 min, TRP concentrations were lower after gelatin than after α-lactalbumin and gelatin+TRP. Plasma ratio of TRP to other large neutral amino acids (LNAA) were, only at t=100 min, lower after gelatin+TRP than after the other breakfasts. Plasma amino acid responses, TRP concentrations and TRP/LNAA ratios were not correlated to hunger. GLP-1 and ghrelin concentrations were similar for all diets. Energy intake during a subsequent lunch was similar for all diets.
Summarized, an α-lactalbumin breakfast suppresses hunger more than a gelatin or gelatin+TRP breakfast. This cannot be explained by (possible) differences found in TRP concentrations and TRP/LNAA ratios in the breakfasts and in plasma, as well as in ciruclating total amino acids, GLP-1 and ghrelin.

KEYWORDS: breakfast, hunger, satiety hormones, amino acid profiles

INTRODUCTION

Of all macronutrients, proteins have repeatedly shown to be the most satiating, either over the short term as well as over 24 h (1-5). The satiating properties of proteins seem to depend on the protein source: for instance, ingestion of whey protein resulted in a more pronounced satiety, and a decreased energy intake during a subsequent meal, than ingestion of casein (6). The authors suggested that the difference between these two protein sources might be mediated by differences in digestion and absorption rate. Thus, compared with the 'slow-absorbable' casein, ingestion of the "fast-absorbable" whey protein resulted in higher peak values of amino acids, which was associated with higher peak values of the 'satiety hormones' glucagons-like peptide-1 (GLP-1) and cholecystokinin (CCK) (6). However, it cannot be excluded that other mechanisms contribute to these differences in satiety, as several alternative mechanisms have been suggested to underlie the satiating capacity of dietary proteins, including the thermic effects of protein consumption or the utilization of amino acids for gluconeogenesis (3).

The amino acid composition may partially influence the satiating properties of a dietary protein. In particular, the amino acid TRP (TRP) has gained specific interest, as it may act as a precursor for the neurotransmitter serotonin (7). Brain serotonin has been suggested to be involved in appetite regulation (8), which is supported by the anorexigenic effects of serotonergic drugs in humans (9, 10). Several dietary intervention studies have attempted to increase brain serotonergic activity, mainly through increasing central TRP availability (7, 11, 12). TRP transport to the brain is facilitated by the so-called L-transporter, which also facilitates the transport of other large neutral amino acids (LNAA), valine, leucine, isoleucine, tyrosine and phenylalanine in a competitive manner (7, 13). Therefore, brain TRP uptake may not only depend on plasma TRP concentrations, but also on the plasma ratio of TRP to the sum of these other LNAA (7, 13).

As protein sources dramatically differ in their TRP content, they may distinctly influence plasma TRP concentrations or plasma TRP/LNAA ratio. The whey peptide, α-lactalbumin, contains relatively high levels of TRP, and has been shown to increase plasma TRP concentrations and plasma TRP/LNAA ratio when ingested alone (11, 12) or as part of a meal (13). In contrast, collagen hydrolysate, or gelatin, contains very low levels of TRP and is even used as an experimental approach to induce central serotonin depletion in laboratory animals (14) as well as in humans (15).

As these differences in TRP content may influence brain serotonin activity, and thereby appetite or energy intake, we investigated the differences in satiating properties of two different breakfasts, one containing α-lactalbumin and one containing gelatin as the only protein source. In order to be able to attribute possible differences to differences in TRP content, we included a third breakfast in which we added TRP (gelatin+TRP) to the gelatin breakfasts, resulting in similar TRP content as the α-lactalbumin-containing breakfast.

SUBJECTS & METHODS

Subjects

Thirty healthy male and female volunteers (age 18-45 year) were recruited by advertisements in local newspapers and on notice boards at the university. They underwent a screening including medical history, measurement of body weight and height and cognitive restrained eating using a Dutch translation of the Three Factor Eating Questionnaire (TFEQ) (16, 17). Twenty-four subjects (11 male, 13 female, age 19-37 years, body mass index 22-29 kg/m^2) were selected on being in good health, non-smokers, non-vegetarian, not cognitively dietary restraint (TFEQ Factor 1 < 9), not using medication apart from oral contraceptives and at most moderate alcohol users (<10 alcoholic consumptions per week). Their mean age was 21 ± 0.8 years, and their BMI was 24.8 ± 0.4 kg/m^2 (**Table 1**). A written informed consent was obtained from these participants and the study protocol was approved by the Medical Ethical Committee of the Academic Hospital Maastricht.

Study design

The study was performed in a randomized, single-blind, within-subject design. Subjects reported to the laboratory for 6 times, separated by at least 3 days. Randomization took place in two blocks of 3 test days: in both blocks, subjects received the 3 different breakfasts (containing either α-lactalbumin, gelatin or gelatin+TRP as the sole protein source) in random order divided over the 3 test days. They were instructed to fast from 22:00 h the night before the test day.

On the first 3 test days, a permanent Teflon catheter was inserted into a dorsal vein of the hand, which was placed in a thermoregulated (60°C) box for arterialized venous blood sampling (18). At 8:30h, the protocol started with scoring hunger ratings. Breakfast was offered (t=0 minutes) and completed within 20 minutes. With the first and the last bite taste perception was scored. Hunger ratings were completed at 20, 40, 60, 80, 100, 120, 180 and 240 minutes after breakfast. Blood samples were withdrawn immediately before breakfast, and 20, 30, 40, 60, 80, 90, 100, 120, 180 and 240 minutes after breakfast for determination of plasma concentrations of amino acids, GLP-1, ghrelin, glucose and insulin.
In the second series of 3 test days, the protocol started at 08:30h with a breakfast as above. At t=180 minutes, subjects were offered an ad libitum lunch. The lunch consisted of Turkish bread (400 g) with egg salad (400 g) with 13/41/46 energy% protein/carbohydrate/fat, and an energy density of 11.4 kJ/g. The bread was prepared in such a way that a thin, homogenous layer of egg salad was placed between two thin layers of bread, so that bites were energetically homogenous. Subjects were instructed to eat till they were comfortably full. Subjects were allowed to drink maximally three glasses of water spread over the entire test period.

Table 1 Subject characteristics at baseline

	Value
Gender (m/f)	11 / 13
Age (yr)	21 ± 0.8
Height (m)	1.75 ± 0.02
Body weight (kg)	76.3 ± 1.7
BMI (kg/m^2)	24.8 ± 0.4
TFEQ 1 (cognitive restraint)	4 ± 0.5
TFEQ 2 (disinhibition)	4 ± 0.4
TFEQ 3 (hunger)	3 ± 0.4
TEE (MJ/day)	12.7 ± 0.3

Values are means ± SEM
TFEQ, Three Factor Eating Questionnaire
TEE, total energy expenditure, is calculated by multiplying the BMR (calculated from the Harris-Benedict equation) by an activity index of 1.75

Breakfast
Breakfast was offered as custard, produced by NIZO Food Research bv. (Ede, the Netherlands), and had a protein/carbohydrate/fat ratio of 10/55/35 energy%, or 2.2/12.4/3.7 weight%. In these studies, 3 different custards were used, containing a single protein source, being either α-lactalbumin (BioPURE –Alpha-lactalbumin™, Davisco Foods International Inc., Eden Prairie, United States of America), gelatin (Solugel LMC/3, PB Gelatins GmbH, Nienburg/Weser, Germany), or gelatin with TRP (Sigma-Aldrich, Steinheim, Germany) added to the level present in α-lactalbumin, as a single protein source. The custard contained tapioca starch (Farinex VA50T and Perfectamyl 3108, AVEBE, Veendam, the Netherlands) and sunflower oil (Reddy NV, Vandemoortele, Roosendaal, the Netherlands) as carbohydrate and fat source, repectively. All 3 custards were citrus-vanilla flavored (J.B. de Lange, Belfeld, The Netherlands). Extensive product development and use of a taste panel lead to custards that did not differ significantly in color, taste, or viscosity. The breakfast contained 20% of daily energy requirement, calculated as basal metabolic rate (BMR), according to the equations of Harris-Benedict, multiplied by an activity index of 1.75 (19). The mean energy content of the breakfast (20% of calculated daily Total Energy Expenditure) was 2.54 ± 0.06 MJ, this resulted in a mean intake of protein, carbohydrate and fat of 14.5 ± 0.4, 81.7 ± 2.1 and 24.4 ± 0.6 g, respectively. The amino acid composition of the 3 different custards is presented in **Table 2**. TRP/LNAA ratios were 88 x 10^{-3}, 6 x 10^{-3} and 280 x 10^{-3} for the custards containing α-lactalbumin, gelatin, and gelatin with added TRP, respectively. All subjects received all 3 breakfasts in random order on the first series of 3 test days, as well as on the second series of 3 test days.

Measurements
Hunger ratings were obtained using a paper version of a 100 mm Visual Analogue Scales (VAS), anchored with 'not at all' and 'extremely'. Subjects were instructed to rate the hunger dimensions by marking the scale, using a pen, at the point that was most appropriate to their feeling at that time.
Energy intake during lunch was calculated by weighing the lunch before and after eating, and multiplying the difference of the weight by the energy value of the lunch as determined by the product labels (11.4 kJ/g).

Table 2 Amino acid composition of the 3 breakfasts (μmol/100 g custard)

	Alpha-lactalbumin	Gelatin	Gelatin + TRP
Cystine	955	6	6
Methionine	186	134	134
Aspartic acid	2722	990	990
Hydroxyproline	0	2155	2155
Threonine	964	367	367
Serine	913	730	730
Glutamic acid	2159	1616	1616
Proline	497	2842	2842
Glycine	793	7707	7707
Alanine	631	2456	2456
Valine	887	454	454
Isoleucine	1040	273	273
Leucine	1973	529	529
Tyrosine	553	61	61
Phenylalanine	578	266	266
γ-amino butyric acid	73	ND	ND
Histidine	420	138	138
Ornithine	57	57	57
Lysine	1690	620	620
Arginine	246	1135	1135
Tryptophan	442	10	442

ND, not detected

Blood analysis
In both experiments, arterialized venous blood was collected in heparinized tubes (Becton Dickinson Vacutainer system), which were kept on ice to minimize enzymatic reactions. Plasma was obtained by centrifugation at 4°C for 10 min at 3000 x g. Subsequently, 250 µl plasma was deproteinized with 20 mg dry sulfosalicylic acid for analysis of plasma AA concentrations and enrichment. Each aliquot of plasma was frozen immediately in liquid nitrogen and stored at –80°C, until analysis. All samples from one subject were run in the same assay. AA concentrations were measured using a HPLC system (Pharmacia, Woerden, The Netherlands) as previously described (C.V. <3%) (20). Urea concentrations were measured spectrophotometrically on a COBAS Mira S (Roche Diagnostica, Hoffmann-La Roche; C.V. < 5%). Concentrations of active ghrelin were measured by radioimmunoassay (Linco Research Inc. St. Charles, Missouri, USA; within assay C.V. 6%, between assay C.V. 16%). Concentrations of active GLP-1 were measured using ELISA (EGLP-35K; Linco Research Inc. St. Charles, Missouri, USA; within assay C.V. 7%, between assay C.V. 8%). Insulin concentrations were measured by an electrochemiluminescence immunoassay (Roche Diagnostica, Hoffmann-La Roche, Basel, Switzerland; C.V. < 10%). Glucose concentrations were measured using enzymatic assay (G6-PDH) (Roche Diagnostica, Hoffmann-La RocheBasel, Switzerland; C.V. < 5%).

Statistical analysis

Data are presented as mean changes from baseline + standard error to the mean (SEM), unless otherwise indicated. In order to perform regression analyses, for hunger scores, plasma concentrations of total and individual amino acids and TRP and for plasma TRP/LNAA ratios, the area under the curve (AUC) of changes from baseline was calculated using the trapezoidal method. A repeated-measures ANOVA, followed by Fisher's PLSD post-hoc test, was carried out to determine possible differences between the different types of protein. Bonferonni correction was used for multiple comparisons. Regression analysis was performed to determine the relationships between the AUC of hunger scores and the AUC from baseline of plasma concentrations of total amino acids and of plasma TRP/LNAA ratios for all 3 breakfasts. Stepwise multiple regression analysis was performed to determine the relationships between AUC of hunger scores and the AUC from baseline of all amino acids measured. A p-value <0.05 was regarded as statistically significant. Statistical procedures were performed using StatView 5.0 (SAS Institute Inc., USA, 1998).

RESULTS

Following breakfast, hunger scores drop immediately (**Figure 1**). In the subsequent 240 minutes after ingestion, hunger scores gradually returned to pre-breakfast values. At t=240 min hunger scores were significantly lower in the α-lactalbumin breakfast group compared to the gelatin breakfast groups, either with or without added TRP. The AUC of changes in hunger scores from baseline were -10031 ± 883, -7679 ± 1072 and -8288 ± 1131 mm x min for the α-lactalbumin, gelatin and gelatin+TRP breakfasts, respectively (NS). Energy intake of an ad libitum lunch, provided 180 min after breakfast, was 2.65 ± 0.28, 2.56 ± 0.24 and 2.61 ± 0.26 MJ for the α-lactalbumin, gelatin and gelatin+TRP breakfasts, respectively (NS).

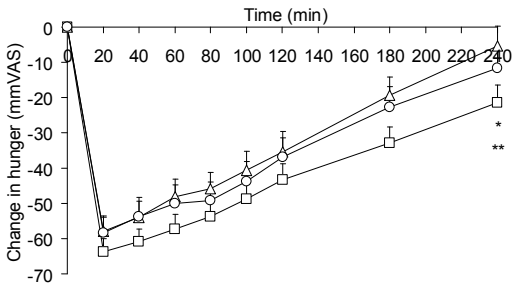

Figure 1 Changes in subjective ratings of hunger, as assessed by visual analogue scales (VAS), after ingestion of a custard based on α-lactalbumin (□), gelatin (△) or gelatin with tryptophan (TRP) addition (○). Values are means, with standard errors represented by vertical bars. *Mean value was significantly different from that following ingestion of the gelatin+TRP custard (p<0.05). **Mean value was significantly different from that following ingestion of the gelatin custard (p<0.01).

Plasma total amino acid concentrations rise immediately after breakfast ingestion, reaching peak values between 60 and 80 min after breakfast (**Figure 2**). After the α-lactalbumin breakfast, total amino acid concentrations returned to pre-breakfast values slightly quicker than after the gelatin breakfasts. Thus, at 180 min after breakfast, total amino acid concentrations were significantly lower after α-lactalbumin than after gelatin without added TRP; and at 240 min after breakfast, total amino acid concentrations were significantly lower after α-lactalbumin than after gelatin with or without added TRP. The AUC of changes in plasma total amino acid concentrations from baseline were 95058 ± 8365, 122788 ± 19326 and 112577 ± 10462 μM x min for the α-lactalbumin, gelatin and gelatin+TRP breakfasts, respectively (n.s.). There was no significant correlation between the AUC of the hunger scores and the AUC of plasma total amino acid concentrations following ingestion of any of the breakfasts.

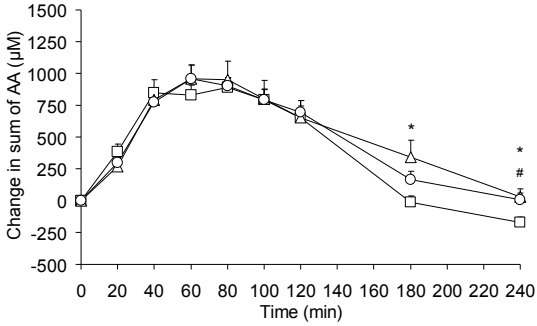

Figure 2 Changes in plasma total amino acid (AA) concentrations after ingestion of a custard based on α-lactalbumin (□), gelatin (△) or gelatin with tryptophan (TRP) addition (○). Values are means, with standard errors represented by vertical bars. *Mean value of the α-lactalbumin custard was significantly different from that following ingestion of the gelatin custard ($p<0.05$). #Mean value of the α-lactalbumin custard was significantly different from that following ingestion of the gelatin+TRP custard ($p<0.05$).

For each individual amino acid, peak plasma concentrations and AUC from baseline are shown in **Table 3**. Peak concentrations of asparagine, tyrosine, isoleucine, phenylalanine, leucine and lysine were higher, and peak concentrations of glycine, arginine and ornithine were lower after the α-lactalbumin breakfast than after the gelatin and gelatin with added TRP breakfasts. Peak plasma concentrations of TRP were higher after ingestion of gelatin with added TRP than after gelatin alone, but not different from the peak values of TRP after ingestion of the α-lactalbumin-containing breakfast. The AUC from baseline of asparagine, histidine, threonine, tyrosine, isoleucine, phenylalanine, leucine and lysine were higher, and the AUC from baseline of serine, glycine, arginine, alanine, taurine and ornithine were lower after the α-lactalbumin breakfast than after the gelatin and gelatin with added TRP breakfasts. In addition, the AUC from baseline of valine was higher after α-lactalbumin than after gelatin with added TRP. The AUC of changes in plasma TRP concentrations from baseline was significant lower after ingestion of the gelatin breakfast than after ingestion of the α-lactalbumin and gelatin with added TRP breakfast. Stepwise multiple regression analysis showed a significant correlation between the AUC of the hunger scores and the AUC from baseline of glutamic acid for the α-lactalbumin breakfast, and between the AUC of the hunger scores and the AUC from baseline of asparagine and taurine for the gelatin with added TRP breakfast. Thus, there was no significant correlation between the AUC of the hunger scores and the AUC of plasma TRP concentrations following ingestion of any of the breakfasts.

In the course of time, plasma levels of TRP were unaffected by the breakfast containing gelatin without added TRP (**Figure 3**). Plasma TRP increased after ingestion of a breakfast containing either α-lactalbumin or gelatin with added TRP, however, from t=20 to t=60 min after breakfast, this increase was significantly larger after the α-lactalbumin breakfast than after the gelatin with added TRP breakfast, despite similar TRP levels in the breakfasts. From 80 to 180 min after breakfast, plasma TRP concentrations were higher after the breakfasts both with α-lactalbumin and with gelatin with added TRP than after the gelatin without TRP breakfast.

Of the different custards, the TRP/LNAA ratios were the highest in the gelatin+TRP custard, followed by the α-lactalbumin custard and the lowest in the gelatin custard (88×10^{-3}, 6×10^{-3} and 280×10^{-3} for the custards containing α-lactalbumin, gelatin, and gelatin with added TRP, respectively). In the plasma, the delta TRP/LNAA ratios were not different between the conditions at any time point, with the exception of t=100 min, where delta TRP/LNAA was significantly lower after ingestion of gelatin+TRP custard when compared to the gelatin or α-lactalbumin custards (**Figure 4**). The AUC of changes in plasma TRP/LNAA ratios were 191 ± 185, 2 ± 25 and -66 ± 13 x min for the α-lactalbumin, gelatin and gelatin+TRP breakfasts, respectively (n.s.). There was no significant correlation between the AUC of the hunger scores and the AUC of plasma TRP/LNAA ratios following ingestion of any of the breakfasts.

Table 3 Amino acid responses to ingestion of a custard containing either α-lactalbumin, gelatin or gelatin with added tryptophan as the single protein source

	Alpha-lactalbumin		Gelatin		Gelatin + tryptophan	
	Peak (μmol/L)	AUC (μM x min)	Peak (μmol/L)	AUC (μM x min)	Peak (μmol/IL	AUC (μM x min)
Glutamic acid	118 ± 15	-740 ± 685	125 ± 9	1660 ± 803	115 ± 4	174 ± 1062
Asparagine	112 ± 6	7148 ± 362	59 ± 5[a]	-554 ± 527[a]	58 ± 2[a]	-1193 ± 245[a]
Serine	176 ± 12	1954 ± 834	188 ± 14	8827 ± 1761[a]	180 ± 9	8005 ± 593[a]
Glutamine	515 ± 23	-4508 ± 2027	516 ± 25	-173 ± 2361	513 ± 24	-555 ± 1823
Histidine	116 ± 3	2264 ± 340	97 ± 7	-67 ± 569[a]	98 ± 3	-847 ± 357[a]
Glycine	259 ± 21	-1290 ± 796	624 ± 71[a]	55300 ± 8371[a]	592 ± 38[a]	54237 ± 3582[a]
Threonine	220 ± 10	8651 ± 620	189 ± 7	4356 ± 1328[a]	188 ± 6	3269 ± 725[a]
Citruline	30 ± 4	-1043 ± 216	33 ± 2	33 ± 207[a]	33 ± 3	-457 ± 196
Arginine	104 ± 7	-1075 ± 349	148 ± 11[a]	7053 ± 1448[a]	142 ± 8[a]	6040 ± 483[a]
Alanine	578 ± 34	27812 ± 3480	647 ± 36	41904 ± 4232[a]	646 ± 31	42795 ± 4634[a]
Taurine	35 ± 2	63 ± 149	39 ± 3	1254 ± 219[a]	39 ± 2	1129 ± 115[a]
γ-amino butyric acid	20 ± 1	68 ± 88	19 ± 2	135 ± 94	19 ± 2	262 ± 60
Tyrosine	87 ± 7	2993 ± 372	61 ± 6[a]	-2173 ± 786[a]	58 ± 3[a]	-3248 ± 212[a]
Valine	221 ± 10	1094 ± 507	196 ± 4	-1268 ± 1150	188 ± 8	-2292 ± 532[a]
Methionine	28 ± 1	-393 ± 198	26 ± 1	-525 ± 302	24 ± 1	-537 ± 91
Isoleucine	140 ± 6	7971 ± 494	78 ± 4[a]	-1253 ± 1172[a]	72 ± 3[a]	-2681 ± 367[a]
Phenylalanine	68 ± 3	1440 ± 186	57 ± 2[a]	-485 ± 402[a]	55 ± 2[a]	-1010 ± 179[a]
Tryptophan	107 ± 5	8562 ± 520	53 ± 5[a]	-1202 ± 999[a]	104 ± 7[b]	6640 ± 393[b]
Leucine	218 ± 10	12007 ± 733	133 ± 8[a]	-2469 ± 1833[a]	130 ± 5[a]	-4412 ± 608[a]
Ornithine	63 ± 4	26 ± 257	80 ± 6[a]	4527 ± 710[a]	72 ± 5[a]	3755 ± 368[a]
Lysine	307 ± 15	20262 ± 1074	219 ± 11[a]	6734 ± 1902[a]	202 ± 8[a]	3512 ± 722[a]

Values are means ± SEM; responses are shown as peak plasma concentrations and as area under the curve (AUC) from baseline
[a] Mean value was significantly different from that following ingestion of the α-lactalbumin custard ($p<0.05$)
[b] Mean value was significantly different from that following ingestion of the gelatin custard ($p<0.05$)

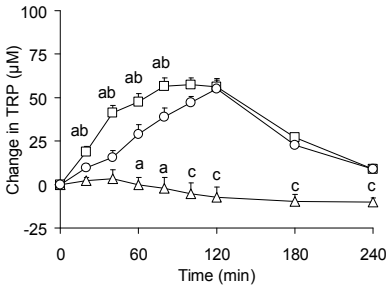

Figure 3 Change in tryptophan (TRP) concentrations in plasma samples after ingestion of a custard based on α-lactalbumin (□), gelatin (△) or gelatin with TRP addition (○). Values are means, with standard errors represented by vertical bars. [a]Mean value was significantly different from that following ingestion of the gelatin+TRP custard ($p<0.001$). [b]Mean value was significantly different from that following ingestion of the gelatin custard ($p<0.001$). [c]Mean value was significantly different from those following ingestion of the α-lactalbumin and gelatin+TRP custards ($p<0.001$).

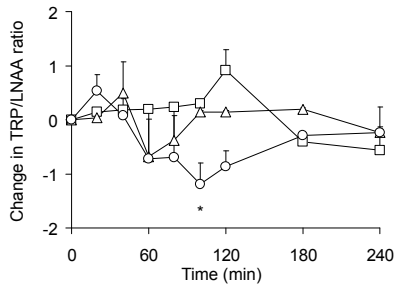

Figure 4 Change in plasma tryptophan/large neutral amino acids (TRP/LNAA) ratio after ingestion of a custard based on α-lactalbumin (□), gelatin (△) or gelatin with TRP addition (○). Included in LNAA are valine, isoleucine, leucine, tyrosine and phenylalanine. Values are means, with standard errors represented by vertical bars. *Mean value of the gelatin+TRP custard was significantly different from that following ingestion of the gelatin and α-lactalbumin custards ($p<0.05$).

Plasma GLP-1 concentrations increased immediately after ingestion of the custard, reaching peak levels at 30 min, which is followed by a gradual decline towards pre-breakfast levels (**Figure 5A**). There were no differences in plasma GLP-1 responses between any of the custards. Plasma ghrelin concentrations decreased after ingestion of the custard, reaching lowest levels between 40 and 60 min, which is followed by a gradual return towards, or even slightly above, pre-breakfast levels (**Figure 5B**). There were no differences in plasma ghrelin responses between any of the custards.

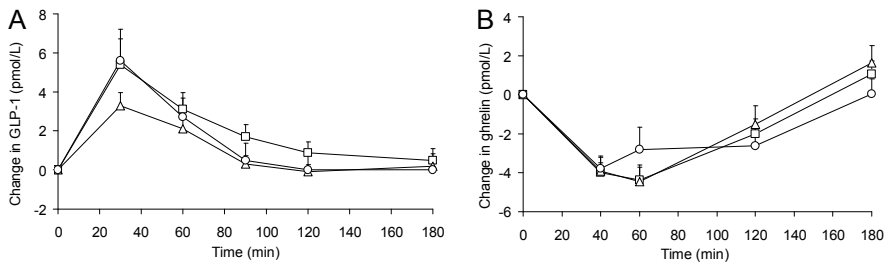

Figure 5 Plasma glucagon-like peptide-1 (GLP-1) (A) and ghrelin (B) concentrations after ingestion of a custard based on α-lactalbumin (□), gelatin (△) or gelatin with tryptophan (TRP) addition (○). Values are means, with standard errors represented by vertical bars.

Plasma concentrations of glucose increased after ingestion of the custard, with peak values at 40 min, where after glucose concentrations gradually declined, reaching pre-breakfast levels between 120 and 180 min after meal intake. At t=40 min, glucose concentrations after ingestion of the α-lactalbumin-containing breakfast (7.0 ± 0.2 mmol/l) were significantly lower than after ingestion of the gelatin (7.8 ± 0.2 mmol/l) or after the gelatin + TRP –containing breakfast (7.5 ± 0.3 mmol/l) breakfast. At other time points, there were no differences in plasma glucose concentrations between any of the custards. Plasma insulin concentrations also rose after ingestion of the custard, reaching peak values at 40 min. At t=180 min, plasma insulin levels have returned to pre-breakfast levels for all 3 custards. There were no differences in plasma insulin responses between any of the custards.

DISCUSSION

This study shows that ingestion of a breakfast containing α-lactalbumin as the only protein source results in a more prolonged suppression of hunger than a breakfast containing gelatin, although the hunger-suppressive effect was insufficient to decrease energy intake during a subsequent lunch. The proteins were compared in a normal-sized meal with a normal macronutrient composition; and although a higher protein load may have resulted in a more pronounced suppression of hunger and a decrease in energy intake, this may not reflect a physiological situation. Also, it cannot be excluded that the lack of an effect on energy intake is due to the timing of the lunch (180 min after breakfast), as the differences in hunger ratings did not reach statistical significance until 240 min after breakfast. There are only a few human studies that compare the satiating properties of different protein sources (3, 6, 21-25).

The faster recurrence of hunger after a gelatin-containing breakfast was not due to its low TRP content, as addition of TRP did not affect subjective ratings of hunger or satiety. It is therefore unlikely that the observed differences in hunger scores are related to the TRP content or the TRP/LNAA ratio. The rise in plasma TRP concentrations after ingestion of α-lactalbumin occurred faster than after ingestion of gelatin with added TRP, but those differences disappeared 100 min after breakfast. From that time on, plasma TRP responses were significantly lower after gelatin ingestions than after gelatin with added TRP, even though hunger ratings were similar. Nevertheless, even though the plasma responses of tryptophan were not correlated to hunger scores in any of the breakfasts offered, it cannot be excluded that the higher TRP levels in the first hour may have contributed to the lower perception of hunger 240 min after the α-lactalbumin-containing breakfast.

Still, in view of the competitive fashion of TRP and LNAA transport across the blood brain barrier, TRP/LNAA ratios, rather than TRP concentrations, are suggested to determine brain TRP uptake. The changes in TRP/LNAA ratios were similar between the 3 breakfasts, with the exception of t=100 min, where the TRP/LNAA ratio after ingestion of gelatin with added TRP was significantly lower when compared to the other 2 breakfasts. Since the hunger responses were similar after ingestion of gelatin with added TRP and after ingestion of gelatin without added TRP, despite these differences in TRP/LNAA ratio; and since hunger ratings were lower in α-lactalbumin versus gelatin without added TRP, despite similar TRP/LNAA ratios, the TRP/LNAA ratios provide no straightforward explanation for the observed differences in hunger ratings. Accordingly, hunger ratings did not correlate to plasma TRP concentrations or plasma ratios of TRP over LNAA for any of the breakfasts.

Remarkably, the TRP/LNAA ratios in the different breakfast are not reflected by plasma TRP/LNAA ratios. As the plasma TRP concentrations largely reflect the TRP concentrations in the breakfasts (high in α-lactalbumin and gelatin with added TRP, low in gelatin without added TRP), LNAA responses should account for the incongruence between TRP/LNAA ratios in plasma and breakfast. In this respect, insulin may pay a role, as insulin selectively stimulates the uptake of LNAA (7, 26). However, as insulin concentrations were similar in all three groups at all time points, other mechanisms, such as amino acid metabolism in intestinal cells, must be involved.

As the differences in hunger ratings between the 3 breakfasts cannot straightforwardly be explained by changes in TRP concentrations or the TRP/LNAA ratios in the custard or plasma, other mechanisms must be involved. Unfortunately, these mechanisms are not elucidated by this study. Already in 1956, Mellinkoff proposed an aminostatic regulation of food intake, implicating that increased plasma amino acid concentrations provide a satiety signal (27). Still, such a mechanism cannot explain the observed differences in subjective hunger ratings: total plasma amino acid concentrations were similar for all 3 breakfasts, with the exception of the period between 180 and 240 min after breakfast, when total plasma amino acid concentrations were the lowest after α-lactalbumin ingestion – which was associated with lower hunger ratings. Other individual amino acids, besides TRP, have also been suggested to be involved in the regulation of appetite and food intake. The responses of the individual amino acids to the α-lactalbumin- and gelatin-containing breakfasts showed marked differences, largely reflecting differences in amino acid composition. For instance, the leucine responses (both as peak values and AUV from baseline) were higher after α-lactalbumin-containing breakfast than after the gelatin-containing breakfasts. In fasted rats, leucine has been shown to decrease food intake, presumably by hypothalamic activation of the mammalian Target of Rapamycin (mTOR) (28). This may suggest that the increased leucine response may be involved in the prolonged suppression of hunger after α-lactalbumin ingestion. However, our studies did not reveal a

significant relationship between the hunger scores and the plasma responses of leucine (as AUC) in any of the breakfasts provided. Therefore, the role of leucine (and other individual amino acids) in the observed difference in hunger perception between the different protein sources remains to be elucidated.
Plasma responses of GLP-1 and ghrelin, a “satiety” and “hunger” hormone, respectively, which are implicated to play a role in regulation of appetite and energy intake (29), were similar for all 3 breakfasts – indicating that these hormones are not involved in the differences in hunger rates. In addition, recent studies showed that the increased satiety after a high-protein meal compared to a non-protein meal are not mediated by changes in circulating ghrelin and/or GLP-1 concentrations (1, 2, 30). Therefore, the satiating properties of dietary protein are likely to be mediated by a more complex set of mechanisms, and this may even be protein specific, as has recently been suggested (3).
All in all, this study shows that a breakfast containing α-lactalbumin as the only protein source results in a more prolonged suppression of hunger than a breakfast containing gelatin. Although the mechanism behind this difference has not been elucidated, TRP concentrations and TRP/LNAA ratios in the breakfasts and the circulation, as well as total amino acid, GLP-1 and ghrelin responses do not seem to be involved.

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AGN, AH-W, MABV, KRW, MPKJE, NEPD and MSW-P designed the study. MABV and AH-W collected and analyzed the data. AGN wrote the manuscript and AH-W, MABV, KRW, MPKJE, NEPD and MSW-P contributed to interpretation of the data and reviewed the manuscript. The study was executed under supervision of AGN, KRW, and MSW-P. None of the authors had a personal or financial conflict of interest.

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Chapter 3

Comparison of 2 diets with either 25% or 10% of energy as casein on energy expenditure, substrate balance, and appetite profile

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ABSTRACT

Background: An increase in the protein content of a diet results in an increase in satiety and energy expenditure. It is not clear to what extent a specific type of protein has such effects.
Objective: The objective was to compare the effects of 2 diets with either 25% or 10% of energy from casein (25En% and 10En% casein diets), as the only protein source, on energy expenditure, substrate balance, and appetite profile.
Design: During a 36-h stay in a respiration chamber, 24 healthy subjects [12 men and 12 women; body mass index (in kg/m^2): 22.4±2.4; age: 25±7 y] received isoenergetic diets according to subject-specific energy requirements: 25En% diet (25%, 20%, and 55% of energy as protein, fat, and carbohydrate, respectively) and 10En% diet (10%, 35%, and 55% of energy as protein, fat, and carbohydrate, respectively) in a randomized crossover design. Three days before the diets began, the subjects consumed a similar diet at home. Energy expenditure, substrate oxidation, and appetite scores were measured.
Results: The 25En% casein diet resulted in a 2.6% higher 24-h total energy expenditure (9.30±0.24 compared with 9.07±0.24 MJ/d; $P<0.01$) and a higher sleeping metabolic rate (6.74±0.16 compared with 6.48±0.17 MJ/d; $P<0.001$) than did the 10En% casein diet. With the 25En% casein diet, compared with the 10En% casein diet, the subjects were in positive protein balance (0.57±0.05 compared with -0.08±0.03 MJ/d; $P<0.0001$) and negative fat balance (-0.83±0.14 compared with 0.11±0.17 MJ/ d; $P<0.0001$), whereas positive carbohydrate balances were not significantly different between diets. Satiety was 33% higher with the 25En% casein diet than with the 10En% casein diet ($P<0.05$).
Conclusion: A 25En% casein diet boosts energy expenditure, protein balance, satiety, and negative fat balance, which is beneficial to body weight management.

KEYWORDS: energy metabolism, substrate oxidation, high protein diet, satiety, (an)orexigenic hormones

INTRODUCTION

Obesity is a major health concern worldwide and treatment is necessary (1). Body weight management requires a multifactorial approach, because several pathways are involved in the system of body weight regulation. Recent findings suggest that an elevation of protein intake affects both short- and long-term mechanisms by increasing satiety despite similar or lower energy intake, by increased thermogenesis, by contributing to storage of fat free mass, and by a lower energy efficiency during overfeeding (2-5). The measured thermic effect of the three separate macronutrients is the highest for protein (20-30%), followed by carbohydrate (5-10%), while fat is having the lowest thermic effect (0-3%) (6). Previous studies found a higher diet induced thermogenesis (DIT) (7, 8) and a higher sleeping metabolic rate (SMR) (8) while on a relatively high protein diet. Moreover, proteins are the most satiating, followed by carbohydrates, and fats being the least satiating (2, 5). Mechanisms that may contribute to protein-induced satiety are increases in energy expenditure (9, 10) and anorexigenic hormone concentrations (10). At this moment it is not clear to which extent these effects hold for specific types of protein. This study investigated possible differences between two diets, with either 25% or 10% of energy (En%) from the diet as protein, and with casein as the only protein source; respectively the 25En% casein diet and the 10En% casein diet. Casein is known as a 'slow' protein, meaning that it clots in the stomach resulting in a delayed gastric emptying (11). In a previous study we compared two breakfasts, both being 20% of daily energy intake and consisting of either 25En% or 10En% casein as the only protein source, with respect to satiety, relevant blood parameters and subsequent energy intake (12). A breakfast with 25En% from casein was more satiating than a breakfast with 10En% from casein, coinciding with prolonged elevated concentrations of plasma amino acids. To confirm this observation over 24 hours, and to determine energy expenditure at the same time we undertook the actual respiration chamber study. We investigated two diets in which we exchanged energy from protein (25En% vs. 10En%) with energy from fat (respectively 20En% vs. 35En%), while the carbohydrate content (55En%) remained the same. This because carbohydrate ingestion results in insulin secretion, and insulin is involved in protein metabolism (13).
The aim of this study was to compare a 25En% casein diet with a 10En% casein diet with respect to energy expenditure, substrate balances, appetite profile and relevant blood parameters in order to determine a possible effect of one single protein, namely casein, and to determine the magnitude of the difference.

SUBJECTS & METHODS

Subjects

Thirty subjects with an age between 18 and 55 years and a body mass index (BMI) between 20 and 33 kg/m^2 were recruited by advertisements on notice boards of Maastricht University and in local newspapers. Recruitment started at the 1st of September 2006. The actual study started in October 2006. Subjects underwent a medical screening and 24 subjects (12 male, 12 female) were selected on the following inclusion criteria: good health, non-smokers, not using medication (except the use of contraceptive), no cow milk allergy, at most moderate alcohol users, stable in weight during the last months, not following a diet and not cognitively dietary restrained. The Dutch translation of the Three Factor Eating was used for assessing eating behaviour of the subjects (14). The power calculation of this study was based on diet induced thermogenesis (DIT) measured in a respiration chamber under high and adequate protein feeding conditions as published by Lejeune et al (8). In this study, DIT values were 0.91±0.25 MJ/day and 0.69±0.24 MJ/day on respectively high protein and adequate protein diets. With a sensitivity of 0.80 and a significance level of 0.05, power calculation (within subjects, two-tailed) revealed a sample size of 12 for each group. The power calculation from our previous study (12) on satiety with casein custard for breakfast revealed, with a sensitivity of 0.80 and a significance level of 0.05, a sample size of 24 in total. Subject characteristics are presented in **Table 1**. Subjects signed an informed consent before participating in the study. The study protocol was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Table 1 Subject characteristics

	Value		
Gender (M/F)	12/12		
Age (y)	25	±	7
Height (m)	1.75	±	0.09
Body weight (kg)	68.5	±	10.5
BMI (kg/m^2)	22.4	±	2.4
Fat mass (kg)	15.4	±	6.7
Fat-free mass (kg)	53.0	±	9.3
TFEQ1 (cognitive restraint)[1]	6	±	3
TFEQ2 (disinhibition)[2]	4	±	2
TFEQ3 (hunger)[3]	5	±	3

Values are means ± SD, n=24
TFEQ, Three Factor Eating Questionnaire
[1]Factor 1 of the TFEQ
[2]Factor 2 of the TFEQ
[3]Factor 3 of the TFEQ

Experimental design
The study had a randomised single-blind crossover design. Subjects came to the university two times, to stay each time for 36 hours in a respiration chamber to measure energy expenditure and substrate oxidation. For women it was important to be in the same phase of their menstrual cycle (15), so the two stays in the respiration chamber were separated by a period of four weeks. In random order the subjects received one of two diets in the chamber: a 10En% or a 25En% protein diet with casein as the only protein source. The macronutrient distribution for the diets was as follows: 10En% casein diet - 10/35/55En% protein/fat/carbohydrate, 25En% casein diet - 25/20/55En% protein/fat/carbohydrate. The two diets were mainly offered as a custard (one custard with 10En% from casein and one custard with 25En% from casein) produced by NIZO Food Research b.v. (Ede, The Netherlands). The protein content of the custards consisted only of casein (Calcium Caseinate S; DMV International, Veghel, The Netherlands), while the carbohydrate and fat content consisted of respectively tapioca starch (Farinex VA50T; AVEBE, Veendam, The Netherlands and Perfect-amyl 3108; AVEBE, Veendam, The Netherlands) and sunflower-seed oil (Reddy; NV Vandemoortele, Roosendaal, The Netherlands). Both custards were citrus–vanilla flavoured (Citrus, Vanilla; J.B. de Lange, Belfeld, The Netherlands). Extensive product development and use of a taste panel led to custards that did not differ significantly in colour, taste or viscosity. Three days before their stay in the respiration chamber subjects were supplied with a diet at home. This diet had the same macronutrient distribution as the diet they received during the subsequent stay in the respiration chamber, but it consisted of normal food products and various protein sources. During the stay in the respiration chamber blood samples, (24-hour) urine samples and appetite scores on Visual Analogue Scales (VAS) were obtained.

Energy intake
Calculations for both the diet at home and the diet in the respiration chamber were based on average daily energy requirements. The daily energy requirement for the diet at home was estimated as 1.75 times the Basal Metabolic Rate (BMR) (16). BMR was calculated with the formula of Harris-Benedict (17). The energy requirement in the respiration chamber was estimated as 1.35 times the BMR. Daily energy intake was divided over three meals: breakfast 20%, lunch 40%, and dinner 40%. Breakfast was given at 0900h, lunch at 1345h and dinner at 1930h.

Energy expenditure and substrate oxidation
Subjects stayed in the respiration chambers from 2000h in the evening of the third day of their diet at home (day 3) until 0800h in the morning of day 5. The respiration chamber is a 14 m^3 room, furnished with a bed, chair, desk with computer, TV, DVD-player, video recorder, telephone, intercom, sink and toilet. During the 36-hour stay in the respiration chamber oxygen (O_2) consumption and carbon dioxide (CO_2) production were measured. The room was

ventilated with fresh air at a rate of 70 – 80 L/min. Flow was measured using electronically modified dry gasmeters (G6, gasmeterfabriek Schlumberger, Dordrecht, The Netherlands). The concentrations of oxygen and carbon dioxide were measured with dual pairs of infrared carbon dioxide analysers (ABB/Hartman&Braun Uras, Frankfurt a.M., Germany) and paramagnetic oxygen analysers (Servomex 4100, Crowborough, England and ABB/Hartman&Braun Magnos, Frankfurt a.M., Germany). During each 15-min period, 6 samples of outgoing air for each chamber, 1 sample of fresh air, zero gas, and calibration gas were measured. The gas samples to be measured were selected by a computer that also stored and processed the data (18). With the exception of strenuous exercise and sleeping, subjects were allowed to move freely from 0700-2300h. Total energy expenditure over 24 hours (TEE) and 24-hour respiratory quotient (RQ) were calculated from 0730h on the first morning until 0730h on the second morning in the respiration chamber. A radar system based on the Doppler principle was used to measure the physical activity of the subjects in the chamber. The following components of energy expenditure were calculated: sleeping metabolic rate (SMR), diet induced thermogenesis (DIT), resting metabolic rate (RMR), and activity induced energy expenditure (AEE). TEE was calculated by the following formula of Carpenter, as published by Brouwer (19): TEE (kJ/day) = $+16*O_2$ (L/day) + $5*CO_2$ (L/day) - 0.95*P (= oxidized protein in g/day). SMR was calculated by assessing the lowest mean activity of the subjects during three consecutive hours between 0000h and 0700h during the second night of their stay in the respiration chamber. Sleeping metabolic rate was the mean energy expenditure during the three consecutive hours in which activity was the lowest. RMR was calculated by plotting energy expenditure (y-axis) against radar output (x-axis), both being averaged over 30-min intervals of the last 24 hours of the stay in the respiration chamber. By filling in the earlier mentioned lowest mean activity into the formula of the linear regression line of the plot, RMR was calculated. DIT was calculated by subtracting SMR from RMR. AEE was calculated by subtracting RMR from TEE. Substrate oxidation was calculated from 24-hour urinary nitrogen, O_2 consumption and CO_2 production. 24-Hour urine was collected from the second voiding on day 4 until the first voiding on day 5. To prevent nitrogen loss through evaporation, 24-hour urine was collected in containers with 10 mL H_2SO_4, while total volume was measured afterwards. Nitrogen concentrations were measured with a nitrogen analyzer (CHN-O-Rapid; Heraeus, Hanau, Germany). Protein oxidation (P, gram/day) was calculated by multiplying the 24-hour urinary nitrogen (gram/day) with 6.25. Carbohydrate (C) and fat oxidation (F) were calculated with the following formula of Carpenter, as published by Brouwer (19):

C (gram/day) = $- 2.97*O_2$ (L/day) + $4.17*CO_2$ (L/day) - 0.39*P (gram/day)

F (gram/day) = $+1.72*O_2$ (L/day) - $1.72*CO_2$ (L/day) - 0.32*P (gram/day)

Blood sampling

At the first morning of their stay in the respiration chamber (day 4) a Venflon catheter was placed in the antecubital vein for blood sampling. Blood samples were drawn 15 minutes before each meal, and 45 and 75 minutes after each meal (0845h/t-15, 0945h/t45, 1015h/t75, 1330h/t270, 1430h/t330, 1500h/t360, 1915h/t615, 2015h/t675, 2045h/t705) for measurements of glucose, insulin, ghrelin, glucagon-like peptide 1 (GLP-1) and peptide-tyrosine-tyrosine (PYY) plasma concentrations. The blood for insulin, glucose and ghrelin analysis was collected in EDTA tubes. For PYY analysis, blood was collected in EDTA tubes in which dipeptidyl peptidase IV inhibitor (10µl/mL blood) and aprotinin (500 KIU/mL blood) was added. The blood for GLP-1 was collected in EDTA tubes with added dipeptidyl peptidase IV inhibitor (10µl/mL blood). After collection of the blood in the tubes, blood samples were immediately centrifuged for 10 minutes at 4°C, 3000 rpm. For ghrelin analysis phenylmethylsulfonyl fluorid solved in methanol and hydrochloric acid were added to the plasma. Plasma samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Plasma concentrations of insulin, PYY and active ghrelin were measured by RIA (Linco Research Inc., St Charles, MO, USA). Plasma glucose concentrations were determined using the hexokinase method (Glucose HK 125 kit, ABX diagnostics, Montpellier, France). Plasma active GLP-1 concentrations were analysed by ELISA (EGLP-35K, Linco Research Inc., St Charles, MO, USA).

Appetite profile

During day 4 before and after each meal appetite profiles were scored at the following time points: 0900h (t0), 0930h (t30), 1000h (t60), 1030h (t90), 1100h (t120), 1200h (t180), 1300h (t240), 1345h (t285), 1415h (t315), 1445h (t345), 1515h (t375), 1545h (t405), 1645h (t465), 1745h (t525), 1930h (t630), 2000h (t660), 2030h (t690), 2100h (t720), 2130h (t750), 2230h

(t810). Appetite was scored by 100 mm anchored Visual Analogue Scales (VAS). Four questions were asked, anchored with 'not at all' to 'extremely', namely "How satiated do you feel?", "How full do you feel?", "How hungry are you?", and "How is your desire to eat?".

Body composition
Body composition was determined by the 3 compartment model, with the use of hydrodensitometry and the deuterium dilution (^2H_2O) technique (20, 21), and was calculated by using the combined equation of Siri (22).

Statistical analysis
Data from energy expenditure and substrate balances are presented as means ± SEM, while appetite scores and blood data are presented as mean changes from baseline (Δ) ± SEM, unless otherwise indicated. The area under/above the curve (AUC/AAC) of the changes over time (0900-2230h of appetite scores, 0845-2045h of blood parameters) were calculated using the trapezoidal method. A 2-factor repeated-measures ANOVA was carried out for determination of possible differences between the 25En% and 10En% casein diet. To determine relationships between variables regression analyses were performed. The level of statistical significance was set at P<0.05. Statistical analyses were performed by using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Total energy expenditure was increased with 2.6% on the 25En% casein diet compared with the 10En% casein diet; respectively 9.30±0.24 vs. 9.07±0.24 MJ/day (P<0.01). The components of TEE, SMR and RMR were respectively 6.74±0.16 vs. 6.48±0.17 MJ/day (P<0.001) and 7.44±0.17 vs. 7.24±0.18 MJ/day (P<0.01) on the 25En% casein diet compared with the 10En% casein diet. DIT and AEE were not significantly different between the two diets. DIT was respectively 0.70±0.06 vs. 0.76±0.09 MJ/day (NS) and AEE was respectively 1.86±0.11 vs. 1.83±0.11 MJ/day (NS) on the 25En% casein diet compared with the 10En% casein diet. Radar counts (activity) during sleep were not different between the two diets (p=0.27). TEE and SMR for both diets for each subject are plotted in **Figure 1** in two graphs with the line of identity.

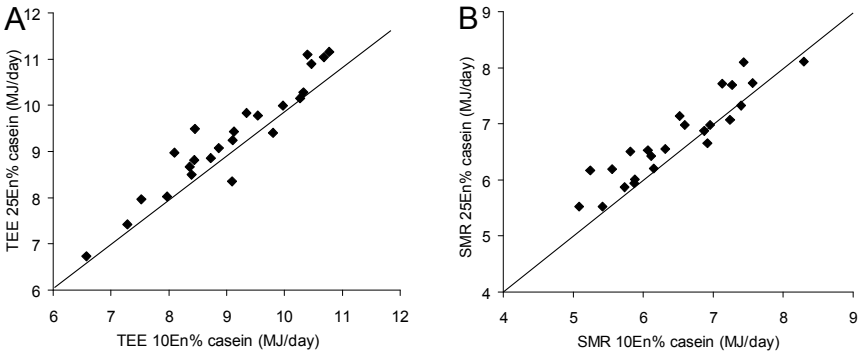

Figure 1 Total energy expenditure over 24 h (TEE; A) and sleeping metabolic rate (SMR; B) for each subject on a diet with 25% of energy (25En%) from casein compared with a diet with 10% of energy (10En%) from casein (n=24). The lines of identity are shown. Two-factor repeated-measures ANOVA was used to determine differences between the 25En% and 10En% casein diets for TEE (mean±SEM: 9.30±0.24 and 9.07±0.24 MJ/d, respectively; P<0.01) and SMR (mean±SEM: 6.74±0.16 and 6.48±0.17 MJ/d, respectively; P<0.001).

Subjects were slightly in a positive energy balance on both diets (p<0.0001, **Figure 2A**). On the 25En% casein diet subjects were in a significantly lower positive energy balance compared with the 10En% casein diet (P<0.01). With respect to macronutrient balances, both the protein and fat balances were significantly different between the two diets (P<0.0001), while carbohydrate balances were not (**Figure 2B**). The 25En% casein diet resulted in a positive protein balance (p<0.0001), a negative fat balance (p<0.0001) and a positive carbohydrate balance (p<0.0001), while the 10En% casein diet resulted in a negative protein balance (p<0.05), fat balance and a positive carbohydrate balance (p<0.0001). RQ did not differ between both diets, which was

0.87±0.00 on the 25En% casein diet and 0.86±0.00 on the 10En% casein diet. On the 25En% casein diet, protein oxidation was higher (P<0.0001) and fat oxidation was lower (P<0.0001), while carbohydrate oxidation was similar compared with the 10En% casein diet (**Table 2**).

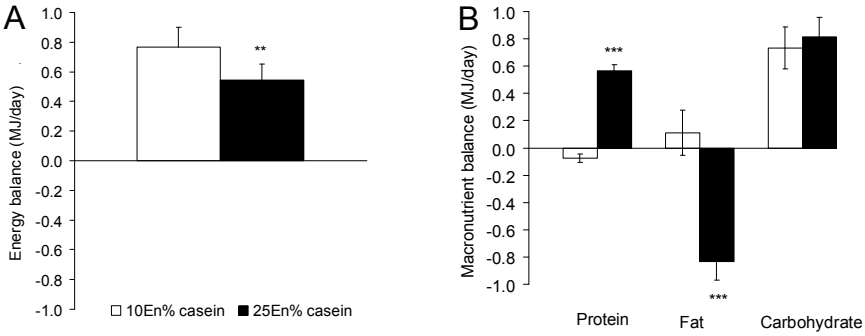

Figure 2 Mean (±SEM) differences in 24-h energy balances (A) and 24-h macronutrient balances (B) between the diet with 25% of energy (25En%) from casein (■) and the diet with 10% of energy (10En%) from casein (□) (n=24) determined by 2-factor repeated-measures ANOVA, **P<0.01, ***P<0.0001. Mean (±SEM) protein balances with the 25En% casein and 10En% casein diets were 30.7±2.6 vs. -4.1±1.7 g/d, respectively. Differences between intake and oxidation or expenditure for each diet (2-factor repeated-measures ANOVA) were as follows: 10En% casein diet [positive energy balance (P<0.0001), negative protein balance (P<0.05), fat balance (NS), positive carbohydrate balance (P<0.0001)] and 25En% casein diet [positive energy balance (P<0.0001), positive protein balance (P<0.0001), negative fat balance (P<0.0001), positive carbohydrate balance (P<0.0001)].

Table 2 Macronutrient intake and expenditure/oxidation measured over 24 h in a respiration chamber

	Macronutrient intake						Expenditure/oxidation					
	10En% casein diet			25En% casein diet			10En% casein diet			25En% casein diet		
Energy (MJ/day)	9.83	±	0.26	9.84	±	0.26	9.07	±	0.24	9.30	±	0.24[1]
Protein (MJ/day)	1.01	±	0.03	2.53	±	0.07[2]	1.09	±	0.04	1.96	±	0.07[2]
Protein (g/day)	54.9	±	1.5	137.3	±	3.6[2]	59.0	±	2.1	106.6	±	3.9[2]
Fat (MJ/day)	3.51	±	0.09	2.01	±	0.05[2]	3.40	±	0.16	2.84	±	0.16[1]
Carbohydrate (MJ/day)	5.31	±	0.14	5.31	±	0.14	4.57	±	0.21	4.49	±	0.15

Values are means ± SEM, n=24
25En%, diet with 25% of energy from casein; 10En%, diet with 10% of energy from casein
[1,2] Significantly different from the 10En% casein diet (2-factor repeated-measures ANOVA): [1]P<0.01, [2]P<0.0001

Hunger was significantly more suppressed on the 25En% casein diet compared with the 10En% casein diet for AAC as well as at various time points for hunger ratings over time (P<0.05, **Figure 3**). With respect to hunger ratings, significant differences of 41% AAC VAS were present. Ratings for desire to eat and hunger were similar, data not shown. In accordance with the hunger ratings subjects reached higher satiety on the 25En% casein diet compared with the 10En% casein diet for AUC as well as at various time points for satiety ratings over time (P<0.05). A significant difference in satiety ratings of 33% AUC VAS was present. Ratings for fullness and satiety were similar, data not shown. In the 25En% condition changes in satiety and changes in hunger were related to changes in SMR (respectively r^2 = 0.261 and r^2 = 0.205, P<0.05). With an increased SMR, satiety was increased while hunger was decreased. Both relationships were not present in the 10En% condition.

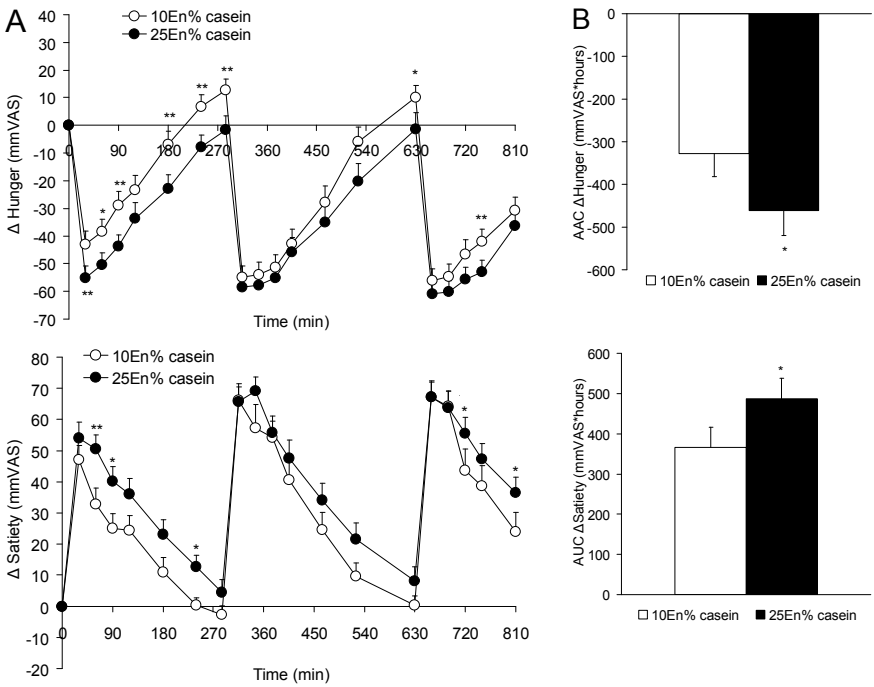

Figure 3 Mean (±SEM) changes from baseline (A) and area above the curve (AAC) and area under the curve (AUC; B) for hunger and satiety scores for the diet with 25% of energy (25En%) from casein and the diet with 10% of energy (10En%) from casein measured with anchored 100-mm visual analogue scales (VAS), n=24. Two-factor repeated-measures ANOVA, * P<0.05, ** P<0.01. For the changes from baseline, significant differences between the 2 diets at the same time point are indicated at various time points.

For both diets glucose, insulin, GLP-1 and PYY plasma concentrations increased after each meal, while ghrelin plasma concentrations decreased (**Figure 4**). Comparing the 25En% casein diet with the 10En% casein diet, glucose concentrations were significantly lower after lunch and dinner, while insulin concentrations were significantly lower after dinner. For glucose the AUC of the plasma concentrations on the 25En% casein diet was significantly lower compared with the 10En% casein diet, respectively 5.1±1.7 vs. 7.3±1.5 mmol/L*hours (P<0.05). After each meal GLP-1 concentrations were significantly lower on the 25En% casein diet compared with the 10En% casein diet, while PYY concentrations were significantly lower after breakfast and lunch. Ghrelin concentrations were not significantly different between the two diets. No correlations between these blood parameters and appetite scores were found.

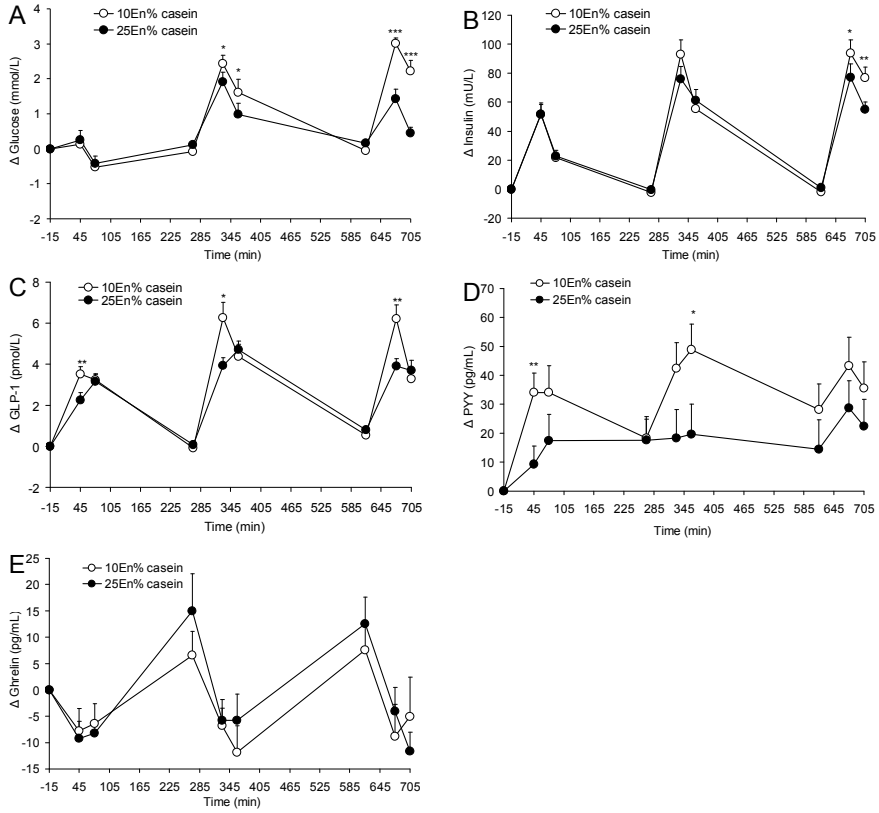

Figure 4 Mean (±SEM) changes from baseline in plasma glucose (A), insulin (B), glucagon-like peptide-1 (GLP-1; C), peptide YY (PYY; D), and ghrelin (E) concentrations for the diet with 25% of energy (25En%) from casein and the diet with 10% of energy (10En%) from casein, n=24. Significant differences between the 2 diets at the same time point are indicated at various time points (2-factor repeated-measures ANOVA): *P<0.05, **P<0.01, ***P<0.0001.

DISCUSSION

After being on a 25En% protein diet for 3 days, a 25En% casein diet on day 4 resulted in a 2.6% higher TEE and higher SMR compared with an isoenergetic 10En% casein diet on day 4 after being on a 10En% protein diet for 3 days. With respect to macronutrient balances, a 25En% casein diet resulted in a positive protein and a negative fat balance compared with the negative protein balance and fat balance on a 10En% casein diet, while carbohydrate balances were the same. On a 25En% compared with a 10En% casein diet hunger was 41% more suppressed and satiety 33% increased. These results were based on isoenergetic diets, indicating that the observed effects were only due to differences in macronutrient composition between the diets.

Increasing the casein content of the diet, while reducing the fat content of the diet and keeping the carbohydrate content the same, resulted in an increase of 2.6% in TEE. Between the 25En% and the 10En% casein diet, protein was exchanged with fat in an energy amount of 1.52MJ/d. The difference in TEE was an increase of 0.23MJ/day on the 25En% casein diet, with similar AEE values between the two diets. This implies that the high protein diet resulted in an additional thermogenesis of 15% of the exchanged energy amount. This is lower than the thermic effect of protein known from literature, namely 20-30% of protein intake (6). Explanations for an apparently lower thermogenic effect are at first that the difference between

the diets was not only an increase in protein intake, but also a decrease in fat intake on the 25En% casein diet. This resulted thus into a decreased thermic effect of fat. Secondly, the theoretical value of 20-30% is calculated on the basis of complete oxidation of all ingested protein. However, the difference in protein balance between the 25En% and 10En% casein diet resulted in a difference in cumulative protein balance of 0.65 MJ/d. Urea synthesis is an energy consuming process, comprising almost one third of the thermic effect of protein (23). Because not all protein was oxidized with accompanying urea excretion, less energy was needed, resulting in a lower net thermic effect.

The higher TEE was mainly due to a higher SMR. We suggest that the increased SMR in the present study may be partly due to the presence of the thermic effect of the diet, as Reed et al (24) found that the thermic response of food may last for more than 6 hours. Because the subjects were already on a 25En% protein diet 3 days before they consumed the 25En% casein diet, the higher SMR may also be partly the result of an adaptation of the body to a high protein diet with respect to an enhanced protein turnover (25). Due to similarity of radar counts, sleeping quality was not different between the diets. The higher SMR on the 25En% casein diet is in accordance with the observations by Lejeune et al (8) who compared a general high protein diet (30/30/40 En% protein/fat/carbohydrate) with an adequate protein diet (10/30/60 En% protein/fat/carbohydrate).

The differences in macronutrient intakes were reflected in the 24-hour oxidations of the macronutrients. 24-Hour carbohydrate oxidations were the same between both diets, while the 25En% casein diet had a significantly higher 24-hour protein oxidation and a significantly lower 24-hour fat oxidation compared with the 10En% casein diet. This resulted in a positive protein balance and a negative fat balance. From the positive protein balance on the 25En% casein diet we may conclude that the body is still in a positive nitrogen balance after being on a high protein diet for 4 days. Although the body is thought to be in a transitory state of positive nitrogen balance, we expect that the positive protein balance may sustain for a longer period of time, however to a lesser extent. Soenen et al (26) observed that, in energy balance, a 3-months dietary intervention with an increased protein intake resulted in a significant increase in fat free mass of 0.73 kg, independently of change in body weight and with similar physical activity levels during the intervention period. With respect to fat balance, lowering the fat content of the diet together with increasing the protein content resulted in a negative fat balance, similar to the finding by Lejeune et al (8). Therefore a high protein diet plays a role in the stimulation of fat oxidation per se, even in energy balance. Because of protein turnover, the result of an increased protein intake on the 25En% casein diet was a higher protein synthesis compared with protein oxidation, resulting in a positive protein balance, the leverage hypothesis (27). This process may go at the expense of fat, resulting in a higher fat oxidation. The observed negative fat balance on a high protein diet may favour fat loss also in the long term, in line with findings of weight maintenance studies (28, 29). With respect to carbohydrate balances, carbohydrate intake and oxidation were the same between both diets, resulting in carbohydrate balances that did not significantly differ between both diets. Due to a slight positive energy balance excess of energy may partly be stored as glycogen.

A 25En% casein diet on day 4 resulted in 41% lower hunger and 33% higher satiety ratings compared with an isoenergetic 10En% casein diet on day 4. In a previous study (12) we already observed higher satiety ratings during 4 hours after identical vanilla custards at breakfast, coinciding with prolonged elevated concentrations of amino acids, indicating a slower gastric emptying. Thus, prolonged elevated concentrations of amino acids may have contributed to the higher satiety ratings, in line with Mellinkoff's amino static theory (30). Moreover, SMR contributed to the higher satiety and lower hunger ratings on the 25En% casein diet, in that a significant positive relationship was found between satiety and SMR, and a significant inverse relationship between hunger and SMR. The 25En% protein condition also resulted in a higher protein oxidation and higher energy expenditure. In a previous study, Westerterp-Plantenga et al (9) found that differences in DIT correlated with differences in satiety over 24 hours. Based upon three other studies that observed satiety scores under limited oxygen availability conditions (31-33), Westerterp-Plantenga et al suggested that with an increased metabolic rate at rest oxygen availability becomes limiting, which seems to be perceived by the subjects as a reduction in the possibility to eat and therefore rated as an increase in satiety. This suggestion might contribute to explain the correlation found between SMR and appetite scores in the present study.

Although the carbohydrate content of both diets was the same (55En%), glucose plasma concentrations were lower on the 25En% casein diet, probably due to slower gastric emptying.

The same was reflected in the insulin responses, with lower insulin concentrations after dinner on the 25En% casein diet. These results are in accordance with our previous research (12) in which subjects also had lower glucose and insulin responses after the same custards for breakfast. With respect to GLP-1 and PYY a similar pattern was found. Significantly lower GLP-1 and PYY plasma concentrations were observed over time on the 25En% casein meals compared with the 10En% casein meals. Again, this may be the result of delayed gastric emptying. However, the less pronounced hormone responses were not reflected in the appetite scores. These results showed that the physiological responses are not always in line with the perceived satiety related feelings, indicating that the regulation of appetite is a complex process in which different mechanisms (like metabolic rate, (an)orexigenic hormones and protein metabolism) may play a role and in which not only one factor can be hold responsible for the perceived satiety.

This was the first time 24-hour measurements for energy expenditure and macronutrient oxidation were performed for studying differences between two diets with different concentrations of casein (25En% vs. 10En%) and with casein as the only protein source. In addition this study had a high power (24 subjects participated in this research) and a broad BMI range. We conclude that after being on a 25En% protein diet for 3 days, a 25En% casein diet on day 4 boosts energy expenditure, protein balance, satiety and negative fat balance compared with an isoenergetic 10En% casein diet on day 4 after being on a 10En% protein diet for 3 days, thus creating opportunities for body weight management. In the 25En% casein condition, changes in SMR were related to changes in satiety and hunger.

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The author's responsibilities were as follows: AHW designed the experiment, collected the data, analysed the data and wrote the manuscript. MABV assisted with the data collection. AGN designed the experiment. MSW-P designed the experiment, contributed to interpretation of the data, reviewed the manuscript and supervised the project. KRW designed the experiment, contributed to interpretation of the data, reviewed the manuscript and supervised the project. None of the authors had any financial or personal interest in any company or organization sponsoring the research.

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Chapter 4

Comparison of 2 diets with either 25 or 10 energy% gelatin on energy expenditure, substrate balances and appetite profile

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ABSTRACT

Background & Aims: Increasing the protein content of a diet results in increased satiety and energy expenditure. It is unknown to what extent gelatin has such effects. We compared satiety and energy expenditure in subjects on 2 single-protein diets with either 25% or 10% of energy (En%) from gelatin.

Methods: During 36 hours in a respiration chamber, 24 healthy subjects (BMI 22.4±2.4 kg/m^2; age 25±7 y) received subject specific isoenergetic diets: 25En% (25/20/55En%-protein/fat/carbohydrate) and 10En% gelatin diet (10/35/55En%-protein/fat/carbohydrate), in a randomised crossover design. Three days before, subjects consumed a diet with similar macronutrient composition at home.

Results: The 25En% compared with the 10En% gelatin diet resulted in a 2.1±1.0% higher 24-hour energy expenditure (9.20±0.23 vs. 9.01±0.23 MJ/d, P<0.05) and higher sleeping metabolic rate (6.70±0.17 vs. 6.53±0.18 MJ/d, P<0.05), whereas subjects were in a higher protein balance (0.30±0.04 vs. -0.16±0.03 MJ/d, P<0.0001), a lower fat balance (-0.87±0.18 vs. 0.22±0.15 MJ/d, P<0.0001), and a more positive carbohydrate balance (1.33±0.18 vs. 0.88±0.16 MJ/d, P<0.01). Hunger and desire to eat were lower after lunch and dinner on the 25En% compared with the 10En% gelatin diet (P<0.05).

Conclusions: In the short-term a 25En% compared with a 10En% gelatin diet boosts energy expenditure, promotes a positive protein and negative fat balance, and reduces hunger.

KEYWORDS: energy metabolism, substrate oxidation, high protein diet, appetite, (an)orexigenic hormones

INTRODUCTION

Obesity is a major health concern worldwide and treatment for this problem is necessary (1). Body weight management requires a multifactorial approach, because several pathways are involved in the system of body weight regulation. Recent findings suggest that an elevated protein intake affects both short- and long-term mechanisms by increasing satiety, despite similar or lower energy intakes, via increased thermogenesis, increased storage of fat free mass, and lower energy efficiency during overfeeding (2-5). The measured thermic effect of the 3 separate macronutrients is the highest for protein (20-30%), followed by carbohydrate (5-10%), and fat (0-3%) (6). Previous studies found a higher diet-induced thermogenesis (DIT) (7, 8), also known as thermic effect of the diet, and a higher sleeping metabolic rate (SMR) (8) during a relatively high-protein diet. Moreover, proteins are highly satiating, because of increases in energy expenditure and anorexigenic hormone concentrations (2, 5, 9, 10).
Currently, it is not clear to what extent these effects hold for one type of protein, since the satiating properties of dietary protein seem to depend on the protein source (11, 12). With respect to gelatin, we observed higher satiety and lower hunger ratings 180 minutes after a breakfast with 10% of energy (10En%) from gelatin compared with 10En% protein breakfasts with casein, whey, whey without glycomacropeptide or soy as the only protein source (13). In addition, we observed a lower energy intake after a 10En% as well as a 25En% gelatin breakfast compared with respectively 10En% and 25En% protein breakfasts with casein, whey without glycomacropeptide or soy as the only protein source (13). Therefore gelatin seems to be an interesting protein as part of a diet.
Gelatin is lacking the essential amino acids tryptophan and cystine and is deficient in for instance methionine, isoleucine, tyrosine and histidine and thus gelatin is regarded as an incomplete protein. The amino acid composition of a protein may be an important determinant for the metabolic efficiency of protein oxidation. Because amino acid catabolism results in a wide variety of carbon chains and cofactors, large differences exist with respect to the efficiency by which amino acids are oxidized (14). Taking into account the amino acid composition of gelatin and the energy expended for ATP synthesis for each amino acid (14), i.e. 15.6 kJ/mol ATP/g for gelatin compared with other proteins ranging from 8.2 kJ/mol ATP/g for casein to 10.9 kJ/mol ATP/g for pork, and therefore we hypothesize that a relatively high energy expenditure is needed for gelatin oxidation.
Moreover, we investigated the satiating properties of gelatin, as the only protein source, over 24 hours in combination with the effects on energy balance. Since gelatin is an incomplete protein, and thus cannot be used as a single-protein source in a diet, this study merely aims to investigate the theoretical proof of concept of the effects of gelatin on energy expenditure and appetite. Energy expenditure over 24 hours and satiety were measured at the same time during a respiration chamber study. We investigated 2 diets in which we exchanged energy from protein (25En% compared with 10En% gelatin) with energy from fat (20En% compared with 35En%), whereas the carbohydrate content (55En%) remained the same. The carbohydrate content was the same with both diets because ingestion of this nutrient results in insulin secretion, and insulin is involved in protein metabolism (15).
The aim of this study was to compare the effects of a 25En% gelatin diet with those of a 10En% gelatin diet on energy expenditure, substrate balance, appetite profile and relevant blood variables to determine a possible effect of one single protein, namely gelatin, and to determine the magnitude of the difference.

SUBJECTS & METHODS

Subjects

Thirty subjects with an age between 18 and 55 years and a body mass index (BMI) between 20 and 33 kg/m^2 were recruited by advertisements on notice boards of Maastricht University and in local newspapers. Recruitment started on 1 September 2006. The experiments began in October 2006. Subjects underwent a medical screening and 24 subjects (12 male, 12 female) were selected on the following inclusion criteria: good health, non-smokers, not using medication (except the use of contraceptive), no cow milk allergy, at most moderate alcohol users, stable in weight during the last 3 months, not following a diet and not cognitively dietary restrained. A Dutch translation of the Three Factor Eating Questionnaire (TFEQ) was used for assessing eating behaviour of the subjects (16). The power calculation of this study was based on DIT measured in a respiration chamber under high and adequate protein feeding conditions

as published by Lejeune et al (8). In this study, DIT values were 0.91±0.25 MJ/d and 0.69±0.24 MJ/d on high protein and adequate protein diets respectively. With a sensitivity of 0.80 and a significance level of 0.05, power calculation (within subjects, two-tailed) revealed a sample size of 12 for each group. The power calculation from our previous study (17) on satiety with casein custard for breakfast revealed, with a sensitivity of 0.80 and a significance level of 0.05, a sample size of 24 in total. Subject characteristics are presented in **Table 1**. Subjects signed an informed consent before participating in the study. The study protocol was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Table 1 Subject characteristics

	Value		
Gender (M/F)	12/12		
Age (y)	25	±	7
Height (m)	1.75	±	0.09
Body weight (kg)	68.5	±	10.5
BMI (kg/m^2)	22.4	±	2.4
Fat mass (kg)	15.4	±	6.7
Fat-free mass (kg)	53.0	±	9.3
TFEQ1 (cognitive restraint, range 0-21)[1]	6	±	3
TFEQ2 (disinhibition, range 0-14)[2]	4	±	2
TFEQ3 (hunger, range 0-14)[3]	5	±	3

Values are means ± SD, n=24
TFEQ, Three Factor Eating Questionnaire
[1]Factor 1 of the TFEQ
[2]Factor 2 of the TFEQ
[3]Factor 3 of the TFEQ

Experimental design

The study had a randomised single-blind crossover design. Subjects came to the university two times, to stay each time for 36 hours in a respiration chamber to measure energy expenditure and substrate oxidation. For women it was important to be in the same phase of their menstrual cycle (18), thus the two stays in the respiration chamber were separated by a period of four weeks. In random order the subjects received 1 of 2 isoenergetic diets in the chamber: a 10En% or a 25En% protein diet with gelatin as the only protein source. The macronutrient distribution for the diets was as follows: 10En% gelatin diet - 10/35/55En% protein/fat/carbohydrate; 25En% gelatin diet - 25/20/55En% protein/fat/carbohydrate. The two diets were mainly offered as a custard (one custard with 10En% from gelatin and one custard with 25En% from gelatin) produced by NIZO Food Research b.v. (Ede, The Netherlands). The protein content of the custards consisted only of gelatin (Solugel LMC/3, PB Gelatins GmbH, Nienburg/Weser, Germany), whereas the carbohydrate and fat content consisted of tapioca starch (Farinex VA50T and Perfect-amyl 3108; AVEBE, Veendam, The Netherlands) and sunflower-seed oil (Reddy; NV Vandemoortele, Roosendaal, The Netherlands) respectively. The amino acid composition of gelatin, the indispensible amino acid requirements based on the WHO report of 2007 (19), and the calculated chemical score of gelatin [5] are shown in **Table 2**. Both custards were citrus–vanilla flavoured (Citrus, Vanilla; J.B. de Lange, Belfeld, The Netherlands). Extensive product development by food technology and use of a taste panel led to custards that did not differ significantly in colour, taste, viscosity and energy density. Three days before their stay in the respiration chamber subjects were supplied with a diet at home. This diet had the same macronutrient distribution as the diet they received during the subsequent stay in the respiration chamber, but it consisted of normal food products and various protein sources. During the stay in the respiration chamber blood samples, (24-hour) urine samples and appetite scores on visual analogue scales (VAS) were obtained.

Table 2 Amino acid composition of gelatin, indispensible amino acid (IAA) requirements (WHO report 2007[19]) and chemical score of gelatin

	Gelatin (g/100 g protein)	IAA requirement (mg/g protein)	Chemical score (%)
Methionine	0.85	16	53
Aspartic acid[a]	5.6		
Hydroxyproline	12.0		
Threonine	1.86	23	81
Serine	3.26		
Glutamic acid[b]	10.1		
Proline	13.9		
Glycine	24.6		
Cystine	0.03	6	**5**[c]
Valine	2.26	39	58
Isoleucine	1.52	30	51
Leucine	2.95	59	50
Tyrosine	0.47	38[d]	62
Phenylalanine	1.87		
γ-amino butyric acid	-		
Histidine	0.91	15	61
Ornithine	0.32		
Alanine	9.3		
Lysine	3.85	45	86
Arginine	8.4		
Thryptophan	<0.10	6	<17

[a] Aspartic acid = asparagine + aspartic acid
[b] Glutamic acid = glutamine + glutamate
[c] Chemical score gelatin
[d] Phenylalanine + tyrosine

Energy intake
Calculations for both, the diet at home and the diet in the respiration chamber, were based on average daily energy requirements. The daily energy requirement for the diet at home was estimated as 1.75 times the basal metabolic rate (BMR) (20). BMR was calculated with the formula of Harris-Benedict (21). The energy requirement in the respiration chamber was estimated as 1.35 times the BMR. Daily energy intake was divided over three meals: breakfast 20%, lunch 40%, and dinner 40%. Breakfast was given at 09:00h, lunch at 13:45h and dinner at 19:30h.

Energy expenditure and substrate oxidation
Subjects stayed in the respiration chambers from 20:00h in the evening of the third day of their diet at home (day 3) until 08:00h in the morning of day 5. The respiration chamber is a 14 m^3 room, furnished with a bed, chair, desk with computer, TV, DVD-player, video recorder, telephone, intercom, sink and toilet. During the 36-hour stay in the respiration chamber oxygen (O_2) consumption and carbon dioxide (CO_2) production were measured. The room was ventilated with fresh air at a rate of 70 – 80 L/min. Flow was measured using electronically modified dry gasmeters (G6, gasmeterfabriek Schlumberger, Dordrecht, The Netherlands). The concentrations of oxygen and carbon dioxide were measured with dual pairs of infrared carbon dioxide analysers (ABB/Hartman&Braun Uras, Frankfurt a.M., Germany) and paramagnetic oxygen analysers (Servomex 4100, Crowborough, England and ABB/Hartman&Braun Magnos, Frankfurt a.M., Germany). During each 15-min period, 6 samples of outgoing air for each chamber, 1 sample of fresh air, zero gas, and calibration gas were measured. The gas samples to be measured were selected by a computer that also stored and processed the data (22). With the exception of strenuous exercise and sleeping, subjects were allowed to move freely from

07:00-23:00h. Total energy expenditure over 24 hours (TEE) and 24-hour respiratory quotient (RQ) were calculated from 07:30h on the first morning until 07:30h on the second morning in the respiration chamber. A radar system based on the Doppler principle was used to measure the physical activity of the subjects in the chamber. The following components of energy expenditure were calculated: SMR, DIT, resting metabolic rate (RMR), and activity induced energy expenditure (AEE). TEE was calculated by the following formula of Carpenter, as published by Brouwer (23): TEE (kJ/day) = +16*O_2 (L/day) + 5*CO_2 (L/day) - 0.95*P (P = oxidized protein in g/d). SMR was calculated by assessing the lowest mean activity of the subjects during three consecutive hours between 00:00h and 07:00h during the second night of their stay in the respiration chamber. Sleeping metabolic rate was the mean energy expenditure during the three consecutive hours in which activity was the lowest. RMR was calculated by plotting energy expenditure (y-axis) against radar output (x-axis), both being averaged over 30-min intervals of the last 24 hours of the stay in the respiration chamber. By filling in the earlier mentioned lowest mean activity into the formula of the linear regression line of the plot, RMR was calculated. DIT was calculated by subtracting SMR from RMR. AEE was calculated by subtracting RMR from TEE. Substrate oxidation was calculated from 24-hour urinary nitrogen, O_2 consumption and CO_2 production. 24-Hour urine was collected from the second voiding on day 4 until the first voiding on day 5. To prevent nitrogen loss through evaporation, 24-hour urine was collected in containers with 10 mL H_2SO_4 (sulfuric acid), whereas total volume was measured afterwards. Nitrogen concentrations were measured with a nitrogen analyzer (CHN-O-Rapid; Heraeus, Hanau, Germany). Protein oxidation (P, gram/day) was calculated by multiplying the 24-hour urinary nitrogen (gram/day) with 6.25. Carbohydrate (C) and fat oxidation (F) were calculated with the following formulas of Carpenter, as published by Brouwer (23):

C (gram/day) = - 2.97*O_2 (L/day) + 4.17*CO_2 (L/day) - 0.39*P (gram/day)

F (gram/day) = +1.72*O_2 (L/day) - 1.72*CO_2 (L/day) - 0.32*P (gram/day)

Blood sampling

At the first morning of their stay in the respiration chamber (day 4) a Venflon catheter was placed in the antecubital vein for blood sampling. Blood samples were drawn 15 minutes before each meal, and 45 and 75 minutes after each meal (08:45h/t-15, 09:45h/t45, 10:15h/t75, 13:30h/t270, 14:30h/t330, 15:00h/t360, 19:15h/t615, 20:15h/t675, 20:45h/t705) for measurements of plasma glucose, insulin, ghrelin, glucagon-like peptide 1 (GLP-1) and peptide-tyrosine-tyrosine (PYY) concentrations. The blood for insulin, glucose and ghrelin analysis was collected in EDTA tubes. For PYY analysis, blood was collected in EDTA tubes in which dipeptidyl peptidase IV inhibitor (10µl/mL blood) and aprotinin (500 KIU/mL blood) was added. The blood for GLP-1 was collected in EDTA tubes with added dipeptidyl peptidase IV inhibitor (10µl/mL blood). After collection of the blood in the tubes, blood samples were immediately centrifuged for 10 minutes at 4°C, 3000 rpm. For ghrelin analysis phenylmethylsulfonyl fluorid solved in methanol and hydrochloric acid were added to the plasma. Plasma samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Plasma concentrations of insulin, PYY and active ghrelin were measured by RIA (Linco Research Inc., St Charles, MO, USA). Plasma glucose concentrations were determined using the hexokinase method (Glucose HK 125 kit, ABX diagnostics, Montpellier, France). Plasma active GLP-1 concentrations were analysed by ELISA (EGLP-35K, Linco Research Inc., St Charles, MO, USA).

Appetite profile

During day 4 before and after each meal appetite profiles were scored at the following time points: 09:00h (t0), 09:30h (t30), 10:00h (t60), 10:30h (t90), 11:00h (t120), 12:00h (t180), 13:00h (t240), 13:45h (t285), 14:15h (t315), 14:45h (t345), 15:15h (t375), 15:45h (t405), 16:45h (t465), 17:45h (t525), 19:30h (t630), 20:00h (t660), 20:30h (t690), 21:00h (t720), 21:30h (t750), 22:30h (t810). Appetite was scored by 100 mm anchored visual analogue scales. Four questions were asked, anchored with 'not at all' to 'extremely', namely "How satiated do you feel?", "How full do you feel?", "How hungry are you?", and "How is your desire to eat?".

Body composition

Body composition was determined by the 3 compartment model, with the use of hydrodensitometry and the deuterium dilution (^2H_2O) technique (24, 25), and was calculated by using the combined equation of Siri (26).

Statistical analysis

Data from energy expenditure and substrate balances are presented as means ± SEM, whereas appetite scores and blood data are presented as mean changes from baseline (Δ) ± SEM, unless otherwise indicated. The area under/above the curve (AUC/AAC) of the changes over time (09:00-22:30h of appetite scores, 08:45-20:45h of blood variables) were calculated using the trapezoidal method. A 2-factor repeated-measures ANOVA was carried out for determination of possible differences between the 25En% and 10En% gelatin diet. To determine relationships between variables regression analyses were performed. The level of statistical significance was set at P<0.05. Statistical analyses were performed by using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Total energy expenditure was increased with 2.1±1.0 % on the 25En% gelatin diet compared with the 10En% gelatin diet; 9.20±0.23 vs. 9.01±0.23 MJ/d respectively (P<0.05). SMR was significantly higher on the 25En% gelatin diet compared with the 10En% gelatin diet, 6.70±0.17 vs. 6.53±0.18 MJ/d respectively (P<0.05). RMR, DIT and AEE were not significantly different between the two diets. RMR was 7.38±0.19 vs. 7.22±0.20 MJ/d respectively (not significant, NS), DIT was 0.69±0.06 vs. 0.69±0.08 MJ/d respectively (NS) and AEE was 1.82±0.07 vs. 1.79±0.07 MJ/d respectively (NS) on the 25En% gelatin diet compared with the 10En% gelatin diet. Radar counts (activity) during sleep were not different between the two diets (p=0.60). TEE and SMR for both diets for each subject are plotted in **Figure 1** in two graphs with the line of identity.

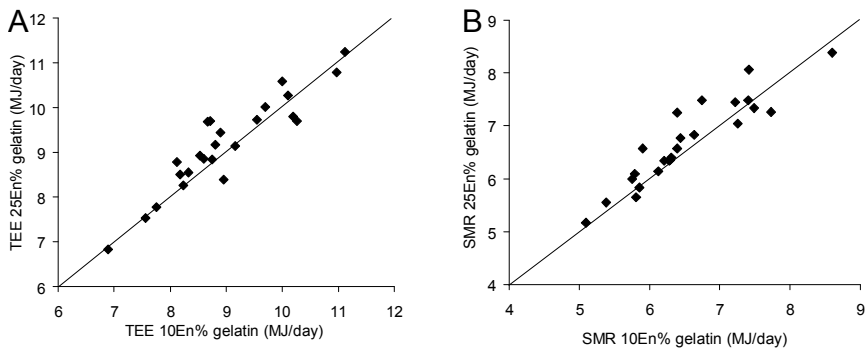

Figure 1 Total energy expenditure over 24-hours (TEE; A) and sleeping metabolic rate (SMR; B) for each subject on a diet with 25% of energy (25En%) from gelatin vs. a diet with 10% of energy (10En%) from gelatin (n=24). Lines represent lines of identity. TEE 25En% gelatin vs. TEE 10En% gelatin (mean±SEM: 9.20±0.23 vs. 9.01±0.23 MJ/d, 2-factor repeated-measures ANOVA, P<0.05); SMR 25En% gelatin vs. SMR 10En% gelatin (mean±SEM: 6.70±0.17 vs. 6.53±0.18 MJ/d, 2-factor repeated-measures ANOVA, P<0.05).

Subjects were slightly in a positive energy balance on both diets (p<0.0001). The difference in energy balance between the two diets just failed to reach significance (P=0.057; **Figure 2A**). With respect to macronutrient balances, the protein, fat and carbohydrate balances were significantly different between the two diets (P<0.0001, P<0.0001 and P<0.01 respectively; **Figure 2B**). The 25En% gelatin diet resulted in a positive protein balance (P<0.0001), a negative fat balance (P<0.0001), and a positive carbohydrate balance (P<0.0001), whereas the 10En% gelatin diet resulted in a negative protein balance (P<0.0001), fat balance (NS) and a positive carbohydrate balance (P<0.0001). On the 25En% gelatin diet, protein oxidation was higher (P<0.0001), whereas fat oxidation and carbohydrate oxidation were lower (P<0.01) compared with the 10En% gelatin diet (**Table 3**). RQ did not differ between both diets, which was 0.86±0.005 on the 25En% gelatin diet and 0.86±0.004 on the 10En% gelatin diet. Proteins, fats and carbohydrates contributed for 24%, 31% and 44% respectively to the RQ of the 25En% gelatin diet, whereas this was 13%, 37% and 50% respectively for the 10En% gelatin diet.

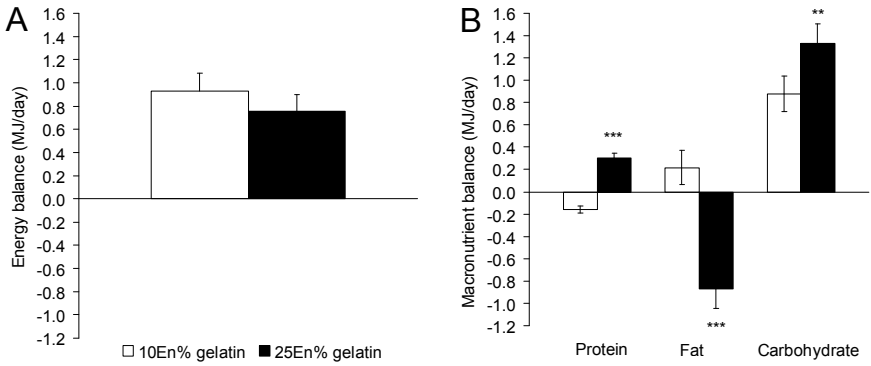

Figure 2 Mean (±SEM) differences in 24-h energy balances (A) and 24-h macronutrient balances (B) between the diet with 25% of energy (25En%) from gelatin (■) and the diet with 10% of energy (10En%) from gelatin (□) (n=24, 2-factor repeated-measures ANOVA, ** P<0.01, *** P<0.0001). Mean (±SEM) protein balance in g/d, 25En% gelatin vs. 10En% gelatin: 16.6±2.1 vs. –8.6±1.8 g/d. Differences between intake and oxidation/expenditure within one diet, 2-factor repeated-measures ANOVA: 10En% gelatin diet: positive energy balance (P<0.0001), negative protein balance (P<0.0001), fat balance (NS), positive carbohydrate balance (P<0.0001); 25En% gelatin diet: positive energy balance (P<0.0001), positive protein balance (P<0.0001), negative fat balance, (P<0.0001), positive carbohydrate balance (P<0.0001).

Table 3 Energy intake/expenditure and macronutrient intake/oxidation measured over 24h in a respiration chamber for subjects on a 25En% and 10En% gelatin diet

	10En% gelatin diet	25En% gelatin diet	10En% gelatin diet	25En% gelatin diet
	Intake	Intake	Expenditure/ Oxidation	Expenditure/ Oxidation
Energy (MJ/d)	9.94 ± 0.29	9.96 ± 0.29	9.01 ± 0.23	9.20 ± 0.23[1]
Protein (MJ/d)	1.02 ± 0.03	2.56 ± 0.08[2]	1.18 ± 0.05	2.25 ± 0.06[2]
Protein (g/d)	55.6 ± 1.6	138.9 ± 4.1[2]	64.2 ± 2.6	122.3 ± 3.0[2]
Fat (MJ/d)	3.55 ± 0.10	2.03 ± 0.06[2]	3.33 ± 0.16	2.90 ± 0.19[3]
Carbohydrate (MJ/d)	5.37 ± 0.16	5.37 ± 0.16	4.49 ± 0.19	4.04 ± 0.17[3]

Values are means ± SEM, n=24
25En%, diet with 25% of energy from gelatin; 10En%, diet with 10% of energy from gelatin
[1,2,3] Significantly different from the 10En% gelatin diet (2-factor repeated-measures ANOVA): [1]P<0.05, [2]P<0.0001, [3]P<0.01

Baseline ratings for hunger and desire to eat were not different between the two diets. Ratings for hunger and ratings for desire to eat were significantly lower at various time points after lunch and dinner on the 25En% gelatin diet compared with the 10En% gelatin diet (P<0.05; **Figure 3**), but no significant differences in AAC for hunger as well as for desire to eat were apparent between the two diets.
Baseline concentrations for the blood variables were not different between the two diets. For both diets plasma glucose, insulin, GLP-1 and PYY concentrations increased after each meal, whereas plasma ghrelin concentrations decreased (**Figure 4**). Glucose and insulin concentrations were significantly lower after dinner on the 25En% gelatin diet compared with the 10En% gelatin diet (P<0.01 or P<0.0001). For glucose the AUC of the plasma concentrations on the 25En% gelatin diet was significantly lower compared with the 10En% gelatin diet, 3.2±1.1 vs. 7.0±1.3 mmol/L*hours respectively (P<0.05). GLP-1, PYY and ghrelin concentrations were not significantly different between the two diets.

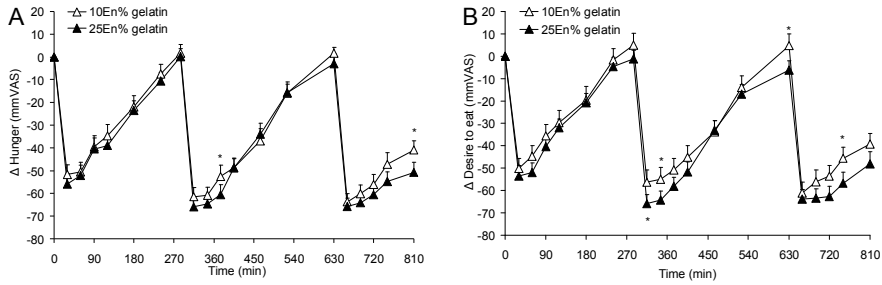

Figure 3 Mean (±SEM) changes from baseline (Δ) of hunger (A) and desire to eat (B) scores for the diet with 25% of energy (25En%) from gelatin (▲) and the diet with 10% of energy (10En%) from gelatin (△) measured with anchored 100-mm visual analogue scales (VAS). Significant differences between the two diets at the same time point are indicated at various time points (n=24, 2-factor repeated-measures ANOVA, * P<0.05).

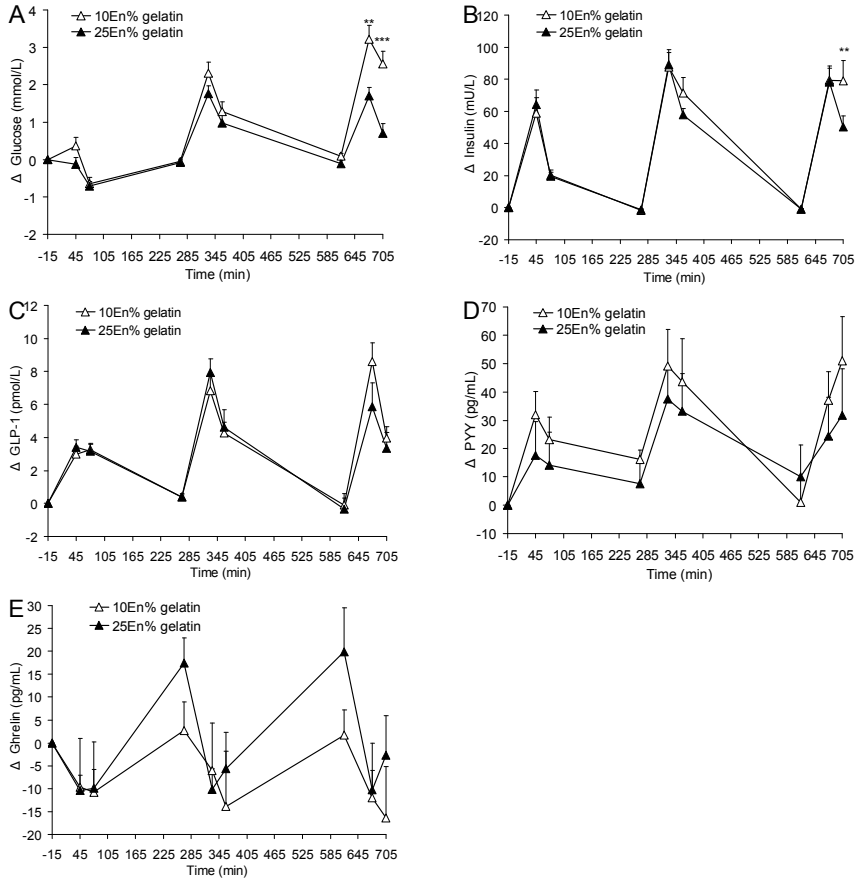

Figure 4 Mean (±SEM) changes from baseline (Δ) of plasma glucose (A), insulin (B), GLP-1 (C), PYY (D) and ghrelin (E) concentrations for the diet with 25% of energy (25En%) from gelatin (▲) and the diet with 10% of energy (10En%) from gelatin (△). Significant differences between the two diets at the same time point are indicated at various time points (n=24, 2-factor repeated-measures ANOVA, **P<0.01, ***P<0.0001)

DISCUSSION

After being on a 25En% protein diet for 3 days, a 25En% gelatin diet on day 4 resulted in a 2.1% higher total energy expenditure and higher sleeping metabolic rate compared with an isoenergetic 10En% gelatin diet on day 4 after being on a 10En% protein diet for 3 days. With respect to macronutrient balances, a 25En% gelatin diet resulted in a higher protein balance, a lower fat balance and a more positive carbohydrate balance compared with the protein, fat and carbohydrate balance on a 10En% gelatin diet. On a 25En% gelatin diet, hunger and desire to eat were lower after lunch and dinner. These findings were based on isoenergetic diets, indicating that the observed effects were only the result of differences in macronutrient composition between the diets.

Increasing the gelatin content of the diet, while reducing the fat content of the diet and keeping the carbohydrate content the same, resulted in an increase of 2.1% in total energy expenditure. Between the 25En% and the 10En% gelatin diet, protein was exchanged with fat in an energy amount of 1.54 MJ/d. The difference in TEE was an increase of 0.19 MJ/d with the 25En% gelatin diet. As AEE values were similar between both diets, this increase in energy expenditure of 0.19 MJ/d can be regarded as an increased thermic effect of the 25En% gelatin diet and is 12% of the 1.54MJ/d of exchanged energy between the two diets. This 12% is lower than the thermic effect of protein known from literature, namely 20-30% of protein intake (6). One explanation for an apparently lower thermic effect of the diet is that the difference between the diets was not only an increase in protein intake, but also a decrease in fat intake on the 25En% gelatin diet. Thus the contribution of fat to the thermic effect of the 25En% gelatin diet was lower than with the 10En% gelatin diet. Secondly, the theoretical thermic effect of 20-30% is calculated on the basis of complete oxidation of all ingested protein. However, the difference in protein balance between the 25En% and 10En% gelatin diet resulted in a difference in cumulative protein balance of 0.46 MJ/d. Urea synthesis is an energy consuming process, which accounts for almost one-third of the thermic effect of protein (27). Because not all protein was oxidized with accompanying urea excretion, less energy was needed, which resulted in a lower net thermic effect.

The higher TEE was mainly due to a higher SMR. We suggest that the increased SMR in the present study may be partly due to the presence of the thermic effect of the diet, as Reed et al (28) found that the thermic response of food may last for more than 6 hours. Because the subjects were already on a 25En% protein diet 3 days before they consumed the 25En% gelatin diet, we speculate that the higher SMR may also be partly the result of an adaptation of the body to a high protein diet with respect to an enhanced protein turnover, as observed in rats, but not yet confirmed in humans (29). Because of the similarity in radar counts, it was concluded that sleeping quality was not different between the diets. The higher SMR on the 25En% gelatin diet is in accordance with the observations by Lejeune et al (8) who executed a similar study with a general high protein diet.

The differences in protein and fat intake between the two diets were reflected in the substrate oxidations. The 25En% gelatin diet had a significantly higher 24-hour protein oxidation and a significantly lower 24-hour fat oxidation compared with the 10En% gelatin diet. This resulted in a positive protein balance and a negative fat balance on the 25En% gelatin diet. Although carbohydrate intake was the same between the two diets, 24-hour carbohydrate oxidation was lower on the 25En% gelatin diet. This resulted in a higher positive carbohydrate balance on the 25En% gelatin diet. Protein oxidation, and thus fat and carbohydrate oxidation, was calculated with the 24-hour urinary nitrogen by using the standard nitrogen-to-protein conversion factor of 6.25. In a review of Mariottti et al (30) they criticize the use of this factor, as it assumes that the nitrogen content of proteins is 16%. However, protein types differ in their nitrogen content. Firstly, as a result from differences in amino acid composition, as the nitrogen content of amino acids can vary considerably. Secondly, the fraction of nitrogenous compounds other than amino acids can vary between protein types. In this review they propose a new set of conversion factors for different protein sources, being 5.55 for gelatin. When using the conversion factor of 5.55 to calculate protein, fat, and carbohydrate oxidations in the present study, compared to the use of 6.25, substrate balances would change by 1) increasing protein balance with +0.13 MJ/d and +0.25 MJ/d, 2) decreasing fat balance with -0.05 MJ/d and -0.09 MJ/d, and 3) decreasing carbohydrate balance with -0.09 MJ/d and -0.17 MJ/d with the 10En% and 25En% gelatin diet respectively. From this point of view protein balance would be less negative/more positive than calculated in the standard way. However, the use of 5.55 as a conversion factor would only be applicable if protein oxidation was totally based upon gelatin oxidation, which is unlikely. On the

other hand, for two reasons protein balance may be lower than calculated. The first reason is that protein oxidation was based on urinary nitrogen only, while nitrogen losses via other ways, for example fecal and dermal nitrogen losses, were not known and thus not taking into account. Therefore, protein oxidation was probably higher than calculated, and protein balance lower. Another reason is that subjects were slightly in a positive energy balance, which could have influenced protein balance due to a higher intake of protein (nitrogen) resulting in a higher protein (nitrogen) balance compared with when being fed in energy balance. Getting back to the results, the negative protein balance in the 10En% protein condition is the result of providing a single-protein diet with a low amount of a protein of very poor quality. From the positive protein balance on the 25En% gelatin diet we may conclude that the body is still in a positive nitrogen balance after being on a high protein diet for 4 days. Soenen et al (31) observed that, in energy balance, a 3-months dietary intervention with an increased protein intake resulted in a significant increase in fat free mass of 0.73 kg, independently of change in body weight and with similar physical activity levels during the intervention period. From their study it is shown that a positive protein balance may sustain for a longer period of time. However, in the study of Soenen et al the protein supplements consisted of complete protein sources, while in the present study gelatin was used as the only protein source. We expect that even a high intake of gelatin in the long-term will not maintain this positive protein balance, as gelatin has a low biological value, which will depress whole body protein synthesis. With respect to fat balance, lowering the fat content of the diet together with increasing the protein content resulted in a negative fat balance. Lejeune et al (8) also observed a negative fat balance in the 30En% protein condition compared with a positive fat balance in the 10En% protein condition, while fat intake was the same. Therefore, a high-protein diet plays a role in the stimulation of fat oxidation per se, even in energy balance. The observed negative fat balance on a high protein diet may favour fat loss in the long term, in line with findings of weight maintenance studies (32, 33). With respect to carbohydrate balances, carbohydrate intake was the same between both diets, whereas carbohydrate oxidation was lower on the 25En% gelatin diet, resulting in a higher positive carbohydrate balance. Because gelatin is an incomplete protein, the lack of and deficiency in several essential amino acids acts as a limiting factor for postprandial protein synthesis. This results in an increase in free amino acids, because they are not used for protein synthesis. Part of these amino acids may be oxidized, but with respect to the 'excess' of protein intake compared with protein oxidation on the 25En% gelatin diet, we suggest that part of the amino acids from the diet were used for gluconeogenesis to be finally stored as glycogen (2).

Although all three macronutrient oxidations were significantly different between the two diets, RQ was not. RQ is the result of a combination of the oxidations of the three macronutrients, i.e. proteins, fats and carbohydrates contributed for 24%, 31% and 44% respectively to the RQ of the 25En% gelatin diet, whereas this was 13%, 37% and 50% respectively for the 10En% gelatin diet, which resulted for both diets in an RQ of 0.86.

With respect to the appetite profile this study shows that hunger was more suppressed and desire to eat was less after lunch and dinner on a 25En% gelatin diet compared with a 10En% gelatin diet. However, no significant differences in the (an)orexigenic hormones GLP-1, PYY and ghrelin were observed between the two diets, so these hormones were not responsible for the perceived satiety related feelings.

Although the carbohydrate content of both diets was with 55En% the same, plasma glucose concentrations were lower on the 25En% compared with the 10En% gelatin diet. Remarkably plasma insulin concentrations were not higher on the 25En% gelatin diet. It is not clear what mechanism is responsible for this observation. However, if the 25En% gelatin diet has the capacity to reduce plasma glucose levels without increased insulin levels, this might be interesting for people with diabetes mellitus, who are dealing with hyperglycemia as a result of insulin deficiency and/or reduced insulin sensitivity (34-36).

Although the 25En% gelatin diet had positive effects on energy expenditure, appetite, protein balance, fat oxidation and glucose and insulin responses, the gelatin diet is merely a theoretical proof of concept. The diet decreased subjective feelings of hunger and increased energy expenditure, however it should be noted that gelatin is an incomplete protein, and therefore it cannot be used as a single-protein source in a diet, because it is deficient in certain essential amino acids. A suggestion may be to use gelatin as a snack, as part of a relatively high protein diet, consisting of complete proteins. This way, overweight people still may benefit from the observed positive effects of gelatin, without lacking the essential amino acids in the diet, thus creating opportunities for body weight management. We conclude that after being on a 25En% protein diet for 3 days, a 25En% gelatin diet on day 4 compared with an isoenergetic 10En%

gelatin diet on day 4, after being on a 10En% protein diet for 3 days, boosts energy expenditure, promotes a positive protein and negative fat balance, and reduces hunger and desire to eat.

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AH-W designed the experiment, collected the data, analysed the data and wrote the manuscript. MABV assisted with the data collection. AGN designed the experiment and reviewed the manuscript. MSW-P and KRW designed the experiment, contributed to interpretation of the data, reviewed the manuscript and supervised the project. All authors read and approved the final manuscript. The research was supported by Top Institute Food and Nutrition, Wageningen, the Netherlands. None of the authors had a financial or personal conflict of interest in any company or organization sponsoring the research.

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Chapter 5

Single-protein casein and gelatin diets affect energy expenditure similarly but substrate balance and appetite differently in adults

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ABSTRACT

Increasing the protein content of a diet results in increased satiety and energy expenditure (EE). It is not clear whether the magnitude of these effects differs between proteins differing in concentrations of indispensable amino acids (IAA). We hypothesized that a protein lacking IAA may stimulate appetite suppression and EE, and may limit positive protein balance. Therefore, we compared appetite, EE, and substrate balances between gelatin (incomplete protein) and casein (complete protein) in single protein diets with either 25% or 10% of energy (En%) from protein. During a 36-h stay in a respiration chamber, 23 healthy men (n=11) and women (n=12) (BMI, 22.2±2.3 kg/m^2; age, 25±7 y) consumed four isoenergetic diets: 25 En% (25/20/55 En% protein/fat/carbohydrate) and 10 En% (10/35/55 En% protein/fat/carbohydrate) casein or gelatin diet, in a randomized crossover design. For 3 d before the study, participants consumed a diet at home with similar macronutrient distribution as the diet they would receive during the subsequent stay in the chamber. Hunger was suppressed 44% more ($P<0.05$) and protein balance was more negative when consuming the 10 En% gelatin (-0.17±0.03 MJ/d) compared with the 10 En% casein diet (-0.07±0.03 MJ/d, $P<0.05$); carbohydrate and fat balances did not differ between the treatments. EE did not differ when participants consumed the 25 En% or 10 En% diets. Participants were in higher protein balance (0.56±0.05 vs. 0.30±0.04 MJ/d, $P<0.0001$), lower carbohydrate balance (0.86±0.14 vs. 1.37±0.17 MJ/d, $P<0.01$), and similar negative fat balance when they consumed the 25 En% casein compared with the 25 En% gelatin diet. In conclusion, when we compared the effects of an incomplete protein (gelatin) and a complete protein (casein) at two concentrations over 36 h, gelatin resulted in a larger appetite suppression; casein caused a larger positive (smaller negative) protein balance, and effects on EE did not differ. In the concept of weight loss for people with obesity, the larger hunger suppressing effect of gelatin may play a role in reducing energy intake, if this effect is maintained when consuming a gelatin diet in the long-term. In addition, long-term use of casein may contribute to preservation of fat free mass.

KEYWORDS: energy metabolism, substrate balance, protein diet, appetite, (an)orexigenic hormones

INTRODUCTION

Obesity is a major health concern worldwide and effective treatment is necessary (1). Body weight management requires a multifactorial approach, because several pathways are involved in the system of body weight regulation. Recent findings suggest that an elevation of protein intake affects both short- and long-term mechanisms (2-7). Firstly, an increased protein intake increases satiety, despite similar or lower energy intake. Secondly, thermogenesis is increased. Thirdly, energy efficiency is lower during overfeeding. Fourthly, fat free mass is preserved. Dietary protein-induced satiety may be due to amino acid composition (6, 8-13), anorexigenic hormone concentrations (6) and energy expenditure (EE) (6, 7).

Currently it is not clear whether such effects differ between proteins differing in concentrations of indispensable amino acids (IAA). Casein and gelatin are two contrasting proteins with respect to their amino acid composition. Casein is a complete protein as it contains all IAA, while gelatin is an incomplete protein as it lacks the essential amino acid tryptophan and contains low amounts of for instance methionine and histidine. Contrasting gelatin and casein is of interest with respect to appetite, EE and protein balance, for the following reasons.

Firstly, in a previous study (11) higher satiety and lower hunger ratings and/or lower energy intake were observed after a single protein breakfast with either 10% or 25% of energy (En%) from gelatin compared with a 10 En% or 25 En% protein breakfast with casein as the only protein source. This may relate to a mechanism observed in metazoans, where it was discovered that the tRNA/GCN2/p-eIF2α system in the brain can detect a deficiency of IAA in the diet from a decline in serum amino acid levels, leading to a behavioral response that rejects consumption of imbalanced diets (14-17), and thus appears as hunger suppression. In addition, the characteristic of casein as a 'slow' protein, indicating that the plasma appearance of dietary amino acids is slower, lower and prolonged after digestion and absorption of casein compared to for example gelatin (18), leading to diminished satiety is relevant as well. Secondly, because amino acid catabolism results in a wide variety of carbon chains and cofactors, large differences exist with respect to the efficacy by which amino acids are oxidized (19). Taking into account the amino acid composition of gelatin and casein, and the energy expended for ATP synthesis (19), being 15.6 $kJ*ATP^{-1}*g^{-1}$ for gelatin and 8.2 $kJ*ATP^{-1}*g^{-1}$ for casein, we hypothesize that gelatin consumption may exert a relatively larger increase in EE. Thirdly, consumption of a complete protein may lead to a more positive protein balance compared with an incomplete protein.

We hypothesize that the use of an incomplete protein (gelatin), compared with a complete protein (casein), in single-protein diets may stimulate appetite suppression and energy expenditure, and may limit a positive protein balance. To test this hypothesis, we aimed to study the effects of gelatin and casein, contrasted in 10En% and 25En% single-protein diets, on appetite profile, EE over 24 hours and substrate balances.

MATERIALS AND METHODS

Participants

The study protocol was approved by the Medical Ethical Committee of the Maastricht University Medical Center. Thirty participants between 18 and 55 years old and a BMI between 20 and 33 kg/m^2 were recruited by advertisements on notice boards of Maastricht University and in local newspapers. Participants underwent a medical screening and 24 participants (12 men, 12 women) were selected on the following inclusion criteria: good health, nonsmokers, no use of medication (except for contraceptives), no cow milk allergy, moderate to no alcohol consumption, stable body weight during the last 3 months, not following a diet and not cognitively dietary restrained. The Dutch translation of the three factor eating questionnaire (TFEQ) was used for assessing eating behavior of the participants (20). Analyses were executed for n=22 (11 men, 11 women) for the comparison between the 10 En% protein diets and n=23 (11 men, 12 women) for the comparison between the 25 En% protein diets, because of dropouts (see **Table 1** for participant characteristics). Participants signed an informed consent form before participating in the study.

Body composition

Body composition was determined according to the 3-compartment model based on body weight, body volume as measured with hydrodensitometry, and total body water as measured with the deuterium dilution (^2H_2O) technique (21, 22), and was calculated by using the combined equation of Siri (23). Body composition measurements were assessed during (deuterium

dilution) and immediately after (body weight and hydrodensitometry) the first stay in the respiration chamber.

Table 1 Participant characteristics

	Value		
Gender (M/F)	11/12		
Age (y)	25	±	7
Height (m)	1.74	±	0.09
Body weight (kg)	67.5	±	9.6
BMI (kg/m^2)	22.2	±	2.3
Fat mass (kg)	15.1	±	6.6
Fat-free mass (kg)	52.4	±	9.0
TFEQ1 (cognitive restraint)[1]	6	±	3
TFEQ2 (disinhibition)[2]	4	±	2
TFEQ3 (hunger)[3]	5	±	3

Values are means ± SD, n=23
TFEQ, Three Factor Eating Questionnaire
[1]Factor 1 of the TFEQ
[2]Factor 2 of the TFEQ
[3]Factor 3 of the TFEQ

Experimental design
The study had a randomized single-blind crossover design. Participants came to the university four times; each time they stayed for 36 hours in a respiration chamber for the measurement of EE and substrate oxidation. For women it was important to be in the same phase of the menstrual cycle (24), so each stay in the respiration chamber was separated by a period of approximately four weeks. In random order the participants received one of the four diets while in the chamber: a 10 En% or a 25 En% protein diet with either casein or gelatin as the only protein source. The macronutrient distribution for the diets was as follows: 10 En% casein diet and 10 En% gelatin diet - 10/35/55 En% protein/fat/carbohydrate, 25 En% casein diet and 25 En% gelatin diet - 25/20/55 En% protein/fat/carbohydrate. Energy from protein (10 En% vs. 25 En%) was exchanged with energy from fat (respectively 35 En% vs. 20 En%), whereas carbohydrate content (55 En%) remained the same, because carbohydrate ingestion results in insulin secretion, and insulin is involved in protein metabolism (25). The four diets were mainly offered as custard (one custard with 10 En% from casein, one custard with 10 En% from gelatin, one custard with 25 En% from casein, and one custard with 25 En% from gelatin) produced by NIZO Food Research b.v. (Ede, The Netherlands). The protein content of the two casein custards consisted only of casein (Calcium Caseinate S; DMV International, Veghel, The Netherlands), whereas the protein content of the gelatin custards consisted only of gelatin (Solugel LMC/3, PB Gelatins GmbH, Nienburg/Weser, Germany) (see **Supplemental Table 1** for amino acid compositions of casein and gelatin. Amino acid compositions were determined as follows: the amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin, using photometric detection. The tryptophan was determined by reversed phase C18 HPLC with fluorescence detection.). The carbohydrate and fat content of all four custards consisted of tapioca starch (Farinex VA50T; AVEBE, Veendam, The Netherlands and Perfect-amyl 3108; AVEBE, Veendam, The Netherlands) and sunflower-seed oil (Reddy; NV Vandemoortele, Roosendaal, The Netherlands) respectively. All custards were citrus–vanilla flavoured (Citrus, Vanilla; J.B. de Lange, Belfeld, The Netherlands). Extensive product development by food technology and use of a taste panel led to custards that did not differ significantly in colour, taste, viscosity and energy density. Three days before their stay in the respiration chamber, the participants were supplied with a diet at home. This diet had the same macronutrient distribution as the diet they received during the subsequent stay in the respiration chamber, but it consisted of normal food products and various protein sources (**Supplemental Table 2** and **3**). During the stay in the respiration chamber blood samples, (24-h) urine samples and appetite scores on visual analogue scales (VAS) were obtained.

Energy intake
Calculations for both the diet at home and the diet in the respiration chamber were based on mean daily energy requirements. The daily energy requirement for the diet at home was estimated by multiplying the basal metabolic rate (BMR) with a physical activity level (PAL) of 1.75, which is the mean PAL for the general population in daily life in the Netherlands (26). BMR was calculated with the equation of Harris-Benedict (27). The energy requirement in the respiration chamber was based on results of a study in which physical activity was determined in confined conditions (a respiration chamber) resulting in a mean PAL of 1.4 (calculated as 24-h EE/sleeping metabolic rate; SMR) (28). The energy requirement in the respiration chamber was estimated as 1.35 times the BMR. Daily energy intake was divided over three meals: 20 % at breakfast, 40 % at lunch, and 40 % at dinner. Breakfast was given at 0900h, lunch at 1345h and dinner at 1930h.

Appetite profile
On day 4, before and after each meal, appetite profiles were scored at the following time points: 0900h (t0), 0930h (t30), 1000h (t60), 1030h (t90), 1100h (t120), 1200h (t180), 1300h (t240), 1345h (t285), 1415h (t315), 1445h (t345), 1515h (t375), 1545h (t405), 1645h (t465), 1745h (t525), 1930h (t630), 2000h (t660), 2030h (t690), 2100h (t720), 2130h (t750), 2230h (t810). Appetite was scored with a 100-mm anchored VAS. Four questions were asked, anchored by 'not at all' to 'extremely', namely "How satiated do you feel?", "How full do you feel?", "How hungry are you?", and "How is your desire to eat?".

Blood sampling
On the first morning of their stay in the respiration chamber (day 4), a Venflon catheter was placed in the antecubital vein for blood sampling. Blood samples were drawn 15 min before each meal, and 45 and 75 min after each meal (0845h/t-15, 0945h/t45, 1015h/t75, 1330h/t270, 1430h/t330, 1500h/t360, 1915h/t615, 2015h/t675, 2045h/t705) for the measurement of plasma glucose, insulin, ghrelin, glucagon-like peptide-1 (GLP-1) and peptide-tyrosine-tyrosine (PYY) concentrations. The blood for insulin, glucose and ghrelin analysis was collected in EDTA tubes. For PYY analysis, blood was collected in EDTA tubes in which dipeptidyl peptidase IV inhibitor (10 mL/L blood) and aprotinin (500,000 KIU/L blood) was added. The blood for GLP-1 was collected in EDTA tubes with added dipeptidyl peptidase IV inhibitor (10 mL/L blood). After the collection of blood into the tubes, blood samples were immediately centrifuged for 10 min at 4°C, 3000 x g. For ghrelin analysis, phenylmethylsulfonyl fluorid dissolved in methanol, and hydrochloric acid were added to the plasma. Plasma samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed further. Plasma concentrations of insulin were measured by RIA (Human Insulin-Specific RIA Kit, Linco Research Inc., St Charles, MO, USA). Plasma concentrations of PYY were measured by RIA (Human PYY (3-36) RIA kit, Linco Research Inc., St Charles, MO, USA). Plasma concentrations of active ghrelin were measured by RIA (Ghrelin (active) RIA kit, Linco Research Inc., St Charles, MO, USA). Plasma glucose concentrations were determined by using the hexokinase method (Glucose HK 125 kit, ABX diagnostics, Montpellier, France). Plasma active GLP-1 concentrations were analyzed by enzyme-linked immunosorbent assay (EGLP-35K, Linco Research Inc., St Charles, MO, USA).

Plasma amino acid concentrations
In a previous study, 24 participants were given the same 10 En% casein, 10 En% gelatin, 25 En% casein and 25 En% gelatin breakfasts and plasma amino acid concentrations were measured during 4 hours after the breakfasts (11).

Energy expenditure and substrate oxidation
Participants stayed in the respiration chambers from 2000h in the evening of the third day of their diet at home (day 3) until 0800h in the morning of day 5. The respiration chamber is a 14 m^3 room, furnished with a bed, chair, desk with computer, TV, DVD-player, video recorder, telephone, intercom, sink and toilet. Measurement of energy expenditure in the respiration chamber was performed as described before (29). With the exception of strenuous exercise and sleeping, participants were allowed to move freely from 0700-2300h. Total energy expenditure over 24 hours (TEE) and 24-h respiratory quotient (RQ) were calculated from 0730h on the first morning until 0730h on the second morning in the respiration chamber. A radar system based on the Doppler principle was used to measure the physical activity of the participants in the chamber. The following components of EE were calculated: SMR, diet induced thermogenesis (DIT), resting metabolic rate (RMR), and activity induced energy expenditure (AEE). TEE was

calculated by the following equation of Carpenter, as published by Brouwer (30): TEE (kJ/d) = +16*O_2 (L/d) + 5*CO_2 (L/d) - 0.95*P, where P is oxidized protein in g/d. SMR was calculated by assessing the lowest mean activity of the participants during three consecutive hours between 0000h and 0700h during the second night of their stay in the respiration chamber. SMR was the mean EE during the three consecutive hours in which activity was the lowest. RMR was calculated by plotting EE (y-axis) against radar output (x-axis), both being averaged over 30-min intervals of the last 24 hours of the stay in the respiration chamber. RMR was calculated by entering the earlier mentioned lowest mean activity into the formula of the linear regression line of the plot. DIT was calculated by subtracting SMR from RMR. AEE was calculated by subtracting RMR from TEE. Substrate oxidation was calculated from 24-h urinary nitrogen, oxygen consumption and carbon dioxide production. Urine samples (24-h) were collected from the second voiding on day 4 until the first voiding on day 5. To prevent nitrogen loss through evaporation, 24-h urine was collected in containers with 10 mL H_2SO_4 (2 mol/L), whereas total volume was measured afterward. Nitrogen concentrations were measured with a nitrogen analyzer (CHN-O-Rapid; Heraeus, Hanau, Germany). Protein oxidation (g/d) was calculated by multiplying 24-h urinary nitrogen (g/d) by 6.25. Carbohydrate (g/d) and fat oxidation (g/d) were calculated with the following equations (30):
Carbohydrate oxidation = - 2.97*O_2 (L/d) + 4.17*CO_2 (L/d) - 0.39*P (g/d)
Fat oxidation = +1.72*O_2 (L/d) - 1.72*CO_2 (L/d) - 0.32*P (g/d)
Energy balance in the respiration chamber was calculated as 24h energy intake minus 24h EE. Substrate balance in the respiration chamber was calculated as 24h substrate intake minus 24h substrate oxidation.

Statistical analysis
Data from EE and substrate balances are presented as means ± SEM, whereas appetite scores and blood data are presented as mean changes from baseline (Δ) ± SEM, unless otherwise indicated. The area under/above the curve (AUC/AAC) of the changes over time (0900-2230h for appetite scores, 0845-2045h for blood variables) were calculated by using the trapezoidal method. A 3-factor ANOVA was carried out to compare men with women for the difference between casein and gelatin under 10 En% as well as under 25 En% protein conditions on all variables. Men and women were not affected differently in any of these variables tested and so their data were combined. A repeated-measures analysis of variance was carried out for determination of possible differences between the 10 En% casein and 10 En% gelatin diets, and between the 25 En% casein and 25 En% gelatin diets on all variables and all time points (for VAS and blood variables). To determine relations between variables regression analyses were performed. The level of statistical significance was set at $P<0.05$. Statistical analyses were performed by using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Energy balance
Participants were slightly in a positive energy balance when they consumed each of the 4 diets ($P<0.0001$). Energy balances did not differ when participants consumed the 10 En% casein (0.82±0.14 MJ/d) and 10 En% gelatin diets (0.87±0.14 MJ/d), or the 25 En% casein (0.56±0.11 MJ/d) and 25 En% gelatin diets (0.70±0.14 MJ/d).

Appetite and plasma hormone concentrations
Baseline ratings for hunger and fullness were not different before each of the diet periods. Hunger was significantly more suppressed when participants consumed the 10 En% gelatin diet compared with the 10 En% casein diet at several time points measured after breakfast and dinner (**Figure 1A**). The AAC for the hunger scores was 44 % lower when participants consumed the 10 En% gelatin diet (-446±43 mmVAS·h) compared with the 10 En% casein diet (-311±54 mmVAS·h, $P<0.05$) (**Figure 1B**). Fullness score was larger at t810 min when participants consumed the 10 En% gelatin diet (38±6 mmVAS) compared with the 10 En% casein diet (25±7 mmVAS, $P<0.05$). The AUC for the fullness scores over time did not differ between the two 10 En% protein diets. When participants consumed the 25 En% gelatin diet compared with the 25 En% casein diet hunger was significantly more suppressed at t810 (-49±4 vs. -34±5 mmVAS, $P<0.01$), but AAC did not differ. Fullness ratings did not differ after they consumed the 25 En% protein diets. Taken together, the gelatin-containing diets, either at a concentration of 10 or 25 En% protein, suppressed hunger more than the casein-containing diets.

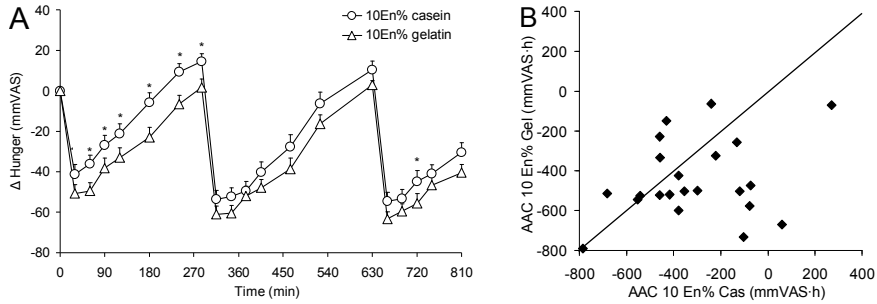

Figure 1

(A) Changes from baseline (Δ) of hunger scores when participants consumed the 10 En% casein diet and the 10 En% gelatin diet measured with anchored 100-mm visual analogue scales. Values are means±SEM, n=22. *Different from the 10 En% gelatin diet at that time, P<0.05.

(B) Area above the curve (AAC) of hunger scores for each participant after consumption of a diet with 10% of energy (En%) from casein vs. a diet with 10% of energy from gelatin. The line of identity is shown, n=22.

When participants consumed the 10 En% gelatin diet, the plasma GLP-1 concentration was significantly higher after dinner compared with after the 10 En% casein diet (P<0.05, **Figure 2A**), whereas plasma PYY (**Figure 2B**), ghrelin (**Figure 2C**), glucose (**Figure 2D**) and insulin (**Figure 2E**) concentrations did not differ after the 2 diet periods. When they consumed the 25 En% gelatin diet compared with the 25 En% casein diet, plasma GLP-1 concentration was significantly higher after lunch (P<0.0001, **Figure 3A**), whereas plasma ghrelin concentrations were significantly lower after lunch and dinner (P<0.05, **Figure 3C**). Plasma PYY (**Figure 3B**), glucose (**Figure 3D**) and insulin (**Figure 3E**) concentrations were not significantly different between the 25 En% casein and 25 En% gelatin diet. Taken together, under 10 En% as well as under 25 En% protein conditions GLP-1 release and/or ghrelin decrease after meals were larger when participants consumed the gelatin diets.

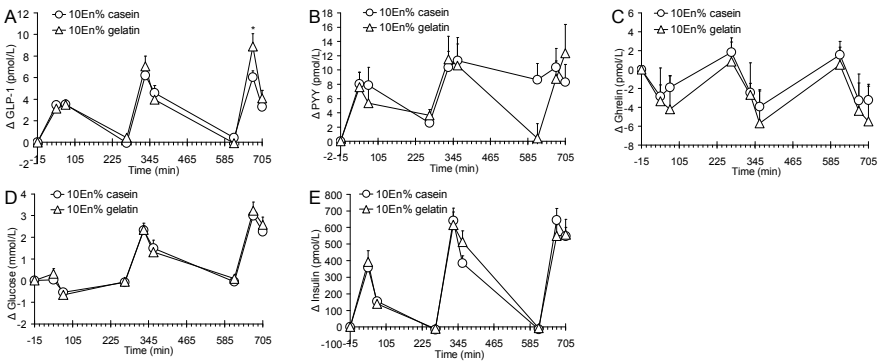

Figure 2 Changes from baseline (Δ) in plasma glucagon-like peptide 1 (GLP-1; A), peptide YY (PYY; B), ghrelin (C), glucose (D) and insulin (E) concentrations when participants consumed the diet with 10% of energy (10 En%) from casein and the diet with 10% of energy from gelatin. Values are means±SEM, n=22. *Different from the 10 En% casein diet at that time, P<0.05.

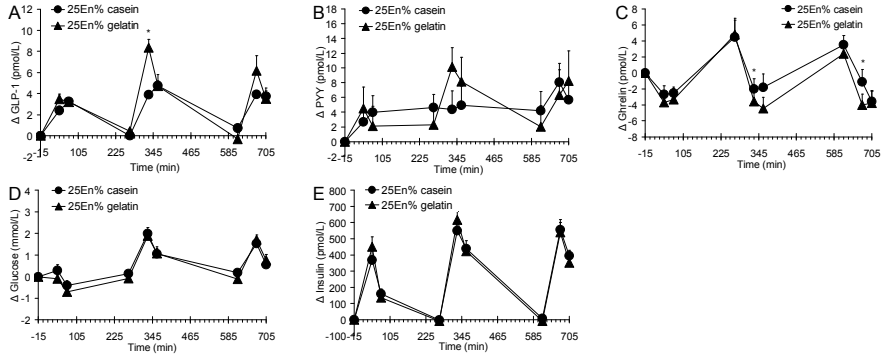

Figure 3 Changes from baseline (Δ) in plasma glucagon-like peptide 1 (GLP-1; A), peptide YY (PYY; B), ghrelin (C), glucose (D) and insulin (E) concentrations when participants consumed the diet with 25% of energy (25 En%) from casein and the diet with 25% of energy from gelatin. Values are means±SEM, n=23. *Difference between the 25 En% casein and 25 En% gelatin diet at that time, P<0.05.

Amino acids

In a previous study, 24 participants were given the same 10 En% casein, 10 En% gelatin, 25 En% casein and 25 En% gelatin breakfasts and plasma amino acid concentrations were measured during 4 hours after the breakfasts (11). The AUC for the changes in plasma amino acid concentrations over 4 hours after consumption of the 10 En% and 25 En% protein breakfasts were calculated (**Figure 4A** and **Figure 4B** respectively). The plasma concentrations of the essential amino acids histidine, valine, methionine, isoleucine, phenylalanine, tryptophan and leucine were decreased, expressed as a negative AUC, and significantly lower (P<0.05) after consumption of the 10 En% gelatin breakfast compared with the 10 En% casein breakfast. Under 25 En% protein conditions, plasma tryptophan concentration was decreased and significantly lower (P<0.05) after consumption of the gelatin compared with the casein breakfast.

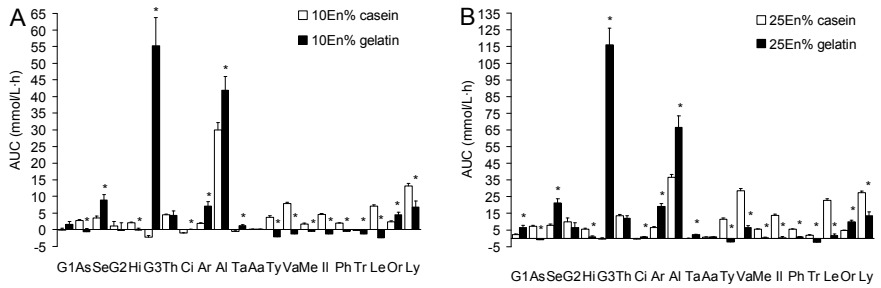

Figure 4 Area under the curve (AUC) for the changes from baseline in plasma amino acid concentrations during 4 h after consumption of a breakfast with 10% of energy (10 En%) from casein or gelatin (A), and after consumption of a breakfast with 25% of energy (25 En%) from casein or gelatin (B). Values are means±SEM, n=24. *Different from the casein diet, P<0.05. G1, glutamate; As, asparagines; Se, serine; G2, glutamine; Hi, histidine; G3, glycine; Th, threonine; Ci, citrulline; Ar, arginine; Al, alanine; Ta, taurine; Aa, alpha-aminobutyric acid; Ty, tyrosine; Va, valine; Me, methionine; Il, isoleucine; Ph, phenylalanine; Tr, tryptophan; Le, leucine; Or, ornithine; Ly, lysine.

Energy expenditure

TEE, RMR, SMR, DIT and AEE did not differ after participants consumed the 10 En% casein and 10 En% gelatin diets, nor when they consumed the 25 En% casein and 25 En% gelatin diets (**Table 2**). Previously, we described the differences between the 10 En% casein and 25 En% casein diets (31) and the differences between the 10 En% gelatin and 25 En% gelatin diets (32).

Table 2 TEE, RMR, SMR, DIT and AEE measured over 24 h in a respiration chamber after consumption of casein or gelatin diets that contained 10 En% or 25 En% protein[1]

	10En%						25En%					
	Casein diet			Gelatin diet			Casein diet			Gelatin diet		
	(MJ/d)											
TEE	9.01	±	0.25	8.97	±	0.24	9.26	±	0.25	9.12	±	0.22
RMR	7.20	±	0.18	7.17	±	0.20	7.39	±	0.17	7.30	±	0.18
SMR	6.45	±	0.18	6.49	±	0.18	6.68	±	0.15	6.62	±	0.16
DIT	0.75	±	0.09	0.68	±	0.08	0.71	±	0.07	0.68	±	0.06
AEE	1.81	±	0.11	1.79	±	0.07	1.87	±	0.11	1.82	±	0.07

Values are means ± SEM, n=22 (10 En% protein diets), n=23 (25 En% protein diets).
[1] No significant differences between consumption of the 10 En% protein diets, and between consumption of the 25 En% protein diets on all variables.

Macronutrient balances

Consumption of both the 10 En% casein and 10 En% gelatin diets resulted in a negative protein balance ($P<0.05$), a neutral fat balance and a positive carbohydrate balance ($P<0.0001$), whereas consumption of both the 25 En% casein and 25 En% gelatin diets resulted in a positive protein balance ($P<0.0001$), a negative fat balance ($P<0.0001$), and a positive carbohydrate balance ($P<0.0001$). Macronutrient balances for both 10 En% protein diets for each participant, and macronutrient balances for both 25 En% protein diets for each participant are plotted in graphs with the line of identity (**Figure 5A-F**). Negative protein balance was less when they consumed the 10 En% casein (-0.07±0.03 MJ/d), compared with the 10 En% gelatin diet (-0.17±0.03 MJ/d, $P<0.05$), whereas neutral fat (0.11±0.17 vs. 0.17±0.15 MJ/d) and positive carbohydrate balances (0.79±0.15 vs. 0.87±0.17 MJ/d) were not significantly different between the diet periods. When participants consumed the 25 En% casein compared with the 25 En% gelatin diet, the positive protein balance (0.56±0.05 vs. 0.30±0.04 MJ/d, $P<0.0001$) was larger, negative fat balances (-0.85±0.15 vs. -0.96±0.16 MJ/d) were not different, and the positive carbohydrate balance (0.86±0.14 vs. 1.37±0.17 MJ/d, $P<0.01$) was lower.

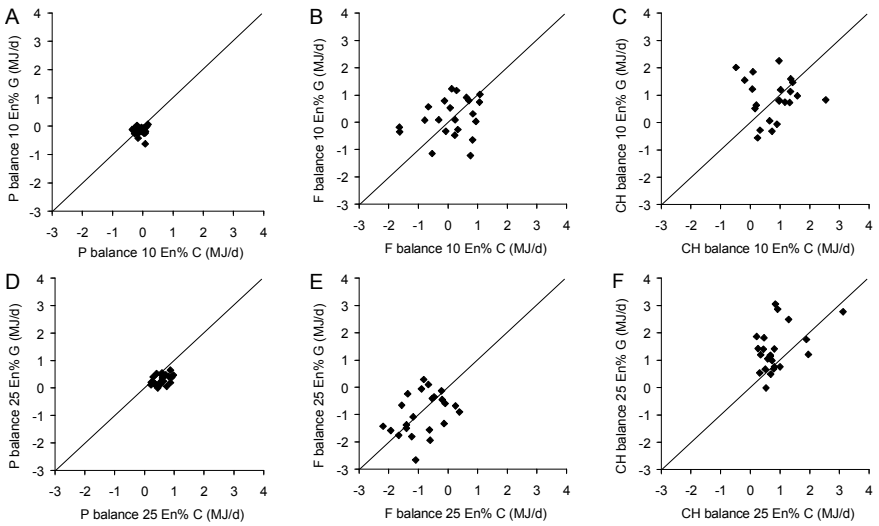

Figure 5 Protein balance (A), fat balance (B) and carbohydrate balance (C) for each participant after consumption of a diet with 10 % of energy (En %) from casein compared with a diet with 10 % of energy from gelatin, and protein balance (D), fat balance (E) and carbohydrate balance (F) for each participant after consumption of a 25 En% casein diet compared with a 25 En% gelatin diet. The lines of identity are shown. 10 En% protein diets, n=22; 25 En% protein diets, n=23.

When they consumed the 10 En% casein diet (1.08±0.04 MJ/d), protein oxidation was lower compared with the 10 En% gelatin diet (1.18±0.05 MJ/d, P<0.05), whereas fat (3.40±0.17 vs. 3.34±0.16 MJ/d) and carbohydrate oxidation rates (4.52±0.22 vs. 4.44±0.19 MJ/d) did not differ between the diet periods. Protein oxidation was lower when participants consumed the 25 En% casein diet compared with the 25 En% gelatin diet (1.96±0.08 vs. 2.22±0.06 MJ/d respectively, P<0.0001), fat oxidation was not different (2.85±0.17 vs. 2.96±0.19 MJ/d respectively), and carbohydrate oxidation was higher (4.43±0.14 vs. 3.93±0.13 MJ/d respectively, P<0.01). RQ was neither significantly different after consumption of the 10 En% casein and 10 En% gelatin diet (0.86±0.0048 vs. 0.86±0.0046, respectively), nor after consumption of the 25 En% casein and 25 En% gelatin diet (0.87±0.0037 vs. 0.86±0.0043, respectively). Taken together, when participants consumed both 10 En% and 25 En% diets, negative and positive protein balances, respectively, were higher when they consumed the casein diets. Additionally, the positive carbohydrate balance was less when participants consumed the 25 En% casein compared with the 25 En% gelatin diet.

DISCUSSION

The present study contrasted an incomplete protein (gelatin) and a complete protein (casein) in 10 En% and 25 En% single-protein diets to measure effects on appetite, 24-h energy expenditure and 24-h macronutrient balances. We hypothesized that the use of gelatin compared with casein may stimulate appetite suppression and EE, and may limit a positive protein balance. We observed significant differences in appetite profile and macronutrient balance, yet no differences in energy expenditure. These results were based on isoenergetic diets, indicating that the observed effects were due to differences in amino acid composition of the proteins used in the diets.

The larger hunger suppression found after consumption of the 10 En% gelatin diet compared with the 10 En% casein diet, is in accordance with the results of our previous research (11), showing higher satiety and lower hunger ratings after consumption of a 10 En% gelatin breakfast compared with a 10 En% casein breakfast. The mechanism mentioned in the introduction, may have contributed to the reduced appetite. Detection in the brain of a deficiency of essential amino acids in the diet from a decline in serum amino acid levels, may lead to a behavioral response rejecting consumption of imbalanced diets (14-17), and thus suppressing hunger. Therefore, the decreased hunger feelings after consumption of a gelatin compared with a casein diet, may be understood as an anorexigenic effect on intake of food that does not contain sufficient IAA (17). Under 10 En% conditions, the plasma concentrations of the essential amino acids histidine, valine, methionine, isoleucine, phenylalanine, tryptophan and leucine were decreased and lower after consumption of the gelatin compared with the casein breakfast, which may indicate a deficiency of these IAA. Under 25 En% conditions, the deficiency of IAA was not so large: only the plasma concentration of tryptophan was decreased and lower after consumption of the gelatin compared with the casein breakfast. As hunger suppression did not differ after participants consumed both 25 En% protein diets, a role of Trp in hunger suppression seems to be unlikely. Moreover, we previously observed that addition of Trp to gelatin did not affect appetite differentially (8). Deficiency of the other IAA may have been involved in the larger hunger suppression observed after consumption of the 10 En% gelatin diet. In addition, the contribution of ghrelin and/or GLP-1 was probably marginal, as the observed differences after consumption of the casein and gelatin diets in the anorexigenic hormone GLP-1 and/or the orexigenic hormone ghrelin were minor and no correlations were observed between these hormones and appetite.

Contrarily to the hypothesis that gelatin oxidation induces a higher EE compared with casein, the higher protein oxidation observed after consumption of the gelatin diets compared with the casein diets did not result in differences in EE. The reason for this may be that in addition to energy costs for protein oxidation, also other factors are involved in the thermic effect of proteins, like protein synthesis (with high ATP costs of peptide bond synthesis), high costs for urea production and gluconeogenesis (4, 33). Although not measured directly, protein synthesis was probably higher after consumption of the casein diets compared with the gelatin diets, as we observed a lower protein oxidation with similar protein intake. The higher protein synthesis may be a factor involved in the similar effect on EE of both protein types.

Compared with the gelatin diet, consumption of the casein diet resulted in a smaller negative and larger positive protein balance, under 10 En% and 25 En% protein conditions respectively. This different effect on protein balance is probably the result of a difference in completeness

between both protein sources, because the absence or low amounts of certain essential amino acids in gelatin may have been a limiting factor for postprandial protein synthesis, resulting in a higher oxidation of the free amino acids. For both casein and gelatin, the negative protein balance under 10 En% conditions and the positive protein balance under 25 En% conditions is due to the presence of the total amount of amino acids in the diet, which turned out to be too low, with respect to total body protein turnover, under 10 En% conditions and to be excessive under 25 En% conditions. Under 25 En% protein conditions the difference in protein balance when participants consumed the two diets was more pronounced than under 10 En% protein conditions. This difference in protein balance probably resulted in a more pronounced difference in carbohydrate balance, as a higher protein oxidation after consumption of the 25 En% gelatin diet may have resulted in a lower carbohydrate oxidation, and thus into a larger positive carbohydrate balance compared with the 25 En% casein diet.
The study results confirm our hypothesis that the use of an incomplete protein (gelatin), compared with a complete protein (casein), in single-protein diets stimulates appetite suppression and limits a positive protein balance. However, in contradiction to the hypothesis, EE was affected similarly. In the concept of weight loss for people with obesity, the larger hunger suppressing effect of gelatin may play a role in reducing energy intake, if this effect is maintained when consuming a gelatin diet in the long-term. In addition, long-term use of casein may contribute to preservation of fat free mass. In conclusion, when we compared the effects of an incomplete protein (gelatin) and a complete protein (casein) at two concentrations over 36 h, gelatin resulted in a larger hunger suppression, casein caused a larger positive (smaller negative) protein balance, and effects on energy expenditure did not differ.

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AH-W, MSW-P, and KRW designed research; AH-W and MABV conducted research; AH-W analyzed data; AH-W wrote paper; AH-W had primary responsibility for final content; MSW-P and KRW supervised the project. All authors read and approved the final manuscript.

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Supplemental table 1 Amino acid composition of casein and gelatin

	Casein	Gelatin
	(g/100 g protein)	(g/100 g protein)
Methionine	2.73	0.85
Aspartic acid[1]	6.4	5.6
Hydroxyproline	-	12.0
Threonine	3.85	1.86
Serine	5.1	3.26
Glutamic acid[2]	20.3	10.1
Proline	9.8	13.9
Glycine	1.69	24.6
Cystine	0.37	0.03
Valine	6.0	2.26
Isoleucine	4.77	1.52
Leucine	8.7	2.95
Tyrosine	5.1	0.47
Phenylalanine	4.66	1.87
γ-amino butyric acid	-	-
Histidine	2.74	0.91
Ornithine	-	0.32
Alanine	2.71	9.3
Lysine	7.3	3.85
Arginine	3.93	8.4
Thryptophan	1.16	<0.10

[1] Aspartic acid = asparagine + aspartic acid
[2] Glutamic acid = glutamine + glutamate

Supplemental table 2 One day-10 En% protein diet for a participant with a daily energy requirement of 10 MJ/d, consumed for 3 consecutive days, before the stay in the respiration chamber

Meal	Food item	Amount	Protein	Fat	Carbohydrate
			(kJ)	(kJ)	(kJ)
Breakfast	Wheat bread	77 g	117	43	547
	Margarine	11 g	0	324	1
	Marmelade	16 g	1	0	167
	Cream cheese	33 g	33	334	17
	Low-fat chocolate milk	164 mL	92	109	357
Lunch	Wheat bread	115 g	176	64	821
	Marmelade	16 g	1	0	167
	Margarine	16 g	0	486	1
	Cream cheese	33 g	33	334	17
	Chocolate spread	16 g	21	216	132
	Low-fat milk	219 mL	130	121	186
Dinner	Chinese Nasi goreng	438 g	372	1134	1861
	Fruit mixture	164 g	11	6	419
	Tomato soup	219 mL	74	130	223
	Low-fat yogurt	109 g	56	0	279
	Orange juice	219 mL	26	0	320
Total diet (kJ)			1144	3301	5516
Macronutrient distribution diet (En%)			11	33	55

Supplemental table 3 One day-25 En% protein diet for a participant with a daily energy requirement of 10 MJ/d, consumed for 3 consecutive days, before the stay in the respiration chamber

Meal	Food item	Amount	Protein	Fat	Carbohydrate
			(kJ)	(kJ)	(kJ)
Breakfast	Wheat bread	83 g	127	46	592
	Butter ('Becel' light)	12 g	0	166	0
	Low-fat cheese	47 g	250	193	0
	Low-fat chocolate milk	237 mL	133	158	515
Lunch	Wheat bread	124 g	190	69	887
	Butter ('Becel' light)	18 g	0	250	0
	Low-fat cheese	71 g	374	289	0
	Low-fat soft curd cheese	355 g	302	13	211
	Orange juice	355 mL	42	0	519
Dinner	Spanish Pilaf	473 g	443	788	1851
	Chicken fillet	71 g	373	100	0
	Low-fat soft curd cheese	355 g	302	13	211
	Orange juice	355 mL	42	0	519
Total diet (kJ)			2578	2085	5306
Macronutrient distribution diet (En%)			26	21	53

Chapter 6

Effects of a supra-sustained gelatin-milk protein diet compared with (supra-) sustained milk protein diets during weight loss

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Submitted for publication

ABSTRACT

Diets higher in protein content result in increased satiety and energy expenditure. In the short-term, gelatin showed stronger hunger suppression and less subsequent energy intake compared with other proteins. This study investigated if a supra-sustained gelatin-milk protein (GMP) diet promotes weight loss compared with a sustained milk protein (SMP) diet and supra-sustained milk protein (SSMP) diet during an 8-wk weight loss period. Seventy-two healthy subjects (31.2±4.8 kg/m ^{2}; 43±10 y) followed one of three subject-specific diets: SMP, SSMP or GMP diet. During week 1-4 energy intake was 100% of individual energy-requirement: protein(P)/fat(F)/carbohydrate(C): 10/40/50% of energy (En%) (SMP) and 20/30/50 En% (SSMP or GMP). During week 5-8 energy intake was 33% of individual energy-requirement: P/F/C: 30/35/35 En% (SMP) and 60/5/35 En% (SSMP or GMP). Thus, absolute protein intake was kept constant throughout per subject. Significant decreases in BMI ($P<0.0001$) were similar between the GMP (-1.7±0.5 kg/m^2) and the SMP (-2.1±0.8 kg/m^2) and SSMP (-1.6±0.5 kg/m^2) diet. Decreases in fat free mass (FFM), fat mass (FM) and FM%, and increases in FFM% were similar between the GMP and both control diets. Changes in RQ differed ($P<0.05$) between the GMP (-0.01±0.06) and the SSMP diet (-0.04±0.04). Changes in HDL concentrations differed ($P<0.05$) between the GMP (-0.21±0.18 mmol/L) and the SMP and SSMP diets (-0.08±0.18 mmol/L and -0.09±0.26 mmol/L respectively). In conclusion, a GMP diet does not induce more beneficial effects during an 8-wk weight loss period compared with a SMP and SSMP diet.

KEYWORDS: protein, weight loss, body composition, appetite

INTRODUCTION

Obesity is associated with disorders such as hypertension, hypercholesterolemia, diabetes, and liver disease (1). Since obesity is a major health concern and the number of people with obesity is still increasing, strategies for weight loss and weight maintenance thereafter are important. Therefore, short-term as well as long-term mechanisms should be affected. Recent findings suggest that an increased protein intake may serve this goal by 1) an increased satiety, despite similar or lower energy intake, 2) an increased thermogenesis, 3) contribution to storage of fat free mass, and 4) lower energy efficiency during overfeeding (2-5).
In previous studies short-term effects of different protein types, represented in normal and high single-protein breakfasts/diets, on satiety, energy intake and energy expenditure were investigated (6-14). Firstly, it was shown that, under 10En% as well as under 25En% protein conditions, energy intake after a single-protein breakfast was less with gelatin compared with casein, soy or whey without glycomacropeptide (10). Under 10En% protein conditions, gelatin decreased hunger more than casein after a single-protein breakfast (10) as well as after a single-protein diet for one day (14). Secondly, over 24 hours it was shown that gelatin compared with casein, under 10En% as well as under 25En% single-protein conditions, resulted in similar effects on total energy expenditure (14). For both protein types total energy expenditure was increased with an increased protein content of the diet (6, 13). At this moment it is not clear whether the beneficial short-term effects of gelatin on hunger and energy expenditure may play a role in the long-term during weight loss.
Since gelatin is an incomplete protein, because it is deficient in certain essential amino acids, i.e. devoid of tryptophan and imbalanced in methionine, it cannot be used as a single-protein source in a long-term diet. To create a relatively high protein diet without lacking the essential amino acids, gelatin should be complemented with a complete protein source. In that case the mechanism of hunger suppression due to gelatin being an incomplete protein (15-18) may not play a role anymore. However, when in our previous experiment gelatin was added to the diet over 36 hrs, VAS ratings on the appetite profile showed a stronger hunger suppression in comparison with casein (14), possibly through increased gluconeogenesis (14).
Therefore, the aim of this study was to investigate if the addition of gelatin to a milk protein diet would promote weight loss during a weight loss period. To investigate this, one intervention diet, a supra-sustained protein diet with gelatin and milk protein as the two protein sources in equal amounts, was compared with two control diets, a sustained and a supra-sustained protein diet with milk protein as the only protein source. The effect of the three diets on body weight, body composition, respiratory quotient (RQ), resting energy expenditure, eating behavior, physical activity, postabsorptive appetite profile and relevant blood parameters were determined before and after an 8 weeks weight loss period.

SUBJECTS & METHODS

Subjects

Eighty-one subjects aged 18-65 y with a body mass index (BMI) of ≥ 25 kg/m^2 were recruited by advertisements on notice boards of Maastricht University and in local newspapers. Subjects underwent a medical screening and were in good health, non-smokers, did not use medication (except for contraceptives), did not have a cow milk allergy and were at most moderate alcohol users. Nine subjects did not complete the weight loss period due to inability to comply to the diet or for personal reasons. Subjects signed an informed consent before participating in the study. The study protocol was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Experimental design

The study had a single blind parallel design. Subjects were randomly assigned to one of three treatment groups: 1) sustained milk protein diet (SMP, control group 1), 2) supra-sustained milk protein diet (SSMP, control group 2), 3) supra-sustained gelatin-milk protein diet (GMP, intervention group). All groups followed an 8-wk weight loss period. At the start and at the end of the weight loss period, subjects visited the university for measurements.

Energy intake

During the first 4 weeks of the weight loss period (wk 1-4) subjects from all 3 diet groups consumed a diet that was 100% of their individual energy requirements for energy balance, while during the last 4 weeks of the weight loss period (wk 5-8) they consumed a diet that was

33% of their individual energy requirements. The energy content of the diet was based on subject-specific average daily energy requirements and calculated as the Basal Metabolic Rate (BMR) multiplied with a Physical Activity Level (PAL) of 1.5. BMR was calculated with the Harris-Benedict formula (19).

Diets

During the complete 8-wk weight loss period subjects from all 3 diet groups consumed a fixed amount of protein each day, referred to as (supra-)sustained protein diets. This implied that the absolute protein content of each of the three protein diets remained the same during the whole period, while each diet differed in percentage of energy from protein between wk 1-4 and wk 5-8 of the weight loss period due to a reduction in energy intake in wk 5-8. Macronutrient compositions of the three diets are shown in **Table 1**. The protein content of the two supra-sustained protein diets was twice the amount of the sustained protein diet. Carbohydrate content was kept similar between the 3 diet groups in order to prevent a possible effect from carbohydrate, as ingestion of this nutrient results in insulin secretion, and insulin is involved in protein metabolism (20). All 3 diets were provided as meal replacements and contained all necessary vitamins, minerals, fatty acids and carbohydrates. The protein content of the sustained and supra-sustained milk protein diets consisted of 100% milk protein, while the protein content of the supra-sustained gelatin-milk protein diet consisted of 50% milk protein and 50% gelatin. In addition, subjects were instructed to eat four portions of fruit and vegetables each day and drink at least 1.5L of water.

Table 1 Macronutrient compositions of the SMP, SSMP and GMP diets during the weight loss period

	Wk 1-4 (100% of E requirements)			Wk 5-8 (33% of E requirements)		
	SMP	SSMP	GMP	SMP	SSMP	GMP
Milk protein (En%)	10	20	10	30	60	30
Gelatin (En%)			10			30
Fat (En%)	40	30	30	35	5	5
Carbohydrate (En%)	50	50	50	35	35	35

E, energy; En%, % of energy; GMP, supra-sustained gelatin-milk protein diet; SMP, sustained milk protein diet; SSMP, supra-sustained milk protein diet

Measurements

At the start (wk 0) and at the end (wk 8) of the weight loss period, subjects visited the university for the following measurements. Subjects came to the university in the morning, after an overnight fast, and were not allowed to eat and drink until all measurements were finished.

Body weight and height. Body weight was measured on a digital scale (BOD POD, Life Measurement Inc., CA, USA) with subjects in their underwear, in a fasted state and after voiding their bladder. Height was measured using a wall-mounted stadiometer (Seca, Model 225, Hamburg, Germany). BMI was calculated as body weight divided by height^2 (kg/m^2).

Waist and hip circumference. Waist circumference was measured at the site of the smallest circumference between the rib cage and the ileac crest, with subjects in standing position. Hip circumference was measured at the site of the largest circumference between the waist and the thighs. Both waist and hip circumferences were measured with an accuracy of 1.0 mm. The waist:hip ratio was calculated by dividing the waist circumference by the hip circumference.

Body composition. Body composition was determined according to the 3-compartment model based on body weight, body volume as measured with the air displacement plethysmograph (21), and total body water as measured with the deuterium dilution (^2H_2O) technique (22, 23), and was calculated by using the combined equation of Siri (24).

Resting energy expenditure (REE) and RQ. REE was measured by means of an open-circuit ventilated hood system, while subjects were lying supine for 40 min. Gas analyses were performed by a paramagnetic oxygen analyzer (Servomex, type 500A, Crowborough, Sussex, UK) and an infrared carbon dioxide analyzer (Servomex, type 500A, Crowborough, Sussex, UK). Calculation of REE was based upon Weir's formula (25). RQ was calculated as CO_2 produced/O_2 consumed.

Blood pressure and heart rate. Diastolic and systolic blood pressure, and heart rate were measured with an upper arm digital blood pressure monitor (OMRON M6, Omron Healthcare Europe BV, Hoofddorp, The Netherlands) while subjects were sitting quietly in a chair.
Eating behavior. The Dutch translation of the Three Factor Eating Questionnaire (TFEQ) (22) was used to determine if attitude towards food intake changed during the weight loss period. The first factor of the TFEQ (F1) measures cognitive restrained eating: control of food intake by thought and will-power. The second factor (F2) represents disinhibition: an incidental inability to resist eating cues, or inhibition of dietary restraint, and emotional eating. The third factor (F3) examines the subjective feeling of general hunger.
Physical activity. To determine if physical activity was kept constant during the weight loss period, subjects filled in the Baecke questionnaire (27) before and after the weight loss period. From this questionnaire 1) physical activity at work, 2) sport during leisure time, and 3) physical activity during leisure time excluding sport, could be determined.
Postabsorptive appetite profile. In the morning, after an overnight fast, appetite was scored by 100 mm anchored Visual Analogue Scales (VAS). Four questions were asked, anchored with 'not at all' to 'extremely', namely "How satiated do you feel?", "How full do you feel?", "How hungry are you?", and "How is your desire to eat?".
Blood parameters. Fasting blood samples were taken for measurements of plasma glucagon-like peptide-1 (GLP-1), peptide-tyrosine-tyrosine (PYY), insulin, glucose, creatinine (serum), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triacylglycerol (TAG) and free fatty acid (FFA) concentrations. The blood for GLP-1 was collected into EDTA-containing tubes to which dipeptidyl peptidase IV inhibitor (10 µl/mL blood) was added. For PYY analysis, blood was collected into EDTA-containing tubes in which dipeptidyl peptidase IV inhibitor (10 µL/mL blood) and aprotinin (500 KIU/mL blood) was added. The blood for insulin, glucose, HDL, LDL, TAG and FFA was collected into EDTA-containing tubes. The blood for creatinine was collected in serum seperator tubes. After the collection of blood into the tubes, blood samples were immediately centrifuged for 10 min (3000rpm at 4°C), except for the creatinine tube, which was centrifuged after staying for 60 minutes on room temperature. Plasma and serum samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed further. Plasma active GLP-1 concentrations were analyzed by enzyme-linked immunosorbent assay (EGLP-35K; Linco Research Inc, St Charles, MO). Plasma concentrations of PYY and insulin were measured by radioimmunoassay (Linco Research Inc, St Charles, MO). Plasma glucose concentrations were determined by using the hexokinase method (Glucose HK CP kit; ABX diagnostics, Montpellier, France). The HOMA-index was calculated as (fasting glucose [mmol/L] x fasting insulin [microU/mL])/22.5. Serum creatinine concentrations were analyzed by means of the Jaffe rate method on the Synchron LX20 Pro (Beckman Coulter, Nyon, Switzerland). Plasma total cholesterol and HDL concentrations were analyzed using CHOD-PAP reagent (Roche Diagnostics GmbH, Mannheim, Germany). LDL was calculated using the Friedewald formula (28). Plasma TAG concentrations were analyzed with the GPO-Trinder kit (Sigma, Missouri, USA). Plasma FFA concentrations were analyzed by using the ACS-ACOD-MEHA method in the Wako-NEFA-C kit (Wako Chemicals GmbH, Neuss, Germany).
Protein intake. At the start and end of the weight loss period subjects collected their urine for 24 hours, which was analysed for nitrogen to check the compliance with protein intake. Protein intake was calculated from the 24-h nitrogen output as follows: protein intake (g/d) = nitrogen output in 24-h urine (g/d) x 6.25.

Statistical analysis
Data are presented as mean ± standard deviation (SD), unless otherwise indicated. For each diet group, a repeated-measures ANOVA was carried out for determination of possible differences between the start (wk 0) and the end (wk 8) of the weight loss period in all measured parameters. To determine possible differences between the supra-sustained gelatin-milk protein diet group and the two control diet groups, a factorial ANOVA was carried out. Post hoc analyses were made with Fisher's PLSD. To determine relationships between variables, simple linear regression analyses were performed. The level of statistical significance was set at $P<0.05$. Statistical analyses were performed by using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS
Baseline subject characteristics for the 3 diet groups are presented in **Table 2**.

Table 2 Subject characteristics and measured variables of the 3 diet groups before and after weight loss

	Baseline (Wk 0)			End weight loss (wk 8)		
	SMP	SSMP	GMP	SMP	SSMP	GMP
N (M/F)	29 (4/25)	22 (7/15)	21 (5/16)	-	-	-
Age (y)	43 ± 10	43 ± 10	44 ± 10	-	-	-
Height (m)	1.67 ± 0.07	1.73 ± 0.08	1.68 ± 0.12	-	-	-
Waist:hip ratio	0.91 ± 0.05	0.92 ± 0.06	0.93 ± 0.06	0.90 ± 0.05	0.90 ± 0.07**	0.91 ± 0.05**
RQ[#]	0.83 ± 0.04	0.84 ± 0.04	0.83 ± 0.06	0.81 ± 0.03**	0.81 ± 0.04**	0.82 ± 0.03
Diastole (mmHg)	81 ± 10	81 ± 8	82 ± 12	76 ± 9**	72 ± 8***	75 ± 11**
Systole (mmHg)	130 ± 17	127 ± 10	129 ± 16	118 ± 10***	117 ± 11***	117 ± 14**
Heart rate (beats/min)	67 ± 10	71 ± 10	69 ± 12	64 ± 9*	64 ± 8***	66 ± 10
TFEQ1[a] (dietary restraint)	9 ± 4	8 ± 5	10 ± 5	11 ± 5**	10 ± 4*	11 ± 5*
TFEQ2[b] (disinhibition)	6 ± 3	6 ± 2	7 ± 3	5 ± 3**	5 ± 3	5 ± 3**
TFEQ3[c] (hunger)	5 ± 3	5 ± 3	6 ± 4	3 ± 2**	4 ± 3	4 ± 3**
Baecke (Work)	2.73 ± 0.57	2.52 ± 0.60	2.87 ± 0.51	2.75 ± 0.56	2.56 ± 0.59	2.85 ± 0.52
Baecke (Sport)	2.58 ± 1.03	2.56 ± 0.63	2.61 ± 1.09	2.64 ± 0.88	2.64 ± 0.77	2.63 ± 0.92
Baecke (Leisure)	3.03 ± 0.62	2.91 ± 0.49	3.00 ± 0.69	3.20 ± 0.61*	3.03 ± 0.62	3.11 ± 0.58
Baecke (Total)	8.34 ± 1.51	7.99 ± 1.23	8.48 ± 1.72	8.58 ± 1.32	8.23 ± 1.38	8.59 ± 1.59
VAS (Satiety)	47 ± 16	41 ± 18	36 ± 25	45 ± 16	41 ± 22	39 ± 18
VAS (Fullness)	40 ± 16	34 ± 18	43 ± 26	44 ± 16	42 ± 22	36 ± 15
VAS (Hunger)	35 ± 17	31 ± 16	38 ± 24	33 ± 17	27 ± 18	35 ± 20
VAS (Desire to eat)	35 ± 20	32 ± 19	47 ± 21	37 ± 18	27 ± 17	38 ± 19

Values are means ± SD

GMP, supra-sustained gelatin-milk protein diet; REE, resting energy expenditure; RQ, respiratory quotient; SMP, sustained milk protein diet; SSMP, supra-sustained milk protein diet; TFEQ, Three Factor Eating Questionnaire; VAS, visual analogue scale

[a, b, c] Factor 1, 2 and 3, respectively, of the TFEQ

[#] Change over time significantly different between SSMP and GMP diet, $P<0.05$

Significant change over time within one diet group: * $P<0.05$, ** $P<0.01$, *** $P<0.0001$

Compliance to the diets
Baseline daily protein intake (DPI) was 0.8±0.3, 0.9±0.3 and 1.0±0.6 g/kg per day for the SMP, SSMP and GMP diet group respectively. At the end of the weight loss period the SSMP and GMP diet groups had a DPI of 1.1±0.3 and 1.2±0.5 g/kg per day respectively, which was for both supra-sustained protein diet groups significantly higher (P<0.01) compared with the DPI of 0.8±0.3 g/kg per day for the SMP diet group. The DPI was not significantly different between both supra-sustained protein diet groups.

Body weight and BMI
In all 3 diet groups body weight and BMI were significantly decreased after the weight loss period (**Figure 1**, P<0.0001). The decreases in body weight and BMI were similar between the GMP diet group and both control groups (NS).

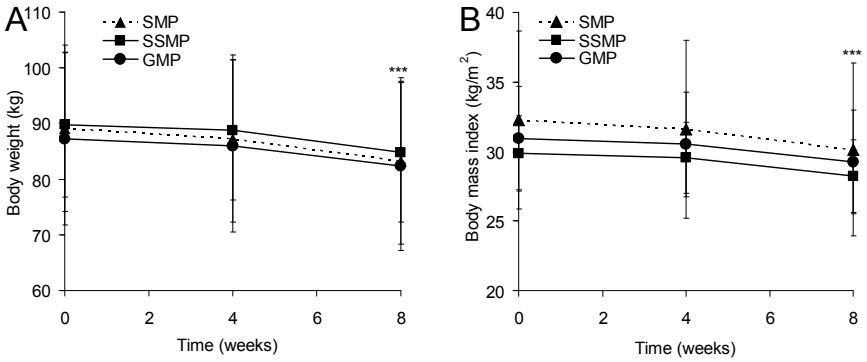

Figure 1 Body weight (A) and body mass index (B) for the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet group before, during and after the 8 weeks weight loss period. Values are mean ± SD. Significant difference over time between wk 8 and wk 0 for each diet group, *** P<0.0001. No significant difference in change over time from wk 0 to wk 8 between the GMP diet and the two control diets.

Waist:hip ratio
Waist:hip ratio was significantly decreased after the weight loss period in the SSMP and GMP diet group (P<0.01, **Table 2**), but did not change in the SMP diet group (NS). The changes in waist:hip ratio were similar between the GMP diet group and both control groups (NS).

Body composition
Fat free mass (FFM) in kg, fat mass (FM) in kg, and fat mass expressed as % of body weight were decreased after the weight loss period in all 3 diet groups (**Figure 2A, B, D**), while fat free mass expressed as % of body weight was increased in all 3 diet groups (**Figure 2C**). The changes in FFM (kg or %) and FM (kg or %) were similar between the GMP diet group and both control groups (NS).

RQ
After the weight loss period, RQ significantly decreased in the SMP and SSMP diet group (P<0.01, **Table 2**), but did not change in the GMP diet group (NS). The changes over time in RQ were significantly different between the SSMP and GMP diet group (P<0.05), with a stronger decrease in RQ for the SSMP diet group. The changes over time in RQ were similar between the SMP and GMP diet group (NS).

REE as a function of FFM
REE is plotted as a function of fat free mass at baseline (wk 0) and after the weight loss period (wk 8) for the SMP, SSMP and GMP diet group (**Figure 3A, B** and **C** respectively). In all 3 diet groups a significant linear relation was present between REE (MJ/d) and FFM (kg) at baseline as well as after the weight loss period (P<0.0001). To determine for each diet group if the REE as function of FFM changed significantly over time, as shown by the regression lines at wk 0 and wk 8, the FFM (kg) values from wk 8 were filled in in the slope equation of wk 0 to result in

a calculated REE of wk 8. The calculated and measured REE of wk 8 were analysed with ANOVA repeated measures to determine any changes in REE as function of FFM. In the SMP and the GMP diet group, REE as a function of FFM decreased significantly (P<0.0001 and P<0.01 respectively), while in the SSMP diet group REE as a function of FFM did not change significantly. However, the changes over time in REE as a function of FFM were not significantly different between the GMP diet group and both control groups.

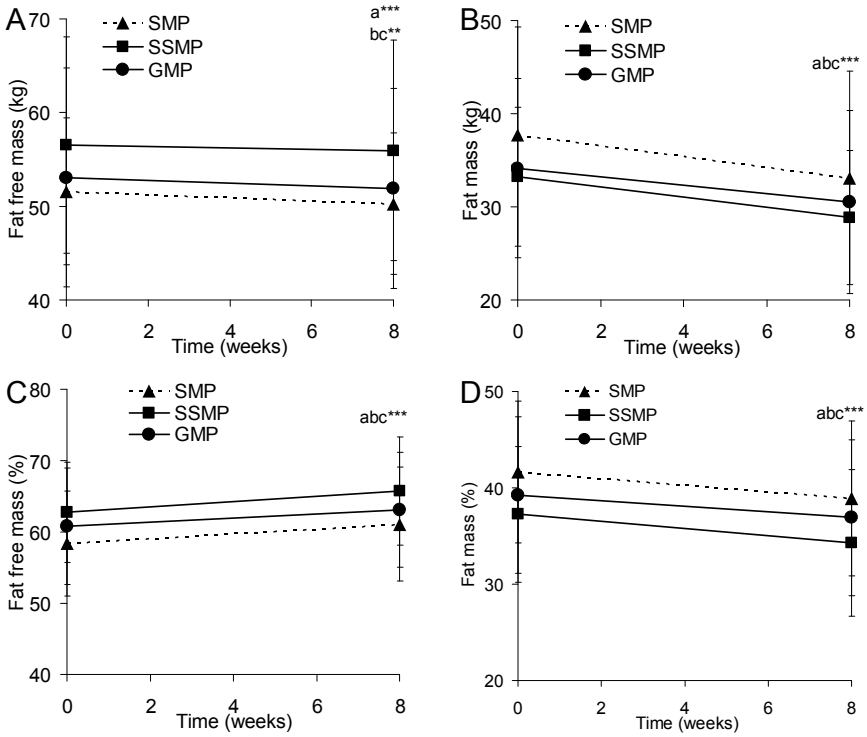

Figure 2 Fat free mass in kg (A), fat mass in kg (B), fat free mass expressed as % of body weight (C) and fat mass expressed as % of body weight (D) for the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet group before and after the 8 weeks weight loss period. Values are mean ± SD. Significant difference over time within one diet group, ** P<0.01, *** P<0.0001, [a] SMP diet, [b] SSMP diet, [c] GMP diet. No significant difference in change over time between the GMP diet and the two control diets.

Blood pressure and heart rate

Diastole and systole significantly decreased after the weight loss period in all 3 diet groups (**Table 2**, P<0.01 or P<0.0001). Heart rate significantly decreased in the SMP and SSMP diet group (**Table 2**, P<0.05 and P<0.0001 respectively) after weight loss, but not in the GMP diet group (NS). The changes over time in diastole, systole and heart rate were similar between the GMP diet group and both control groups (NS).

Eating behavior

Dietary restraint (factor 1 of the TFEQ, **Table 2**) increased significantly in all 3 diet groups (P<0.05 or P<0.01). Disinhibition (factor 2) and general hunger (factor 3) significantly decreased in the SMP and GMP diet groups (P<0.01), but did not reach significance in the SSMP diet group (NS). The changes over time in dietary restraint, disinhibition and general hunger were similar between the GMP diet group and both control groups (NS).

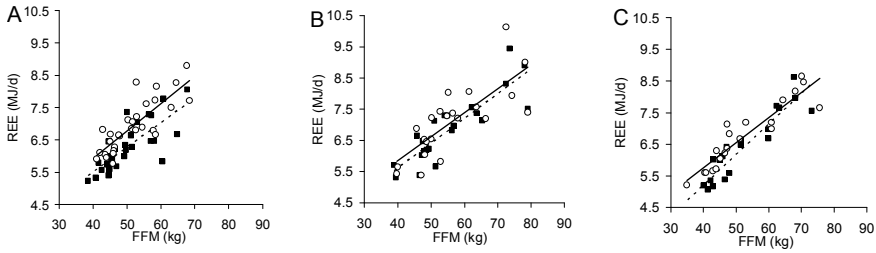

Figure 3 Resting energy expenditure (REE) as a function of fat free mass (FFM) plotted for wk 0 (baseline, ○, trendline —) and for wk 8 (after weight loss, ■, trendline ····) for the sustained milk protein (SMP; A), supra-sustained milk protein (SSMP; B) and supra-sustained gelatin-milk protein (GMP; C) diet group. Regression equation SMP diet, wk 0: REE = 0.085FFM + 2.54 (R^2 = 0.67, P<0.0001). Regression equation SMP diet, wk 8: REE = 0.076FFM + 2.47 (R^2 = 0.60, P<0.0001). Regression equation SSMP diet, wk 0: REE = 0.077FFM + 2.78 (R^2 = 0.60, P<0.0001). Regression equation SSMP diet, wk 8: REE = 0.079FFM + 2.44 (R^2 = 0.75, P<0.0001). Regression equation GMP diet, wk 0: REE = 0.079FFM + 2.61 (R^2 = 0.85, P<0.0001). Regression equation GMP diet, wk 8: REE = 0.097FFM + 1.36 (R^2 = 0.87, P<0.0001).

Physical activity

Physical activity (Baecke Work, Sport, Leisure or Total, **Table 2**) did not significantly change over time in all 3 diet groups, except for leisure time in the SMP diet group, which significantly increased (P<0.05), but did not affect total physical activity in this group. Changes over time in physical acitivity were similar between the GMP diet group and both control groups (NS).

Postabsorptive appetite profile

Postabsorptive scores for satiety, fullness, hunger and desire to eat did not significantly change over time in all 3 diet groups (**Table 2**) and changes over time were similar between the GMP diet group and both control groups (NS).

Blood parameters

Fasting plasma concentrations at baseline and after the weight loss period are presented in **Table 3**. Plasma GLP-1 and glucose concentrations significantly decreased and increased, respectively, after weight loss in the GMP diet group, while no significant changes over time occurred in the SMP and SSMP diet groups. Plasma PYY, insulin and LDL concentrations, and HOMA index significantly decreased after weight loss in all 3 diet groups. Plasma HDL concentrations significantly decreased after weight loss in the SMP and GMP diet groups, but did not significantly change in the SSMP diet group. Plasma TAG concentrations did not significantly change over time in all 3 diet groups. Plasma FFA and serum creatinine concentrations significantly increased in the SMP diet group, but no significant changes occurred in the SSMP and GMP diet groups. Changes over time in all blood variables were similar between the GMP diet group and both control groups (NS), except from the plasma HDL concentrations, which decreased more over time in the GMP diet group compared with both control groups (P<0.05).

Table 3 Fasting blood variables of the 3 diet groups before and after weight loss

	Baseline (Wk 0)									End weight loss (wk 8)								
	SMP			SSMP			GMP			SMP			SSMP			GMP		
GLP-1 (pmol/L)	1.6	±	1.6	2.4	±	2.8	1.5	±	1.0	1.3	±	0.9	2.0	±	2.1	1.3	±	0.7*
PYY (pg/mL)	80	±	37	56	±	49	62	±	31	20	±	13***	18	±	18**	18	±	17***
Insulin (µU/mL)	15.99	±	6.12	14.12	±	4.90	13.35	±	6.01	12.21	±	5.31**	10.09	±	3.90**	10.79	±	4.75**
Glucose (mmol/L)	5.15	±	0.40	5.14	±	0.41	4.97	±	0.37	5.14	±	0.55	5.10	±	0.46	5.22	±	0.51**
HOMA index	3.70	±	1.60	3.25	±	1.23	2.97	±	1.37	2.87	±	1.51*	2.31	±	1.03**	2.53	±	1.20*
Creatinine (µmol/L)	74	±	13	78	±	14	81	±	23	82	±	16*	83	±	15	82	±	14
HDL (mmol/L)[#]	1.42	±	0.38	1.38	±	0.38	1.56	±	0.37	1.34	±	0.33*	1.30	±	0.23	1.35	±	0.31***
LDL (mmol/L)	3.68	±	0.71	3.90	±	0.92	3.65	±	0.95	2.97	±	0.62***	3.09	±	1.08**	3.04	±	0.73**
TAG (mmol/L)	1.27	±	0.71	1.20	±	0.54	1.03	±	0.42	1.18	±	0.34	1.07	±	0.37	1.10	±	0.33
FFA (mmol/L)	480	±	140	466	±	165	508	±	230	582	±	193**	532	±	162	523	±	165

Values are means ± SD
GMP, supra-sustained gelatin-milk protein diet; SMP, sustained milk protein diet; SSMP, supra-sustained milk protein diet
[#] Change over time significantly different between the GMP diet and the two control diets, P<0.05
Significant change over time within one diet group: * P<0.05, ** P<0.01, *** P<0.0001

DISCUSSION

In this study we investigated whether the addition of gelatin to a milk protein diet promotes weight loss during a weight loss period. The results show that this was not observed. Changes over the 8-wk weight loss period in body weight, BMI, waist:hip ratio, body composition, REE as a function of FFM, blood pressure, heart rate, eating behavior, physical activity, postabsorptive appetite profile, plasma/serum GLP-1, PYY, insulin, glucose, creatinine, LDL, TAG, and FFA concentrations and HOMA index were not significantly different between the GMP diet group and the SMP and SSMP diet groups. The GMP diet group differed from the SSMP diet group in showing a smaller decrease in RQ over time, and differed from both the SMP and SSMP diet groups in showing a stronger decrease in plasma HDL concentrations over time.

Compliance in this study was confirmed with the 24h urinary nitrogen results. The DPI of the sustained milk protein diet group at the end of the weight loss period was 0.8 g/kg per day, which is the required minimum amount of daily protein intake as recommended by the World Health Organization (29). The DPI of the supra-sustained protein diet groups, being 1.1 and 1.2 g/kg per day, were significantly higher compared with the sustained protein diet group, while protein intake was similar between both supra-sustained protein diet groups.

In all 3 diet groups body weight and BMI were significantly reduced after the 8-wk weight loss period, while these decreases over time were not different between the GMP diet and both control diets. These results show that adding gelatin to a sustained milk protein diet, while creating a supra-sustained gelatin-milk protein diet, does not result in larger effects on weight loss compared with a sustained and supra-sustained protein diet with milk protein as the only protein source. Thus, the beneficial short-term effect of gelatin on hunger suppression (10) was not present anymore, as expected from the now complete protein that was consumed. Moreover, also the promoting effect from gluconeogenesis on hunger suppression, as hypothesized based upon a previous study (14) did not seem to play a role anymore over the longer term. Although changes in fasting plasma GLP-1 and PYY concentrations over time were observed, these changes in the so-called appetite hormones did not affect the postabsorptive appetite profile in all 3 diets. The addition of gelatin to the diet did not result in a different effect on the postabsorptive appetite profile compared with both control diets, and therefore did not contribute to promote differences in weight loss. With respect to eating behavior, changes over time in dietary restraint, disinhibition and general feelings of hunger were also not different between the GMP diet group and both control groups and therefore did not contribute to promote differences in weight loss. In most weight loss studies relative high protein diets are considered during ad libitum energy intakes, not during energy restriction. In absolute terms the ‘relatively high’ intakes in these studies may just meet the required minimum amount of daily protein intake (0.8 g/kg per day) as recommended by the World Health Organization, and are in fact normal-protein diets. In addition, the ‘relatively normal’ protein intakes in these studies may be lower than the required minimum amount of daily protein intake, and are in fact low-protein diets (30, 31). This may contribute to the observed increases in satiety with the ‘relatively high’ protein diets, resulting in lower energy intakes and thus into higher body weight losses in most weight loss studies. As in our study the SMP diet group had already a protein intake of 0.8 g/kg per day (which is the required minimum amount of daily protein intake), while the supra-sustained protein diets groups had even higher protein intakes of 1.1 and 1.2 g/kg per day during energy restriction, we compared absolutely normal (sustained) with absolutely high (supra-sustained) protein diets. Although during the GMP diet protein intake was higher compared with the SMP diet, no differences in appetite profile and weight loss were observed. This may suggest that the required minimum absolute amount of protein intake is sufficient to accomplish the beneficial effects of protein on satiety and weight loss during energy restriction. The effect of a sustained (absolute) required minimum amount of protein intake on the appetite profile being sufficient is in line with the “protein leverage hypothesis”, which implies that maintaining absolute daily protein intake is prioritized over fat and carbohydrate intakes, regardless of macronutrient composition of the diets (32).

In all 3 diet groups absolute fat free mass and fat mass decreased as a result of body weight loss. However, when expressing fat free mass and fat mass as a percentage of body weight, in all 3 diet groups fat free mass percentage increased, while fat mass percentage decreased. As physical activity did not change over time in all 3 diet groups, physical activity was not involved in the improvement in body composition. This may indicate that in all 3 diet groups body composition improved due to sparing of fat free mass as a result of keeping the daily protein

intake at minimum required levels or even higher. Although body composition improved in all 3 diet groups, the addition of gelatin to a milk protein diet did not result in different effects on body composition compared with the two milk protein diets. Protein intake was 0.4 and 0.1 g/kg per day higher in the GMP diet group compared with the SMP and SSMP diet groups, respectively, but this higher intake did not result in a higher preservation of fat free mass. Thus, the addition of gelatin to a sustained milk protein diet, or the exchange of energy from milk protein with energy from gelatin in a supra-sustained protein diet does not affect body composition differentially. In addition, in both milk protein diet groups RQ decreased, while RQ remained similar in the GMP diet group. The change in RQ over time was different between the SSMP and GMP diet group, which may indicate that gelatin has less potential to increase postabsorptive fat oxidation during weight loss. However, the difference in RQ change over time did not result in a less favourable effect of the GMP diet on body composition.
Resting energy expenditure as a function of fat free mass decreased significantly over time in the SMP and GMP diet groups, while the change over time was not significant in the SSMP diet group. Previous studies already observed that the decrease in REE is the result of compensatory changes in energy expenditure to a decrease in body weight (33). Thus only in the SSMP diet group energy expenditure was sustained despite negative energy balance; this phenomenon was not reached in the SMP and GMP diet groups, and may need a considerably higher protein intake of the quality of a complete protein.
Regarding health benefits, all 3 weight loss diets resulted in beneficial decreases in diastole, systole, heart rate (not significant for the GMP diet group), and fasting plasma insulin and LDL concentrations, while the GMP diet did not show different effects on these parameters over time compared with both milk protein diets. However, the observed higher decrease in fasting plasma HDL concentration with the GMP diet showed a less favourable effect of gelatin compared with the SMP and SSMP diet.
Milk protein, either at the level of SMP, or at the level of SSMP, has beneficial effects on weight loss during negative energy balance, with respect to loss of body mass, body composition, and fasting plasma insulin and LDL concentrations. Addition of gelatin to milk protein is not able to improve these factors under negative energy balance conditions.
In conclusion, this study shows that effects of a diet observed in the short-term do not necessarily result in similar effects in the long-term. The short-term effect of gelatin on hunger suppression did not result in more beneficial effects during weight loss compared with the two milk protein diets. We conclude that a supra-sustained gelatin-milk protein diet does not induce more beneficial effects during an 8-wk weight loss period compared with a sustained and supra-sustained milk protein diet. The larger decrease in HDL with the GMP diet may indicate a less favourable metabolic effect. This effect supports using better quality protein diets.

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Chapter 7

No long-term weight maintenance effects of gelatin in a supra-sustained protein diet

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ABSTRACT

In the short-term, gelatin showed stronger hunger suppression and less energy intake compared with other proteins. This study investigated if a supra-sustained gelatin-milk protein (GMP) diet improves weight maintenance (WM) compared with a sustained milk protein (SMP) diet and supra-sustained milk protein (SSMP) diet during a 4-months WM period after 8-wk weight loss (WL) in sixty-five healthy subjects (28.6±3.4 kg/m^2; 44±10 y). Absolute protein intake was kept constant (sustained) throughout per subject. Diets were: protein(P)/fat(F)/carbohydrate(C): 15/40/45% of energy (En%) (SMP) and 30/25/45 En% (SSMP or GMP) for wk 9-16. Diets wk 17-24: P/F/C: 30/35/35En% (SMP) and 60/5/35 En% (SSMP or GMP). From wk 8 to 16, and wk 16 to 24, changes in BMI were similar between the GMP (-0.4±0.6 and 0.3±0.7 kg/m^2 respectively), and the SMP (-0.7±0.9 and 0.1±0.7 kg/m^2 respectively) and SSMP (-0.6±0.6 and 0.3±0.6 kg/m^2 respectively) diets. Sparing of fat-free mass (FFM): increases/decreases in FFM%/fat-mass% from wk 8 to 16 were similar between the GMP and both control diets, and maintained from wk 16 to 24. In conclusion, all 3 diets resulted in a successful WM period, while a GMP diet does not improve body weight maintenance and related variables after weight loss compared with a SMP and SSMP diet.

KEYWORDS: protein, weight maintenance, body composition, appetite

INTRODUCTION

Obesity is associated with disorders such as hypertension, hypercholesterolemia, diabetes, and liver disease (1). Since obesity is a major health concern and the number of people with obesity is still increasing, strategies for weight loss and weight maintenance thereafter are necessary. Therefore, short-term as well as long-term mechanisms should be affected. Recent findings suggest that an increased protein intake may serve this goal by 1) sustained satiety, despite similar or lower energy intake, 2) sustained thermogenesis, 3) sparing of fat free mass, and 4) lower energy efficiency during the period of weight maintenance (2-5).

In previous studies short-term effects of different protein types, represented in normal and high single-protein breakfasts/diets, on satiety, energy intake and energy expenditure were investigated (6-14). Firstly, it was shown that, under 10En% as well as under 25En% protein conditions, energy intake after a single-protein breakfast was less with gelatin compared with casein, soy or whey without glycomacropeptide (10). Under 10En% protein conditions, gelatin decreased hunger more than casein after a single-protein breakfast (10) as well as after a single-protein diet for one day (14). Since gelatin is an incomplete protein, because it is deficient in certain essential amino acids, i.e. devoid of tryptophan and imbalanced in methionine, these results may relate to a mechanism observed in metazoans, where it was discovered that the tRNA/GCN2/p-eIF2α system in the brain can detect a deficiency of essential amino acids in the diet from a decline in serum amino acid levels, leading to a behavioral response that rejects consumption of imbalanced diets (15-18), and thus appears as hunger suppression. This phenomenon has been observed since the 1970's as excesses or deficiencies of amino acids in the diet depressing food intake in rats (19). Later, Fromentin et al showed that the parabranchial nuclei are involved in the learned aversion to an amino acid devoid diet (20). In a review Gietzen and Rogers explain that given a choice, herbivores and omnivores select a diet containing higher levels of protein or amino-acid mixtures, even if the choices contain balanced indispensable amino acid profiles; this shows that nitrogen per se can become limiting (21). They furthermore show that in addition to rodents, Protozoa, Nematoda, Mollusca, Arthropoda, Vertebrata including fish, birds and mammals have the same indispensable amino acids, except humans, which do not require arginine (21), The inability of an incomplete protein diet to support human life was known already in the early 1800s, when Napoleon's injured soldiers failed to recover on a diet with gelatin as the protein source (22). A general amino acid control system which is activated by deprivation via deacylated tRNA showed conservation of amino acid sensory mechanisms across eukaryotic species (21).

Also, when in our previous experiment, gelatin was added to the diet over 36 hrs VAS ratings on the appetite profile showed a stronger hunger suppression in comparison with casein (14), possibly through increased gluconeogenesis (14).

Moreover, over 24 hours it was shown that gelatin compared with casein, under 10En% as well as under 25En% single-protein conditions, resulted in similar effects on total energy expenditure (14). For both protein types total energy expenditure was increased with an increased protein content of the diet (6, 13, 14). At this moment it is not clear whether the beneficial short-term effects of gelatin on hunger and energy expenditure may play a role in the long-term during a weight maintenance period after weight loss.

Since gelatin is an incomplete protein, it cannot be used as a single-protein source in a long-term diet. To create a relatively high protein diet without lacking the essential amino acids, gelatin should be complemented with a complete protein source. Then the hunger suppression effect of gelatin being an incomplete protein may have disappeared, but a possible hunger suppression effect through increased gluconeogenesis still may sustain. Therefore, the aim of this study was to investigate if the addition of gelatin to a milk protein diet would improve weight maintenance during a 4-months weight maintenance period after weight loss. To investigate this during weight loss and weight maintenance, one intervention diet, a supra-sustained protein diet with gelatin and milk protein as the two protein sources in equal amounts, was compared with two control diets, a sustained and a supra-sustained protein diet with milk protein as the only protein source. The effect of the three diets on body weight (BW), body composition, respiratory quotient (RQ), resting energy expenditure, eating behavior, physical activity, postabsorptive appetite profile and relevant blood parameters were determined at the start, after 8 weeks and after 16 weeks of the weight maintenance period following weight loss.

SUBJECTS & METHODS

Subjects

Eighty-one subjects aged 18-65 y with a body mass index (BMI) of ≥ 25 kg/m^2 were recruited by advertisements on notice boards of Maastricht University and in local newspapers. Subjects underwent a medical screening and all were in good health, were non-smokers, did not use medication (except for contraceptives), did not have a cow milk allergy and were at most moderate alcohol users. Nine subjects did not complete the weight loss period due to inability to comply with the diet or for personal reasons. From the 72 subjects who finished the weight loss period, 65 subjects started the weight maintenance period after a weight loss of 6±2% of initial body weight. Subjects signed an informed consent before participating in the study. The study protocol was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Experimental design

The study had a single blind parallel design. Subjects were randomly assigned to one of three treatment groups: 1) sustained milk protein diet (SMP, control group 1), 2) supra-sustained milk protein diet (SSMP, control group 2), 3) supra-sustained gelatin-milk protein diet (GMP, intervention group). After following an 8-wk weight loss period, based on an energy intake of 33% of individual energy requirements, subjects started a 4-months weight maintenance period. At the end of wk 8, after 2 months (end wk 16) and after 4 months (end wk 24) of the weight maintenance period, subjects visited the university for measurements.

Energy intake

During the complete weight maintenance period (wk 9-24) subjects from all 3 diet groups consumed a diet that was 100% of their individual energy requirements for energy balance. This was calculated as 67% from 100% of energy requirements at the start of the weight loss period. The energy content of the diet at the start of the weight loss period was based on subject-specific average daily energy requirements and calculated as the Basal Metabolic Rate (BMR) multiplied with a Physical Activity Level (PAL) of 1.5. BMR was calculated with the Harris-Benedict formula (23).

Diets

During the complete 4-months weight maintenance period subjects from all 3 diet groups consumed a fixed amount of protein each day, referred to as (supra-)sustained protein diets. This implied that the absolute protein content of each of the three protein diets remained the same during the whole period. During the first 2 months of weight maintenance, the complete diet (100% of energy requirements) was provided. During the last 2 months of weight maintenance half of the diet (50% of energy requirements) was provided, while subjects were able to eat ad libitum, without counselling of the research team, for the other 50% of energy requirements. To keep protein intake the same during the complete weight maintenance period, each diet differed in percentage of energy from protein between wk 9-16 and wk 17-24 (**Table 1**). The protein content of the two supra-sustained protein diets was twice the amount of the sustained protein diet. Carbohydrate content was kept similar between the 3 diet groups in order to prevent a possible effect from carbohydrate, as ingestion of this nutrient results in insulin secretion, and insulin is involved in protein metabolism (24). All 3 diets were provided as meal replacements and contained all necessary vitamins, minerals, fatty acids and carbohydrates. The protein content of the sustained and supra-sustained milk protein diets consisted of 100% milk protein, while the protein content of the supra-sustained gelatin-milk protein diet consisted of 50% milk protein and 50% gelatin. In addition, subjects were instructed to eat four portions of fruit and vegetables each day and drink at least 1.5L of water.

Compliance with protein intake

Before every test day subjects collected their urine for 24 hours, which was analysed for nitrogen to check the compliance with protein intake. Protein intake was calculated from the 24-h nitrogen output as follows: protein intake (g/d) = nitrogen output in 24-h urine (g/d) x 6.25.

Table 1 Macronutrient compositions of the SMP, SSMP and GMP diets during wk 9-16 and wk 17-24 of the weight maintenance period

	Wk 9-16: 100% of E requirements			Wk 17-24: 50% of E requirements (+ ad libitum)		
	SMP	SSMP	GMP	SMP	SSMP	GMP
Milk protein (En%)	15	30	15	30	60	30
Gelatin (En%)			15			30
Fat (En%)	40	25	25	35	5	5
Carbohydrate (En%)	45	45	45	35	35	35

E, energy; En%, % of energy; GMP, supra-sustained gelatin-milk protein diet; SMP, sustained milk protein diet; SSMP, supra-sustained milk protein diet

Measurements

Subjects visited the university for the following measurements at the end of wk 8, after 2 months (end wk 16) and after 4 months (end wk 24) of the weight maintenance period. Subjects came to the university in the morning, after an overnight fast, and were not allowed to eat and drink until all measurements were finished.

Body weight and height. BW was measured on a digital scale (BOD POD, Life Measurement Inc., CA, USA) with subjects in their underwear, in a fasted state and after voiding their bladder. Height was measured using a wall-mounted stadiometer (Seca, Model 225, Hamburg, Germany). BMI was calculated as BW divided by height^2 (kg/m^2).

Waist and hip circumference. Waist circumference was measured at the site of the smallest circumference between the rib cage and the ileac crest, with subjects in standing position. Hip circumference was measured at the site of the largest circumference between the waist and the thighs. Both waist and hip circumferences were measured with an accuracy of 1.0 mm. The waist:hip ratio was calculated by dividing the waist circumference by the hip circumference.

Body composition. Body composition was determined according to the 3-compartment model based on BW, body volume as measured with the air displacement plethysmograph (25), and total body water as measured with the deuterium dilution (^2H_2O) technique (26, 27), and was calculated by using the combined equation of Siri (28).

Resting energy expenditure (REE) and RQ. REE was measured by means of an open-circuit ventilated hood system, while subjects were lying supine for 40 min. Gas analyses were performed by a paramagnetic oxygen analyzer (Servomex, type 500A, Crowborough, Sussex, UK) and an infrared carbon dioxide analyzer (Servomex, type 500A, Crowborough, Sussex, UK). Calculation of REE was based upon Weir's formula (29). RQ was calculated as CO_2 produced/O_2 consumed. This measurement was not performed at wk 24.

Blood pressure and heart rate. Diastolic and systolic blood pressure, and heart rate were measured with an upper arm digital blood pressure monitor (OMRON M6, Omron Healthcare Europe BV, Hoofddorp, The Netherlands) while subjects were sitting quietly in a chair.

Eating behavior. The Dutch translation of the Three Factor Eating Questionnaire (TFEQ) (30) was used to determine if attitude towards food intake changed during the weight loss period. The first factor of the TFEQ (F1) measures cognitive restrained eating: control of food intake by thought and will-power. The second factor (F2) represents disinhibition: an incidental inability to resist eating cues, or inhibition of dietary restraint, and emotional eating. The third factor (F3) examines the subjective feeling of general hunger.

Physical activity. To determine if physical activity was kept constant during the weight maintenance period, subjects filled in the Baecke questionnaire (31). From this questionnaire 1) physical activity at work, 2) sport during leisure time, and 3) physical activity during leisure time excluding sport, could be determined.

Postabsorptive appetite profile. In the morning, after an overnight fast, appetite was scored by 100 mm anchored Visual Analogue Scales (VAS). Four questions were asked, anchored with 'not at all' to 'extremely', namely "How satiated do you feel?", "How full do you feel?", "How hungry are you?", and "How is your desire to eat?".

Blood parameters. Fasting blood samples were taken for measurements of plasma glucagon-like peptide-1 (GLP-1), peptide-tyrosine-tyrosine (PYY), insulin, glucose, creatinine (serum), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triacylglycerol (TAG) and free fatty acid (FFA) concentrations. The blood for GLP-1 was collected into EDTA-containing tubes to

which dipeptidyl peptidase IV inhibitor (10 µl/mL blood) was added. For PYY analysis, blood was collected into EDTA-containing tubes in which dipeptidyl peptidase IV inhibitor (10 µL/mL blood) and aprotinin (500 KIU/mL blood) was added. The blood for insulin, glucose, HDL, LDL, TAG and FFA was collected into EDTA-containing tubes. The blood for creatinine was collected in serum seperator tubes. After the collection of blood into the tubes, blood samples were immediately centrifuged for 10 min (3000rpm at 4°C), except for the creatinine tube, which was centrifuged after being kept 60 minutes at room temperature. Plasma and serum samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed further. Plasma active GLP-1 concentrations were analyzed by enzyme-linked immunosorbent assay (EGLP-35K; Linco Research Inc, St Charles, MO). Plasma concentrations of PYY and insulin were measured by radioimmunoassay (Linco Research Inc, St Charles, MO). Plasma glucose concentrations were determined by using the hexokinase method (Glucose HK CP kit; ABX diagnostics, Montpellier, France). The HOMA-index was calculated as (fasting glucose [mmol/L] x fasting insulin [microU/mL])/22.5. Serum creatinine concentrations were analyzed by means of the Jaffe rate method on the Synchron LX20 Pro (Beckman Coulter, Nyon, Switzerland). Plasma total cholesterol and HDL concentrations were analyzed using CHOD-PAP reagent (Roche Diagnostics GmbH, Mannheim, Germany). LDL was calculated using the Friedewald formula (32). Plasma TAG concentrations were analyzed with the GPO-Trinder kit (Sigma, Missouri, USA). Plasma FFA concentrations were analyzed by using the ACS-ACOD-MEHA method in the Wako-NEFA-C kit (Wako Chemicals GmbH, Neuss, Germany). No blood measurements were performed at wk 24.

Statistical analysis

Data are presented as mean ± standard deviation (SD), unless otherwise indicated. For each diet group, a repeated-measures ANOVA was carried out for determination of possible differences between wk 8 and wk 16, and between wk 16 and wk 24 in all measured parameters. To determine possible differences between the supra-sustained gelatin-milk protein diet group and the two control diet groups, a factorial ANOVA was carried out. Post hoc analyses were made with Fisher's PLSD. To determine relationships between variables, simple linear regression analyses were performed. The level of statistical significance was set at $P<0.05$. Statistical analyses were performed by using StatView 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Subject characteristics for the 3 diet groups at the start of the weight maintenance period are presented in **Table 2**.

Compliance to the diets

A good compliance was reached during the complete weight maintenance period. At week 8 and 16 the daily protein intake (DPI) was significantly higher in both supra-sustained protein diet groups compared with the sustained milk protein diet group (**Table 2**). During the last 2 months of the weight maintenance period, when subjects were allowed to eat ad libitum in addition, protein intake increased in all 3 diet groups, indicating a good compliance during this period.

Body weight and BMI

At the end of the complete 4-months weight maintenance period, no significant weight regain occurred in all 3 diet groups. Weight loss at wk 8 was -6.1±2.2 kg, -5.3±1.5, and -4.7±1.5 kg in the SMP, SSMP and GMP diet groups respectively, while weight loss at wk 24 was -7.5±5.3 kg, -6.4±2.6, and -4.5±3.3 kg respectively. In all 3 diet groups BW and BMI significantly decreased from wk 8 to 16 (**Figure 1**, $P<0.05$); after that BW and BMI were sustained. The changes in BW and BMI were similar between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

Waist:hip ratio

Waist:hip ratio significantly decreased from wk 8 to 16 in the SMP and GMP diet group ($P<0.05$, **Table 2**), and remained the same from wk 16 to 24 in all 3 diet groups. The changes in waist:hip ratio were similar between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

Table 2 Subject characteristics and measured variables of the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet groups at the start, at wk 16, and at the end of weight maintenance

	SMP			SSMP			GMP		
	Wk 8	Wk 16	Wk 24	Wk 8	Wk 16	Wk 24	Wk 8	Wk 16	Wk 24
N (M/F)	26 (4/22)	-	-	19 (6/13)	-	-	20 (5/15)	-	-
Age (y)	43 ± 11	-	-	43 ± 10	-	-	44 ± 10	-	-
Height (m)	1.67 ± 0.06	-	-	1.74 ± 0.07	-	-	1.67 ± 0.12	-	-
DPI (g/kg per day)	0.77 ± 0.23	0.78 ± 0.27	0.97 ± 0.39	1.15 ± 0.32[a]	1.16 ± 0.25[a]	1.31 ± 0.37[a]	1.17 ± 0.48[a]	1.21 ± 0.43[a]	1.26 ± 0.67
Waist:hip ratio[#]	0.89 ± 0.05	0.87 ± 0.06[b]	0.87 ± 0.06	0.90 ± 0.07	0.89 ± 0.07	0.90 ± 0.07	0.91 ± 0.05	0.88 ± 0.07[b]	0.88 ± 0.07
RQ[#]	0.81 ± 0.03	0.84 ± 0.04[b]	-	0.80 ± 0.03	0.83 ± 0.05[b]	-	0.82 ± 0.03	0.83 ± 0.05	-
Diastole[#] (mmHg)	75 ± 9	77 ± 9	75 ± 9	72 ± 9	74 ± 7	74 ± 7	75 ± 11	75 ± 12	72 ± 11
Systole[#] (mmHg)	118 ± 10	120 ± 13	118 ± 12	116 ± 11	117 ± 10	119 ± 9	116 ± 14	118 ± 15	116 ± 15
Heart rate[#] (beats/min)	64 ± 9	65 ± 10	69 ± 8[c]	64 ± 8	66 ± 8	71 ± 10[c]	66 ± 10	65 ± 10	71 ± 11[c]
TFEQ1[1,#] (dietary restraint)	11 ± 5	11 ± 5	12 ± 5	11 ± 4	12 ± 4	12 ± 5	12 ± 5	12 ± 4	12 ± 5
TFEQ2[2,#] (disinhibition)	5 ± 2	5 ± 2	4 ± 2	5 ± 3	5 ± 3	5 ± 3	5 ± 3	4 ± 2	5 ± 2
TFEQ3[3,#] (hunger)	3 ± 3	3 ± 2	3 ± 3	4 ± 3	3 ± 2	3 ± 2	3 ± 3	2 ± 2	3 ± 4
Baecke[#] (Work)	2.77 ± 0.53	2.69 ± 0.58	2.70 ± 0.58	2.55 ± 0.62	2.56 ± 0.62	2.55 ± 0.63	2.90 ± 0.49	2.85 ± 0.47	2.90 ± 0.46
Baecke[#] (Sport)	2.69 ± 0.91	2.66 ± 1.00	2.40 ± 0.93	2.63 ± 0.82	2.67 ± 0.82	2.58 ± 0.73	2.69 ± 0.91	2.62 ± 0.96	2.57 ± 0.79
Baecke[#] (Leisure)	3.17 ± 0.63	3.19 ± 0.56	3.33 ± 0.65	3.07 ± 0.67	3.05 ± 0.60	2.96 ± 0.55	3.16 ± 0.56	3.19 ± 0.64	3.18 ± 0.68
Baecke[#] (Total)	8.63 ± 1.36	8.54 ± 1.43	8.44 ± 1.49	8.25 ± 1.45	8.28 ± 1.47	8.09 ± 1.42	8.74 ± 1.48	8.66 ± 1.34	8.65 ± 1.30
VAS[#] (Satiety)	44 ± 16	46 ± 16	46 ± 21	39 ± 23	40 ± 16	40 ± 20	37 ± 16	38 ± 17	40 ± 20
VAS[#] (Fullness)	43 ± 16	47 ± 12	49 ± 17	41 ± 23	37 ± 18	40 ± 13	34 ± 14	32 ± 14	42 ± 22
VAS[#] (Hunger)	33 ± 17	35 ± 17	28 ± 18	25 ± 19	26 ± 18	32 ± 17	34 ± 20	33 ± 18	34 ± 20
VAS[#] (Desire to eat)	37 ± 19	39 ± 15	33 ± 20	24 ± 17	30 ± 21	36 ± 25	37 ± 19	38 ± 21	39 ± 22

Values are means ± SD

DPI, daily protein intake; REE, resting energy expenditure; RQ, respiratory quotient; TFEQ, Three Factor Eating Questionnaire; VAS, visual analogue scale

[a] Significantly different from SMP diet group at same week, P<0.05

[b,c] Significantly different from [b]wk 8, [c]wk 16 within a diet group, P<0.05

[1,2,3] Factor 1, 2 and 3, respectively, of the TFEQ

[#] No significant difference between the GMP diet and the two control diets for changes in the variable from wk 8 to 16, and wk 16 to 24

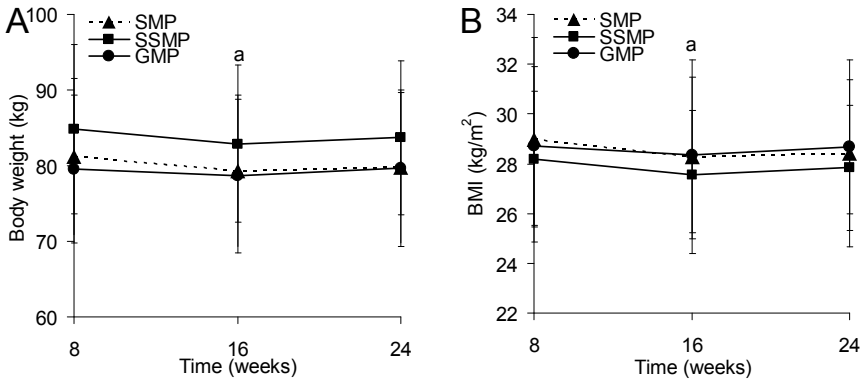

Figure 1 Body weight (A) and body mass index (BMI; B) for the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet group before (wk 8), during (wk 16) and after (wk 24) the weight maintenance period. Values are means ± SD. [a]Significantly different from wk 8 for all 3 diet groups, P<0.05. Changes in body weight and BMI from wk 8 to wk 16, and from wk 16 to wk 24, were not significantly different between the GMP diet and the two control diets.

Body composition

Fat free mass (FFM) in kg did not significantly change from wk 8 to 16, and from wk 16 to 24, in all 3 diet groups (**Figure 2A**). FFM% significantly increased, and fat mass (FM) in kg and expressed as % of BW were significantly decreased from wk 8 to 16 in all 3 diet groups (P<0.05, **Figure 2B-D**), while sustained from wk 16 to wk 24. The changes in FFM (kg or %) and FM (kg or %) were similar between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

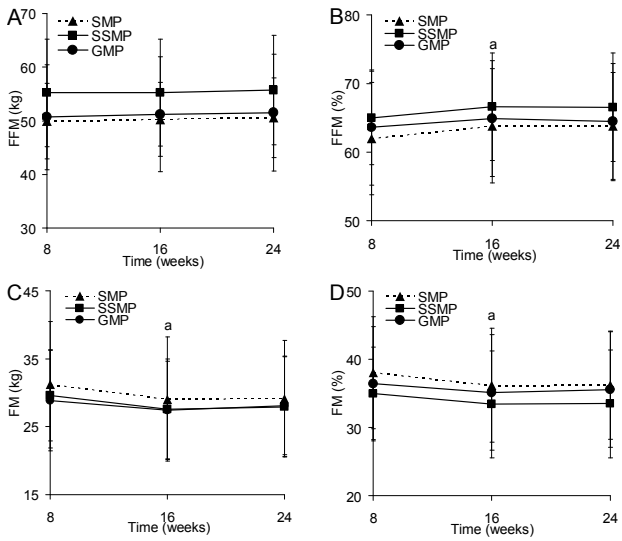

Figure 2 Fat free mass (FFM) in kg (A) and expressed as % of body weight (B), and fat mass (FM) in kg (C) and expressed as % of body weight (D) for the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet group before (wk 8), during (wk 16) and after (wk 24) the weight maintenance period. Values are means ± SD. [a]Significantly different from wk 8 for all 3 diet groups, P<0.05. Changes in FFM (kg or %) and FM (kg or %) from wk 8 to wk 16, and from wk 16 to wk 24, were not significantly different between the GMP diet and the two control diets.

RQ
RQ significantly increased from wk 8 to 16 in the SMP and SSMP diet group (P<0.05, **Table 2**), and changes in RQ were similar between the GMP diet group and both control groups.

REE as a function of FFM
REE is plotted as a function of fat free mass at the start (wk 8) and after 2 months of the weight maintenance period (wk 16) for the SMP (**Figure 3A**), SSMP (**Figure 3B**) and GMP (**Figure 3C**) diet group. In all 3 diet groups a significant linear relation was present between REE (MJ/d) and FFM (kg) at wk 8 and wk 16 (P<0.0001). To determine for each diet group if REE as function of FFM changed significantly over time, as shown by the regression lines at wk 8 and wk 16, the FFM (kg) values from wk 16 were filled in in the slope equation of wk 8 to obtain a calculated REE of wk 16. The calculated and measured REE of wk 16 were analysed with ANOVA repeated measures to determine any changes in REE as function of FFM. In all 3 diet groups, REE as a function of FFM appeared not to have changed significantly from wk 8 to 16, and possible small but not significant differences in these relationships did not differ between the GMP diet group and both control diet groups.

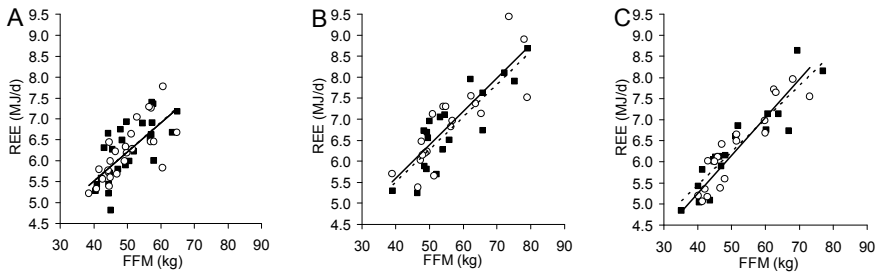

Figure 3 Resting energy expenditure (REE) as a function of fat free mass (FFM) plotted for wk 8 (start weight maintance, ○, trendline —) and for wk 16 (after 8 weeks of weight maintenance, ■, trendline ····) for the sustained milk protein (SMP; A), supra-sustained milk protein (SSMP; B) and supra-sustained gelatin-milk protein (GMP; C) diet group. Regression equation SMP diet, wk 8: REE = 0.069FFM + 2.747 (R^2 = 0.559, P<0.0001). Regression equation SMP diet, wk 16: REE = 0.071FFM + 2.68 (R^2 = 0.499, P<0.0001). Regression equation SSMP diet, wk 8: REE = 0.079FFM + 2.425 (R^2 = 0.717, P<0.0001). Regression equation SSMP diet, wk 16: REE = 0.077FFM + 2.42 (R^2 = 0.740, P<0.0001). Regression equation GMP diet, wk 8: REE = 0.09FFM + 1.631 (R^2 = 0.864, P<0.0001). Regression equation GMP diet, wk 16: REE = 0.079FFM + 2.292 (R^2 = 0.838, P<0.0001).

Blood pressure and heart rate
Diastole and systole were similar between wk 8 and 16, and wk 16 and 24 in all 3 diet groups (**Table 2**). Heart rate was similar between wk 8 and 16, and significantly increased from wk 16 to 24 (P<0.05) in all 3 diet groups (**Table 2**). The changes in blood pressure and heart rate were similar between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

Eating behavior
Dietary restraint (factor 1 of the TFEQ), disinhibition (factor 2) and hunger (factor 3) did not significantly change from wk 8 to 16, and from wk 16 to 24 in all 3 diet groups (**Table 2**). The changes in dietary restraint, disinhibition and hunger from wk 8 to 16 and from wk 16 to 24 were similar between the GMP diet group and both control groups.

Physical activity
Physical activity (Baecke Work, Sport, Leisure or Total, **Table 2**) did not significantly change from wk 8 to 16, and wk 16 to 24 in all 3 diet groups. The changes in physical activity were similar between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

Postabsorptive appetite profile
Postabsorptive scores for satiety, fullness, hunger and desire to eat were similar between wk 8 and 16, and wk 16 and 24 in all 3 diet groups. The changes in these scores were similar

between the GMP diet group and both control groups during the first 2 months and during the last 2 months of the weight maintenance period.

Blood parameters

Fasting blood concentrations were measured at wk 8 and 16 (**Table 3**). Plasma GLP-1, PYY, insulin and TAG concentrations, serum creatinin concentrations and HOMA index did not significantly change from wk 8 to 16 in all 3 diet groups. Plasma glucose concentrations significantly decreased in the GMP diet group ($P<0.05$), and did not significantly change in the SMP and SSMP diet groups. Plasma HDL concentrations significantly increased in the GMP diet group ($P<0.05$), and did not significantly change in the SMP and SSMP diet groups. Plasma LDL concentrations significantly increased in all 3 diet groups ($P<0.05$). Plasma FFA concentrations significantly decreased in the SMP and GMP diet groups ($P<0.05$), and did not significantly change in the SSMP diet group. The changes in all blood variables were similar between the GMP diet group and both control groups.

Table 3 Fasting blood variables of the sustained milk protein (SMP), supra-sustained milk protein (SSMP) and supra-sustained gelatin-milk protein (GMP) diet groups at the start and at wk 16 of weight maintenance[1]

	SMP		SSMP		GMP	
	Wk 8	Wk 16	Wk 8	Wk 16	Wk 8	Wk 16
GLP-1 (pmol/L)	1.3 ± 0.9	1.5 ± 1.3	2.1 ± 2.2	1.9 ± 2.2	1.3 ± 0.7	1.3 ± 0.7
PYY (pg/mL)	19 ± 11	16 ± 16	17 ± 17	15 ± 9	18 ± 18	25 ± 30
Insulin (µU/mL)	11.31 ± 3.92	11.03 ± 3.84	9.84 ± 3.91	10.51 ± 3.68	10.31 ± 4.31	10.31 ± 3.08
Glucose (mmol/L)	5.10 ± 0.51	4.99 ± 0.35	5.13 ± 0.48	5.08 ± 0.41	5.21 ± 0.52	4.94 ± 0.34[a]
HOMA index	2.60 ± 1.06	2.47 ± 0.98	2.27 ± 1.06	2.40 ± 0.94	2.41 ± 1.08	2.27 ± 0.68
Creatinine (µmol/L)	83 ± 16	84 ± 21	83 ± 16	75 ± 13	82 ± 15	83 ± 21
HDL (mmol/L)	1.36 ± 0.34	1.41 ± 0.40	1.30 ± 0.22	1.36 ± 0.28	1.38 ± 0.29	1.45 ± 0.32[a]
LDL (mmol/L)	2.91 ± 0.59	3.26 ± 0.82[a]	2.93 ± 0.99	3.44 ± 0.73[a]	3.05 ± 0.75	3.39 ± 0.85[a]
TAG (mmol/L)	1.15 ± 0.33	1.29 ± 0.45	1.00 ± 0.29	1.10 ± 0.29	1.06 ± 0.27	1.09 ± 0.31
FFA (mmol/L)	576 ± 198	440 ± 150[a]	542 ± 154	476 ± 115	514 ± 164	410 ± 166[a]

Values are means ± SD
[1] No significant difference in change over time between the GMP diet and the two control diets in all variables
[a] Significantly different from wk 8 within a diet group, $P<0.05$

DISCUSSION

In this study we investigated whether the addition of gelatin to a milk protein diet results in a better weight maintenance during a 4-months weight maintenance period after weight loss. All 3 diets resulted in a successful weight maintenance period, as no weight was significantly regained after weight loss at the end of the 4-months weight maintenance period. No significant differences between the GMP diet group and the SMP and SSMP diet groups were observed in changes over the first and last 8 weeks of the weight maintenance period in BW, BMI, waist:hip ratio, body composition, RQ, REE as a function of FFM, blood pressure, heart rate, dietary restraint, disinhibition, subjective feeling of general hunger, physical activity, postabsorptive appetite profile, plasma/serum GLP-1, PYY, insulin, glucose, creatinine, HDL, LDL, TAG, and FFA concentrations, and HOMA index. For successful weight maintenance, a sustained milk protein diet is sufficient, while addition of gelatin has no additional effects on weight maintenance. Compliance in this study was confirmed with the 24h urinary nitrogen results. The DPI of the sustained milk protein diet group at wk 8 and 16 was 0.8 g/kg per day, which is the required minimum amount of daily protein intake as recommended by the World Health Organization (33). The DPI of the supra-sustained protein diet groups, being 1.2 g/kg per day at wk 8 and 16, were significantly higher compared with the sustained protein diet group, while protein intake was similar between both supra-sustained protein diet groups. During the last 8 weeks of weight maintenance 50% of energy requirements was provided as a diet, which contained the same absolute amount of protein they had to consume during the first 8 weeks of weight maintenance. As subjects were allowed the eat ad libitum in addition, from the increases in protein intake in all 3 diet groups can be concluded that subjects were also compliant during the last 8 weeks of the weight maintenance period.

The beneficial short-term effect of gelatin on hunger suppression (10, 14) did not play a role in the long-term during weight maintenance. As expected the hunger suppression effect of an incomplete protein had disappeared by adding the complete milk-protein to the gelatin in a sufficient amount, and the supposed gluconeogenesis did not affect appetite in a way that satiety would be increased (19-22). This was confirmed by the postabsorptive appetite profiles and fasting plasma concentrations of the so-called satiety hormones GLP-1 and PYY. These variables did not significantly change over time and were not different between the GMP diet and both control diets. In addition, no significant changes in dietary restraint, disinhibition and general feelings of hunger as determined by the TFEQ were observed over time in all 3 diet groups, and as these changes over time were not different between the GMP diet and both control diets they could not have contributed to possible differences in weight change during this period. In all 3 diet groups the decrease in body weight from wk 8 to 16 was the result of a loss in absolute fat mass, while absolute fat free mass did not change. This resulted in an improved body composition, as in all 3 diet groups FFM% increased, and FM% decreased. From wk 16 to 24 the improved body composition was maintained in all 3 diet groups. No changes in FFM (kg or %) and FM (kg or %) were observed in all 3 diet groups. As physical activity did not change over time in all 3 diet groups, physical activity was not involved in the improvement in body composition due to sparing of fat free mass. Although from wk 8 to 16, protein intake was 0.43 and 0.05 g/kg per day higher in the GMP diet group compared with the SMP and SSMP diet groups, this did not result in a higher preservation of fat free mass. Thus, the addition of gelatin to a sustained milk protein diet, or the exchange of energy from milk protein with energy from gelatin in a supra-sustained protein diet did not affect body composition differently. Accordingly, resting energy expenditure as a function of fat free mass did not change from wk 8 to 16 in all 3 diet groups. In addition, changes in RQ over time were not different between the GMP diet group and both milk protein diet groups.

Regarding health benefits, during the first 8 weeks of the weight maintenance period beneficial reductions in waist:hip ratio and FFA were observed. Although after weight loss plasma LDL concentrations were decreased (34), from wk 8 to 16 of weight maintenance non-favourable increases in plasma LDL concentrations were observed in all 3 diet groups. The beneficial decreases in diastole, systole and heart rate after weight loss (34) were maintained throughout the 4 months weight maintenance period, except from an increase in heart rate during the last 8 weeks of weight maintenance in all 3 diet groups.

In summary, this study shows that effects of a diet observed in the short-term do not necessarily result in similar effects in the long-term, partly because it was not possible to give just gelatin as a protein over the long-term, partly because the possible additional effect due to additional

gelatin intake, namely gluconeogenesis, did not support body weight maintenance in a distinguishable way. The new aspect of this study was that the supposition that additional gelatin used in the long term could have a weak effect was ruled out.
We conclude that a supra-sustained gelatin-milk protein diet did not induce more beneficial effects on body weight maintenance and related variables during a 4-months weight maintenance period after weight loss compared with a sustained and supra-sustained milk protein diet.

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Chapter 8

General discussion

Increasing dietary protein from a relatively normal to a relatively high protein intake affects both short- and long-term mechanisms of body weight regulation by increased satiety and increased thermogenesis during energy balance, sustained satiety and energy expenditure during negative energy balance, preservation of fat-free mass during negative energy balance, and lower energy efficiency during positive energy balance. Protein types differ in their amino acid composition and metabolism and thus may affect these mechanisms differently. Therefore hypotheses were tested under normal and higher protein conditions with respect to appetite, energy expenditure, substrate balances, and body weight loss and maintenance, making use of different types of protein.

Effects on appetite may differ between dietary proteins in the short-term. First, we hypothesized that a protein high in tryptophan content, i.e. α-lactalbumin, leads to a higher satiety response than a protein low in tryptophan content, i.e. gelatin. Although tryptophan is a precursor for brain serotonin, which is involved in appetite regulation, the differences in tryptophan content or tryptophan/LNAA ratio between α-lactalbumin and gelatin did not seem to be involved in the more prolonged hunger suppression after the α-lactalbumin breakfast (1). Second, we hypothesized that an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, suppresses appetite when consumed in single-protein diets. For instance, gelatin suppressed hunger more than casein, which may be related to a system in the brain that can detect a deficiency of essential amino acids in the diet from a decline in serum amino acid levels, leading to a behavioral response that rejects consumption of imbalanced diets and thus appears as hunger suppression (2). Third, following the aminostatic hypothesis by Mellinkoff, the increased satiety with a protein that shows prolonged elevated concentrations of amino acids, for instance a relatively high-casein diet compared with a relatively normal-casein diet, may support this hypothesis (3).

We hypothesized that a diet relatively high in casein/gelatin content results in higher energy expenditure than a diet relatively normal in casein/gelatin content, because the body has a limited storage capacity for protein when ingested in amounts that exceed the minimum requirement and therefore it has to be processed metabolically, which involves high ATP costs. In addition, we hypothesized that the use of an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, increases energy expenditure due to a surplus of amino acids that could not be used for protein synthesis. Energy expenditure was higher with the relatively high-protein diets compared with the relatively normal-protein diets, regardless of differences in amino acid composition (casein or gelatin), although the magnitude of the effect was higher with casein (3, 4). The increased energy expenditure results from high ATP costs for postprandial protein synthesis and oxidation, urea production and/or gluconeogenesis. When effects on energy expenditure are compared between protein types, a complete protein (casein) induces similar effects on 24-h energy expenditure compared with an incomplete protein (gelatin), despite differences in inefficiency of protein oxidation between protein types (2). With respect to protein balance, we hypothesized that the use of an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, limits a positive protein balance when consumed in single-protein diets. It was shown that an incomplete protein limits a positive protein balance, probably because a lower availability of essential amino acids limits postprandial protein synthesis, while amino acid oxidation is increased (2). Since protein oxidation rates were different between a complete and incomplete protein, while energy expenditure was not, this shows that effects on energy expenditure are not only determined by differences in costs and rate of protein oxidation. Energy costs for protein synthesis, urea production and/or gluconeogenesis also contribute.

Finally, short-term effects of proteins do not necessarily result in similar effects in the long-term. The addition of gelatin to a sustained milk-protein diet does not improve body weight loss and related variables during a weight loss and subsequent weight maintenance period compared with (supra-)sustained milk-protein diets (5, 6). Since the effect of gelatin on hunger in the short-term may result from the incompleteness as a protein source, the effect on hunger disappeared by complementation of gelatin with a complete protein source in a long-term diet, and no residual effect of gelatin remained.

APPETITE: PROTEIN-INDUCED SATIETY

It was shown that effects on appetite differ between protein breakfasts/diets in the short-term, because of differences in amino acid composition and protein metabolism (1, 2). We hypothesized that the tryptophan content of proteins may contribute to differences in appetite. Tryptophan is a precursor for serotonin (7, 8), and competes with the other large neutral amino

acids (LNAA) valine, leucine, isoleucine, tyrosine and phenylalanine for transport across the blood-brain barrier (9). Brain serotonin seems to play a role in food regulation, since suppressive effects on food intake and body weight are observed with serotonergic stimulation, and the opposite effect, an increased food intake, when reducing serotonergic activity (7, 10). Therefore, α-lactalbumin, gelatin and gelatin with added tryptophan were compared. A more prolonged suppression of hunger after a breakfast containing α-lactalbumin than after a breakfast containing gelatin or gelatin with added tryptophan was observed (1). However, the differences in hunger ratings between the three breakfasts could not straightforwardly be explained by changes in tryptophan concentrations or the tryptophan/LNAA ratio in the custard or plasma. In another study an α-lactalbumin supplement was consumed after a relatively normal-protein breakfast and compared with a placebo supplement (9). Although changes in plasma tryptophan/LNAA ratio differed between the two supplements, since the plasma ratio increased after the α-lactalbumin supplement and decreased after the placebo supplement, appetite and food intake were not different after both supplements. In addition to proteins, which may affect plasma tryptophan/LNAA ratio by their tryptophan and LNAA content, carbohydrates may affect plasma tryptophan/LNAA ratio by their accompanying insulin response, since insulin selectively promotes peripheral uptake of the LNAA, thereby increasing the tryptophan/LNAA ratio in the plasma (11). In the long-term, no evidence was found for the hypothesis that diets modified in macronutrient distribution cause a negative energy balance by increasing the availability of circulating tryptophan for serotonin synthesis in the brain, in that 24-h integrated plasma tryptophan concentrations and tryptophan/LNAA ratio were not changed by different diets and did not mediate the negative energy balance (12). In addition to our results, this suggests that tryptophan/LNAA ratio in the plasma is not directly linked to appetite via the serotonin-pathway, also since no significant correlation was found between the AUC of the hunger scores and the AUC of plasma tryptophan/LNAA ratios or plasma tryptophan concentrations following ingestion of α-lactalbumin, gelatin or gelatin with added tryptophan breakfasts (1). Other mechanisms must have played a role in the differences in protein-induced hunger suppression between gelatin and α-lactalbumin, however these have not yet been elucidated (1).

In the comparison between the incomplete protein gelatin and the complete protein casein, a larger hunger suppression after gelatin than after casein was observed, when consumed as single-protein diets for 1 day (2). This is in accordance with previous results on the same single-protein breakfasts: higher satiety and lower hunger ratings were observed after a 10 En% gelatin breakfast compared with a 10 En% casein breakfast (13). In this respect the completeness of the protein source may have played a role, since the complete protein casein contains all indispensable amino acids (IAA) in sufficient amounts, while the incomplete protein gelatin lacks the essential amino acid tryptophan and contains relatively low amounts of the other indispensable amino acids, i.e. histidine, threonine, valine, methionine, isoleucine, phenylalanine, leucine, and lysine (2, 13). We hypothesize that the difference in hunger suppression may relate to a mechanism observed in metazoans, where it was discovered that the tRNA/GCN2/p-eIF2α system in the brain can detect a deficiency of IAA in the diet from a decline in serum amino acid levels, leading to a behavioral response that rejects consumption of imbalanced diets (14-17) and thus appears as hunger suppression. Although our study protocol was not designed to test this hypothesis, the amino acid concentrations measured after the breakfasts may support a possible role of the ‘indispensable amino acid deficiency hypothesis’. The effect of gelatin, compared with casein, on overall hunger response was observed under relatively normal protein conditions, but not under relatively high protein conditions (2). Under relatively normal conditions this coincided with decreased and lower 4 h-concentrations of the essential amino acids histidine, valine, methionine, isoleucine, phenylalanine, tryptophan, and leucine after the gelatin compared with the casein breakfast, while only tryptophan was reduced and lower under relatively high protein conditions (2). The reduced concentrations of the IAA after gelatin consumption may indicate a deficiency of these IAA, which can be detected by the tRNA/GCN2/p-eIF2α system in the brain, resulting in a higher hunger suppression compared with casein consumption.

Increasing the protein content in a meal/diet results in an increased satiety in energy balance (18-21). In a high-casein breakfast, higher satiety ratings coincided with prolonged elevated concentrations of amino acids, indicating a slower gastric emptying (22). These prolonged elevated concentrations of amino acids may have contributed to the higher satiety ratings, in agreement with the amino static theory of Mellinkoff et al (23). They suggest that a satiety center in the brain exists, which is sensitive to serum amino acid levels, and once amino acid

concentrations reach a certain point, appetite decreases (18, 23). It was shown that the magnitude of the effect on appetite with increased protein content differs due to differences in amino acid composition of the diet, since the effect of an increased casein intake on appetite was stronger than the effect of an increased gelatin intake (3, 4). Casein showed an overall increased satiety and decreased hunger response, while gelatin did not. The mechanisms contributing to effects on appetite may differ between protein types. For instance, also an increased soy content in a single-protein breakfast increases satiety, which is related to increased taurine responses and coincides with increased insulin responses (24).
In addition to this mechanism, changes in satiety and hunger were related to changes in SMR with the high-casein diet (3). Since the high-casein diet resulted in a higher SMR, a suggestion for this relation, based on 3 other studies that observed satiety scores under limited oxygen availability conditions (25-27), is that oxygen availability becomes limiting with an increased metabolic rate at rest, which seems to be perceived by the subjects as a reduction in the possibility to eat and therefore is rated as an increase in satiety (21).

ENERGY EXPENDITURE

It was shown that a complete protein (casein) and an incomplete protein (gelatin) induce similar effects on energy expenditure, regardless of consumption under relatively normal or high-protein feeding conditions (2). Possible mechanisms that contribute to protein-induced energy expenditure are postprandial protein synthesis with high ATP costs for peptide bond synthesis and high ATP costs for urea production or gluconeogenesis (18, 28-30). In addition to these mechanisms the metabolic efficiency of protein oxidation, i.e. amino acid oxidation, also contributes to effects on energy expenditure, which depends on the amino acid composition of the protein. We hypothesized that gelatin oxidation is more inefficient than casein oxidation, i.e. 15.6 and 8.2 kJ/ATP per gram protein respectively (31), and in this way that gelatin will increase energy expenditure more than casein. Gelatin and casein induced similar effects on energy expenditure, even though protein oxidation was higher with the gelatin diets than with the casein diets (2). Therefore other mechanisms than protein oxidation alone must have contributed to the effects on energy expenditure. Although not measured directly, protein synthesis was probably higher after consumption of the casein diets compared with the gelatin diets, since a more positive/less negative protein balance with the casein diets compared with the gelatin diets was observed (2). Studies observed that large doses of essential amino acids (like valine, leucine, and phenylalanine) increase muscle protein synthesis, while non-essential amino acids (like serine, alanine or proline) do not (32, 33). Since gelatin is an incomplete protein with respect to the amount of essential amino acids, while casein is a complete protein, a higher protein synthesis after casein consumption is suggested to have contributed to the similar effect on EE of both protein types. Thus, the contribution of mechanisms involved in postprandial protein metabolism, e.g. protein synthesis, protein oxidation, urea synthesis, and gluconeogenesis, depends on the amino acid composition of proteins, and therefore energy expenditure can be affected similarly or differently dependent on protein sources consumed. Protein oxidation contributed more to energy expenditure with gelatin, since oxidation of this incomplete protein was higher and is hypothesized to be more inefficient, while the contribution of protein synthesis to energy expenditure is hypothesized to be higher with casein, since casein is a complete protein and resulted in higher protein balances.
Increasing the protein content in a meal/diet results in increased energy expenditure, i.e. an increased 24-h EE, DIT or SMR (19, 21, 29, 34-38). We showed that energy expenditure, i.e. 24-h EE and SMR, was higher with the relatively high-protein diets compared with the relatively normal-protein diets, regardless of differences in amino acid composition (casein or gelatin), although the effect on energy expenditure was stronger with casein than with gelatin as protein source (3, 4). As will be discussed in a later section, differences in substrate balances between casein and gelatin under relatively normal as well as under relatively high protein conditions were observed. Therefore, contribution of mechanisms that increase energy expenditure with an increased protein content of the diet differ between proteins. As discussed previously, the contribution of protein synthesis to increased energy expenditure was probably higher with an increased complete protein (casein) content than with an increased incomplete protein (gelatin) content in the diet, while the contribution of protein oxidation to increased energy expenditure was higher with an increased gelatin content than with an increased casein content in the diet. In addition, we hypothesize that the contribution of gluconeogenesis to increased energy expenditure is higher with an increased incomplete protein (gelatin) content than with an

increased casein content (4). The rationale behind this hypothesis is that the lack of and deficiency in several essential amino acids acts as a limiting factor for postprandial protein synthesis. This results in an increase in free amino acids, because they are not used for protein synthesis. Part of these 'extra' free amino acids may be oxidized, but part of these amino acids can also be used for gluconeogenesis to be finally stored as glycogen. In a recent study Veldhorst et al observed that, after a decrease in body glycogen stores, the increased energy expenditure after a high-protein, carbohydrate-free diet compared with a normal-protein diet was for 42% explained by an increased gluconeogenesis (39). This study showed that gluconeogenesis contributes to an increased EE with an increased protein intake.

SUBSTRATE BALANCE

It was shown that a complete protein (casein) and an incomplete protein (gelatin) induce different effects on substrate balances as a result of differences in amino acid composition and protein metabolism between the protein sources (2). Under relatively normal as well as relatively high protein conditions, protein oxidation was higher with the gelatin than with the casein diets, resulting in lower protein balances. The completeness of a protein regarding content of essential amino acids is important, since these results suggest that protein synthesis was lower with the gelatin than with the casein diets. It is observed that muscle protein anabolism is stimulated by oral ingestion of a mixture of amino acids similar to that of meat proteins (40) and by a solution of only essential amino acids plus carbohydrate (41). A comparison between types of amino acids showed in young subjects that large doses of essential amino acids (like valine, leucine, and phenylalanine) increase muscle protein synthesis, while non-essential amino acids (like serine, alanine or proline) do not (32, 33). The suggestion that essential amino acids are primarily responsible for amino acid induced stimulation of muscle protein synthesis, was confirmed in elderly subjects (42). Addition of non-essential amino acids to a supplement of essential amino acids did not have an additional effect on muscle protein anabolism above the effect of the essential amino acids alone (42). Thus, the absence or low amounts of certain essential amino acids in an incomplete protein are a limiting factor for postprandial protein synthesis. The larger negative protein balance under relatively normal protein conditions with the gelatin diet shows that when the total amount of amino acids in the diet is too low, with respect to total body protein turnover, protein loss from the body is higher with an incomplete than with a complete protein (2). Thus, for prevention or reduction of muscle mass loss the use of a complete protein is prioritised over an incomplete protein source. Under relatively high protein conditions, when amino acid supply from the diet is excessive with respect to total body protein turnover, a complete protein may increase muscle mass more than an incomplete protein, which can be important for improving body composition in the long-term. In addition to differences in completeness, differences in digestion rate between protein sources also have an effect on protein synthesis (43-46). After ingestion of whey, a 'fast' protein, plasma appearance of dietary amino acids is fast, high, and transient. On the contrary, after ingestion of casein, a 'slow' protein, plasma appearance of dietary amino acids is slower, lower, and prolonged. Fastly digested proteins strongly increase amino acid availability, resulting in a significant stimulation of protein synthesis and protein oxidation, but no change in protein breakdown, while slowly digested proteins slightly increase protein synthesis and oxidation, but markedly inhibit protein breakdown (43). In this way, in young men slowly digested protein meals (only consisting of protein) are more efficient for postprandial protein deposition than fastly digested protein meals, independent of amino acid composition (43, 44, 46). When young men ingest a slow or a fast protein together with fats and carbohydrates as mixed meal, whey is still more rapidly absorbed and induces higher and more transient hyperaminoacidemia than casein (47). However, the difference between the proteins in mixed meals is less pronounced than when the proteins are given alone, which is mainly the result of a slower digestion rate of whey in the presence of carbohydrate and fat (47). Since casein is slowly digested, also in the presence of fats and carbohydrates, this aspect may also have contributed to the more positive and less negative protein balance, i.e. higher protein synthesis, observed with the casein compared with the gelatin diets.

Since fat balances are not different between casein and gelatin under relatively normal as well as relatively high protein conditions (2), this suggests that differences in amino acid composition between protein types do not affect overall fat storage and oxidation. On the contrary, differences in completeness between protein sources do influence overall carbohydrate storage and oxidation, since carbohydrate balance was more positive with gelatin than with casein

under relatively high protein conditions (2). As discussed previously, we hypothesize that part of the explanation for this effect is an increased gluconeogenesis with the gelatin diet, which is directed to glycogen storage, and which is due to an increase in free amino acids that are not used for protein synthesis.
Although the magnitude of macronutrient balances differ between a complete protein casein and an incomplete protein gelatin (2), both proteins induce a positive protein balance, negative fat balance and positive carbohydrate balance when the amount of protein is increased from 10 En% to 25 En% in a single-protein diet (3, 4). Thus, a relatively high content of protein in a diet is important to prevent protein loss from the body and to promote protein storage, while the use of a complete protein is prioritized over an incomplete protein. Over a period of 4 days, consumption of a relatively high-protein diet still results in a positive nitrogen balance, regardless of protein type used (3, 4). Using complete protein sources it is observed that, in energy balance, a 3-mo dietary intervention with an increased protein intake results in a significant increase in fat free mass of 0.73 kg, independently of change in body weight and with similar physical activity levels during the intervention period (48). Therefore we suggest that with an increased intake of a complete protein a positive protein balance may be sustained for a longer period of time, but to a lesser extent than observed over 4 days. However, we expect that even a high intake of gelatin in the long term will not maintain this positive protein balance, as gelatin has a low biological value, which will depress whole body protein synthesis. With respect to fat balance, similar negative fat balances are observed with increased protein content in a single-protein diet from either casein or gelatin (3, 4). When consuming a relatively high-protein diet, in which protein is exchanged by carbohydrate, while fat content is kept similar compared with an adequate-protein diet, this also results in a negative fat balance (19). Thus, a relatively high-protein diet plays a role in the stimulation of fat oxidation per se, and this does not depend on protein type (casein or gelatin) used. Indeed, in the long-term, high-protein diets favor fat loss above loss of fat free mass during weight maintenance after weight loss (49, 50).

WEIGHT LOSS AND MAINTENANCE

It was observed that the addition of gelatin to a sustained milk protein diet does not improve weight loss and body composition more during a weight loss period and does not result in a better weight maintenance period after weight loss compared with a sustained or supra-sustained milk protein diet (5, 6). This shows that effects of proteins observed in the short-term do not necessarily result in similar effects in the long-term. The larger hunger suppression and lower energy intake after gelatin than after casein, soy or whey without glycomacropeptide in the short-term (2, 13) does not result in a higher weight loss or a better weight maintenance in the long-term when adding gelatin to a sustained milk protein diet. We hypothesized that the incompleteness of gelatin is involved in the larger hunger suppression after gelatin compared with the other proteins through the tRNA/GCN2/p-eIF2α system in the brain (14-17). When consuming a diet with a mixture of gelatin and milk protein, a complete protein, the deficiency in essential amino acids in gelatin is complemented with the essential amino acids present in milk protein. Thus, the beneficial effect of gelatin on hunger in the short-term does not play a role anymore in the long-term and therefore the gelatin diet does not result in a higher weight loss and/or better weight maintenance. Consumption of a diet with a sustained amount of milk protein is sufficient for a successful weight loss and weight maintenance period. A sustained milk protein diet and a supra-sustained milk protein diet result in similar decreases in body weight, fat mass and fat percentage during a weight loss and subsequent weight maintenance period, while sparing of fat free mass is more successful with the extra protein intake from the supra-sustained milk protein diet over 6 months (51). Addition of the incomplete protein gelatin does not improve body composition more during weight loss as well as weight maintenance compared with sustained and supra-sustained milk protein diets (5, 6). In this respect, extra intake of a complete protein is more beneficial for sparing of fat free mass than extra intake of an incomplete protein. In addition to the studies that observed higher weight losses (52, 53) with relatively high-protein diets, and less weight regain during weight maintenance (49, 50), we showed that a sustained protein intake from milk protein is sufficient to induce similar weight losses when compared with extra protein intake from an incomplete protein. This suggests that the required minimum absolute amount of protein intake is sufficient to accomplish the beneficial effects of protein on satiety and weight loss. This is in line with the “protein leverage hypothesis”, which implies that maintaining absolute daily protein intake is prioritized over fat and carbohydrate intakes, regardless of macronutrient composition of the diets (54).

The sustained and both supra-sustained protein diets all resulted in a successful weight loss and complete weight maintenance thereafter. Satiety was sustained during weight loss and maintained during weight maintenance, which may be a possible mechanism for the success. During weight loss and weight maintenance fat free mass was (partly) preserved and fat mass was reduced, which improved body composition and which is an energy inefficient process. With the supra-sustained milk protein diet resting energy expenditure as a function of fat free mass was sustained during negative energy balance, while it decreased with the sustained milk protein diet and supra-sustained gelatin-milk protein diet. Metabolic targets were affected during weight loss, with less reversal effects during weight maintenance. Thus, all 3 diets result in a successful weight loss and complete weight maintenance thereafter, while a protein intake of 0.8 g/kg per day of milk protein is sufficient to achieve this.

CONCLUSIONS

1. The tryptophan/LNAA ratio in the plasma is not directly linked to appetite via the serotonin-pathway.
2. Gelatin suppresses hunger more than casein, when consumed as a single-protein diet for one day.
3. Higher satiety ratings under high-casein, compared with normal-casein, conditions coincide with prolonged elevated concentrations of amino acids, which contribute to satiety according to the 'the amino static theory'.
4. Increased satiety ratings under high-casein conditions relate to increases in sleeping metabolic rate.
5. The contributions of mechanisms to increased energy expenditure with increased protein content differ between a complete protein (casein) and incomplete protein (gelatin). Protein synthesis contributes more to increased energy expenditure with a complete protein, while protein oxidation contributes more with an incomplete protein.
6. An incomplete protein (gelatin) results in a lower protein balance than a complete protein (casein), because of a higher protein oxidation together with lower, limited, protein synthesis.
7. Carbohydrate balance is higher with an incomplete protein under relatively high protein conditions, suggesting to be partly explained by increased gluconeogenesis.
8. Fat balances are not affected differently between conditions with an incomplete or complete protein diet.
9. Short-term effects of protein diets do not necessarily result in similar effects in the long-term.
10. A minimum required daily protein intake from milk protein is sufficient for succesfull weight loss and maintenance, while addition of gelatin does not improve this.

FUTURE RESEARCH

A positive protein balance over a period of 4 days of consumption of relatively high-protein diets was observed. It is of interest to study if this short-term effect still holds in the long-term. Subjects should be tested in energy balance while consuming a high-protein diet over a period of 3 months. Substrate balances should be measured in the respiration chamber on several occasions during this period. In addition, body composition can be measured to observe if body composition improves during this period. If a positive protein balance is maintained longer than the 4 days, body composition may give more information whether the positive protein balance actually results in an increased fat free mass, indicating protein synthesis.
Similar energy expenditure between gelatin and casein were observed, while protein and/or carbohydrate balances differed. It is of interest to study what processes in the protein metabolism, i.e. protein synthesis, protein oxidation, urea production or gluconeogenesis, contributed to energy expenditure and how the contributions of these mechanisms differ between casein and gelatin. This provides more information about how protein metabolism differs between protein types and to what extent these mechanisms contribute to energy expenditure.

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Summary

Regulation of body weight implies a balance between energy intake and energy expenditure. Nowadays many people struggle with the balance between intake and expenditure as reflected in the increasing incidence of obesity, requiring strategies for weight loss and weight maintenance thereafter. Increasing dietary protein from a relatively normal to a relatively high protein intake affects both short- and long-term mechanisms of body weight regulation by increased satiety and increased thermogenesis during energy balance, sustained satiety and energy expenditure during negative energy balance, preservation of fat-free mass during negative energy balance, and lower energy efficiency during positive energy balance. Protein types differ in their amino acid composition and metabolism and thus may affect these mechanisms differently. Therefore several hypotheses were tested under normal and higher protein conditions with respect to appetite, energy expenditure, substrate balances, and body weight loss and maintenance, making use of different types of protein.

Regarding effects on appetite, we hypothesized that 1) a protein high in tryptophan (TRP) content (α-lactalbumin) leads to a higher satiety response than a protein low in TRP content (gelatin), due to its higher TRP content; 2) an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, suppresses appetite when consumed in single-protein diets. Regarding effects on energy expenditure, we hypothesized that 1) a diet relatively high in casein/gelatin content results in higher energy expenditure, and higher satiety response, than a diet relatively normal in casein/gelatin content; 2) the use of an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, increases energy expenditure due to a surplus of amino acids that could not be used for protein synthesis. Regarding effects on substrate balance, we hypothesized that the use of an incomplete protein, i.e. gelatin, compared with a complete protein, i.e. casein, limits a positive protein balance when consumed in single-protein diets. Regarding effects on weight loss and weight maintenance, we hypothesized that the addition of gelatin to a milk protein diet will promote weight loss during a weight loss period and will improve weight maintenance during a 4-months weight maintenance period after weight loss.

The hypotheses were tested in subsequent studies. Three relatively normal single-protein breakfasts (10/55/35% of energy from protein/carbohydrate/fat), containing either α-lactalbumin (high in TRP), gelatin (low in TRP) or gelatin with added TRP (gelatin+TRP, high in TRP), were consumed by healthy subjects according to subject-specific energy requirements and were compared on appetite and energy intake (**chapter 2**). Subsequent, relatively normal and high single-protein diets (10/55/35% and 25/55/20% of energy, respectively, from protein/carbohydrate/ fat), with casein or gelatin as protein source, were consumed by healthy subjects according to subject-specific energy requirements, and were compared with respect to their effects on 24-h energy expenditure, substrate balances and appetite (**chapter 3-5**). Last, a supra-sustained gelatin-milk protein (GMP, protein content: 50% milk protein and 50% gelatin) diet was compared with a sustained milk protein (SMP, protein content: 100% milk protein) diet and supra-sustained milk protein (SSMP, protein content: 100% milk protein) diet in overweight and obese subjects during an 8-wk weight loss period, followed by an 4 months weight maintenance period (**chapter 6 and 7**). Absolute protein intake was kept constant (sustained) per subject during the complete 6 months dietary intervention.

The α-lactalbumin breakfast suppressed hunger more prolonged than the gelatin or gelatin+TRP breakfasts, but this could not be explained by differences found in TRP concentrations or TRP/LNAA ratios in the breakfasts or plasma. No significant correlation was found between the AUC of the hunger scores and the AUC of plasma tryptophan/LNAA ratios or plasma TRP concentrations following ingestion of α-lactalbumin, gelatin or gelatin+TRP breakfasts. In the comparison between the incomplete protein gelatin and the complete protein casein, larger hunger suppression after gelatin than after casein was observed. We hypothesize that the difference in hunger suppression may relate to a mechanism observed in metazoans, where it was discovered that the tRNA/GCN2/p-eIF2α system in the brain can detect a deficiency of indispensable amino acids in the diet from a decline in serum amino acid levels, leading to a behavioral response that rejects consumption of imbalanced diets and thus appears as hunger suppression. Finally, it was shown that the magnitude of the effect on appetite with increased protein content differs due to differences in amino acid composition of the diet, since the effect of an increased casein intake on appetite was stronger than the effect of an increased gelatin intake. Mechanisms contributing to effects on appetite may differ between protein types. Prolonged elevated concentrations of amino acids may have contributed to the higher satiety ratings with an increased casein intake, in agreement with the amino static theory of Mellinkoff

et al. In addition, changes in satiety and hunger were related to changes in sleeping metabolic rate (SMR) with the high-casein diet.
Energy expenditure (EE), i.e. 24-h EE and SMR, was higher with the relatively high-protein diet compared with the relatively normal-protein diet for both casein and gelatin. In addition, a complete protein (casein) and an incomplete protein (gelatin) induce similar effects on energy expenditure, regardless of consumption under relatively normal or high-protein feeding conditions. Contribution of mechanisms involved in postprandial protein metabolism, e.g. protein synthesis, protein oxidation, urea synthesis, and gluconeogenesis, depends on the amino acid composition of proteins, and therefore energy expenditure can be affected similarly or differently dependent on protein sources consumed. Regarding protein balances, under relatively normal as well as relatively high protein conditions, protein oxidation was higher with the gelatin than with the casein diets, resulting in lower protein balances. The absence or low amounts of certain essential amino acids in an incomplete protein are a limiting factor for postprandial protein synthesis. This suggests that protein oxidation contributed more to energy expenditure with gelatin, while the contribution of protein synthesis to energy expenditure is hypothesized to be higher with casein. Fat balances were not different between casein and gelatin under relatively normal as well as relatively high protein conditions, which suggests that differences in amino acid composition between protein types do not affect overall fat storage and oxidation. Carbohydrate balance was more positive with gelatin than with casein under relatively high protein conditions. We hypothesize that part of the explanation for this effect is an increased gluconeogenesis with the gelatin diet, which is directed to glycogen storage, and which is due to an increase in free amino acids that are not used for protein synthesis.
Addition of gelatin to a sustained milk protein diet does not improve weight loss and body composition more during a weight loss period and does not result in a better weight maintenance period after weight loss compared with a sustained or supra-sustained milk protein diet. This shows that effects of proteins observed in the short-term do not necessarily result in similar effects in the long-term. The sustained and both supra-sustained protein diets all resulted in a successful weight loss and complete weight maintenance thereafter, while a protein intake of 0.8 g/kg per day of milk protein is sufficient to achieve this.
In conclusion, 1) the tryptophan/LNAA ratio in the plasma is not directly linked to appetite via the serotonin-pathway; 2) gelatin suppresses hunger more than casein, when consumed as a single-protein diet for one day; 3) higher satiety ratings under high-casein, compared with normal-casein, conditions coincide with prolonged elevated concentrations of amino acids, which contribute to satiety according to the 'the amino static theory'; 4) increased satiety ratings under high-casein conditions relate to increases in sleeping metabolic rate; 5) the contributions of mechanisms to increased energy expenditure with increased protein content differ between a complete protein (casein) and incomplete protein (gelatin). Protein synthesis contributes more to increased energy expenditure with a complete protein, while protein oxidation contributes more with an incomplete protein; 6) an incomplete protein (gelatin) results in a lower protein balance than a complete protein (casein), because of a higher protein oxidation together with lower, limited, protein synthesis; 7) carbohydrate balance is higher with an incomplete protein under relatively high protein conditions, suggesting to be partly explained by increased gluconeogenesis; 8) fat balances are not affected differently between conditions with an incomplete or complete protein diet; 9) short-term effects of protein diets do not necessarily result in similar effects in the long-term; 10) a minimum required daily protein intake from milk protein is sufficient for successful weight loss and maintenance, while addition of gelatin does not improve this.

Samenvatting

Regulatie van het lichaamsgewicht houdt in dat er een balans moet zijn tussen energie-inname en energiegebruik. Gezien de toename van de incidentie van overgewicht en obesitas gedurende de laatste drie decennia, hebben veel mensen moeite met het handhaven van deze balans. Dit betekent dat er wetenschappelijk onderbouwde strategieën nodig zijn om gewicht te verliezen en daarna dit gewicht te behouden. Een relatieve verhoging van de hoeveelheid eiwit in het dieet beïnvloedt zowel de korte als de lange termijn mechanismen voor lichaamsgewichtregulatie. Deze zijn een toename in verzadiging en energiegebruik in energiebalans, het handhaven van verzadiging en energiegebruik in negatieve energiebalans, het behouden van de vetvrije massa in negatieve energiebalans, en een lagere energetische efficiëntie in positieve energiebalans. Eiwitten verschillen in hun aminozuursamenstelling en metabolisme, waardoor zij verschillen in deze mechanismen. Hieruit volgen verschillende hypotheses gericht op eetlust, energiegebruik, substraatbalans en verlies en behoud van lichaamsgewicht. Deze werden getoetst voor verschillende eiwitten onder condities van normale en relatief hoge eiwitinname.

Met betrekking tot de effecten op eetlust werden de volgende hypotheses getoetst: 1) Een eiwit met een hoog tryptofaan (TRP)-gehalte (α-lactalbumine) resulteert in een hogere verzadiging dan een eiwit met een laag TRP-gehalte (gelatine) ten gevolge van het hogere TRP-gehalte; 2) een incompleet eiwit (gelatine) onderdrukt de eetlust meer dan een compleet eiwit (caseïne) als deze in een dieet worden genuttigd bestaande uit slechts één eiwitsoort. Met betrekking tot de effecten op energiegebruik werden de volgende hypotheses getoetst: 1) Een dieet met een relatief hoog caseïne/gelatinegehalte resulteert in een hoger energiegebruik, en hogere verzadiging, dan een dieet met een relatief laag caseïne/gelatinegehalte; 2) het nuttigen van een incompleet eiwit (gelatine) in vergelijking met een compleet eiwit (caseïne) verhoogt het energiegebruik door een overschot aan aminozuren die niet gebruikt kunnen worden voor eiwitsynthese. Met betrekking tot de effecten op substraatbalans werd de hypothese getoetst dat het nuttigen van een incompleet eiwit (gelatine) in vergelijking met een compleet eiwit (caseïne) een positieve eiwitbalans beperkt als deze in een dieet worden genuttigd bestaande uit slechts één eiwitsoort. Met betrekking tot de effecten op gewichtsverlies en gewichtsbehoud werd de hypothese getoetst dat het toevoegen van gelatine aan een melkeiwitdieet gewichtsverlies zal bevorderen gedurende een gewichtsverliesperiode, en na deze periode gewichtsbehoud zal bevorderen gedurende een periode van 4 maanden waarin naar gewichtsbehoud wordt gestreefd.

Hiertoe werden de volgende studies uitgevoerd. Drie ontbijten met een relatief normaal eiwitgehalte (10/55/35 energieprocenten van eiwit/koolhydraat/vet) bestaande uit slechts één eiwitsoort, namelijk α-lactalbumine (hoog in TRP), gelatine (laag in TRP) of gelatine met toegevoegd TRP (gelatine+TRP, hoog in TRP), werden genuttigd door gezonde proefpersonen op basis van proefpersoonspecifieke energiebehoeftes. De drie ontbijten werden vergeleken ten aanzien van hun effecten op eetlust en energie-inname (**hoofdstuk 2**). Vervolgens werden diëten met een relatief normaal of hoog eiwitgehalte (respectievelijk 10/55/35 of 25/55/20 energieprocenten van eiwit/koolhydraat/vet) bestaande uit slechts één eiwitsoort, namelijk caseïne of gelatine, genuttigd door gezonde proefpersonen op basis van proefpersoonspecifieke energiebehoeftes. De diëten werden vergeleken ten aanzien van hun effecten op 24-uurs energiegebruik, substraatbalans en eetlust (**hoofdstuk 3-5**). Tenslotte werden drie verschillende eiwitdiëten genuttigd door proefpersonen met overgewicht en obesitas gedurende een gewichtsverliesperiode van 8 weken, gevolgd door een periode van 4 maanden waarin werd gestreefd naar gewichtsbehoud (**hoofdstuk 6 en 7**). Gedurende de hele periode van 6 maanden werd de absolute dagelijkse eiwitinname voor elke proefpersoon constant gehouden. Deze was gelijk aan de aanbevolen dagelijkse hoeveelheid eiwitinname (het SMP-dieet, waarvan het eiwitgehalte voor 100% uit melkeiwit bestond) of een hogere inname dan deze aanbevolen dagelijkse hoeveelheid eiwitinname (het SSMP-dieet, waarvan het eiwitgehalte voor 100% uit melkeiwit bestond, of het GMP-dieet, waarvan het eiwitgehalte voor 50% uit melkeiwit en 50% uit gelatine bestond). Het gelatinedieet werd vergeleken met beide melkeiwitdiëten met betrekking tot effecten op gewichtverlies en gewichtsbehoud.

Het α-lactalbumine-ontbijt onderdrukte de honger meer gedurende langere tijd dan de gelatine- en gelatine+TRP-ontbijten. Echter, dit kon niet worden verklaard door waargenomen verschillen in TRP-concentraties of TRP/LNAA-verhoudingen in de ontbijten of het bloedplasma. Er werd geen significante correlatie gevonden tussen de AUC van de hongerscores en de AUC van de TRP/LNAA-verhoudingen of TRP-concentraties in het bloedplasma na het nuttigen van het α-lactalbumine-, gelatine- of gelatine+TRP-ontbijt. In de vergelijking tussen het incomplete eiwit gelatine en het complete eiwit caseïne, onderdrukte gelatine de honger meer dan caseïne.

Onze verklaring is dat het verschil in hongeronderdrukking gerelateerd zou kunnen zijn aan een mechanisme dat is waargenomen in metazoa. Hierbij werd ontdekt dat het tRNA/GCN2/p-eIF2α-systeem in de hersenen een gebrek aan essentiële aminozuren in het dieet kan detecteren door middel van een afname aan aminozuurgehaltes in het serum. Dit leidt tot een gedragsrespons die het nuttigen van niet gebalanceerde diëten tegengaat, wat als hongeronderdrukking kan worden ervaren. Tenslotte werd gevonden dat de grootte van het effect op eetlust bij een verhoogde eiwitinname verschilt ten gevolge van verschillen in aminozuursamenstelling van het dieet, aangezien het effect van een verhoogde caseïne-inname groter was dan het effect van een verhoogde gelatine-inname. Mechanismen die bijdragen aan de effecten op eetlust kunnen mogelijk verschillen tussen eiwitsoorten. Langdurig verhoogde aminozuurconcentraties hebben mogelijk bijgedragen aan de hogere verzadiging bij een verhoogde caseïne-inname, wat in overeenstemming is met de 'aminostatische theorie' van Mellinkoff et al. Verder waren veranderingen in honger en verzadiging gerelateerd aan veranderingen in het slaapmetabolisme bij het dieet met een hoog caseïnegehalte.

Voor zowel caseïne als gelatine was het energiegebruik, d.w.z. het 24-uurs energiegebruik en het slaapmetabolisme, hoger bij het dieet met een relatief hoog eiwitgehalte ten opzichte van het dieet met een normaal eiwitgehalte. Bovendien hebben een compleet eiwit (caseïne) en een incompleet eiwit (gelatine) vergelijkbare effecten op energiegebruik, ongeacht of deze genuttigd worden onder condities van een relatief normale of een relatief hoge eiwitinname. De mechanismen die betrokken zijn bij het postprandiale eiwitmetabolisme (b.v. eiwitsynthese, eiwitoxidatie, ureumsynthese en gluconeogenese) zijn afhankelijk van de aminozuursamenstelling van de eiwitten. Afhankelijk van de genuttigde eiwitsoorten kan het energiegebruik daarom vergelijkbaar of verschillend beïnvloed worden. Met betrekking tot eiwitbalansen werd gevonden dat, ongeacht of de diëten genuttigd werden onder condities van een relatief normale of relatief hoge eiwitinname, de eiwitoxidatie hoger is bij gelatinediëten dan bij caseïnediëten, wat resulteert in lagere eiwitbalansen. De afwezigheid of de lage hoeveelheden van bepaalde essentiële aminozuren in een incompleet eiwit zijn een beperkende factor voor postprandiale eiwitsynthese. Dit suggereert dat bij gelatine eiwitoxidatie meer bijdraagt aan het energiegebruik, terwijl bij caseïne eiwitsynthese meer lijkt bij te dragen aan het energiegebruik. Onder condities van zowel een relatief normale als een relatief hoge eiwitinname verschilden de vetbalansen niet tussen gelatine en caseïne. Dit suggereert dat verschillen in aminozuursamenstelling tussen eiwitsoorten geen invloed hebben op de totale vetopslag en vetoxidatie. Bij een relatief hoge eiwitinname was de koolhydraatbalans meer positief bij gelatine dan bij caseïne. Dit effect kan gedeeltelijk worden verklaard doordat bij het gelatinedieet de vrije aminozuren toenemen, omdat deze niet gebruikt kunnen worden voor eiwitsynthese, waardoor de gluconeogenese toeneemt en de gevormde glucose vervolgens als glycogeen wordt opgeslagen.

Het toevoegen van gelatine aan een melkeiwitdieet dat gebaseerd is op de aanbevolen dagelijkse hoeveelheid eiwitinname, zorgt niet voor een hoger gewichtsverlies of betere lichaamssamenstelling gedurende een gewichtsverliesperiode en zorgt niet voor een beter gewichtsbehoud gedurende de periode na gewichtsverlies in vergelijking met melkeiwitdiëten die gebaseerd zijn op de aanbevolen dagelijkse hoeveelheid eiwitinname of een verhoogde inname hiervan. Dit laat zien dat waargenomen effecten van eiwitten op de korte termijn niet vanzelfsprekend resulteren in vergelijkbare effecten op de lange termijn. De drie diëten (beide melkeiwitdiëten en het melkeiwitdieet waaraan gelatine was toegevoegd) resulteerden in een succesvol gewichtsverlies en volledig gewichtsbehoud daarna, terwijl een inname van melkeiwit van 0.8 g/kg per dag voldoende is om dit resultaat te bereiken.

Samengevat hebben deze onderzoeken geleid tot de volgende conclusies: 1) De TRP/LNAA-verhouding in het plasma is niet direct gerelateerd aan eetlust via het serotoninesysteem; 2) gelatine onderdrukt de honger meer dan caseïne als deze in een 1-daags dieet worden genuttigd bestaande uit slechts één eiwitsoort; 3) de hogere verzadiging bij een hoge caseïne-inname, vergeleken met een normale caseïne-inname, valt samen met langdurig verhoogde aminozuurconcentraties, die volgens de 'aminostatische theorie' bijdragen aan verzadiging; 4) toename in verzadiging is gerelateerd aan toename in het slaapmetabolisme bij een dieet met een hoog caseïnegehalte; 5) de bijdragen van mechanismen aan een toegenomen energiegebruik bij een verhoogde eiwitinname verschillen tussen een compleet eiwit (caseïne) en een incompleet eiwit (gelatine). Eiwitsynthese draagt meer bij aan een verhoogd energiegebruik bij een compleet eiwit, terwijl eiwitoxidatie meer bijdraagt bij een incompleet eiwit; 6) een incompleet eiwit (gelatine) resulteert in een lagere eiwitbalans dan een compleet eiwit (caseïne) ten gevolge van een hogere eiwitoxidatie in samenhang met een lagere,

beperkte, eiwitsynthese; 7) bij een relatief hoge eiwitinname is de koolhydraatbalans hoger bij een incompleet eiwit, waarbij de suggestie is dat dit gedeeltelijk verklaard kan worden door een toegenomen gluconeogenese; 8) het effect op vetbalans is niet verschillend tussen het nuttigen van een compleet of incompleet eiwit; 9) effecten van eiwitdiëten op de korte termijn resulteren niet vanzelfsprekend in vergelijkbare effecten op de lange termijn; 10) een inname van melkeiwit gelijk aan de minimaal aanbevolen dagelijkse eiwitinname is voldoende voor een succesvol gewichtsverlies en gewichtsbehoud erna, terwijl het toevoegen van gelatine dit resultaat niet verbetert.

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Curriculum Vitae

Ananda Hochstenbach-Waelen was born on December 2nd 1979 in Ubach over Worms, the Netherlands. She completed secondary school at 'College Rolduc' in Kerkrade in 1998. In the same year she started the study Health Sciences, with the specialization Movement Sciences, at Maastricht University. She obtained her Master Degree in 2002.
In 2003 and 2004 she worked at the iRv (Institute for Rehabilitation Research) on a project about Functional Electrical Stimulation.
In June 2005 she started working as a PhD student at the department of Human Biology of Maastricht University under supervision of Prof. M.S. Westerterp-Plantenga and Prof. K.R. Westerterp. Her research was part of the project 'The role of dietary protein in weight management' of the Top Institute Food and Nutrition (TIFN). The PhD research performed during this period is described in this thesis.