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Running title: Adiposity and age effect on protein metabolism in rats

Abstract

Purpose: Physiological parameters such as adiposity and age are likely to influence protein digestion and utilization. The aim of this study was to evaluate the combined effects of age and adiposity on casein protein and amino acid true digestibility and its postprandial utilization in rats.

Methods: Four groups were included (n = 7/8): 2 months/normal adiposity, 2 months/high adiposity, 11 months/normal adiposity and 11 months/high adiposity. Rats were given a calibrated meal containing ^{15}N-labeled casein (Ingredia, Arras, France) and were euthanized 6 h later. Digestive contents were collected to assess protein and amino acid digestibilities. ^{15}N enrichments were measured in plasma and urine to determine total body deamination. Fractional protein synthesis rate (FSR) was determined in different organs using a flooding dose of ^{13}C valine.

Results: Nitrogen and amino acid true digestibility of casein was around 95-96% depending on the group and was increased by 1% in high adiposity rats (P = 0.04). Higher adiposity levels counteracted the increase in total body deamination (P = 0.03) that was associated with older age. Significant effects of age (P = 0.006) and adiposity (P = 0.002) were observed in the muscle FSR, with age decreasing it and adiposity increasing it.

Conclusion: This study revealed that a higher level of adiposity resulted in a slight increase in protein and individual amino acid true digestibility values and seemed to compensate for the metabolic postprandial protein alterations observed at older age.

Keywords: Aging, obesity, Protein metabolism, amino acid digestibility, rat model

Introduction

Protein quality can be defined as a protein's ability to satisfy the metabolic demand for amino acids and nitrogen [1]. It not only depends on its composition in amino acids, but is also conditioned by its digestibility value and the bioavailability of its individual amino acids. To adapt protein intakes to meet the needs of the individual, factors such as age, health status, physiological status, and energy requirements should also be considered when addressing protein quality [2]. Factors influencing protein digestibility have not been entirely elucidated.

In humans, the aging process has been associated with modulations to the gastro-intestinal tract, notably with delayed gastric emptying, an increase in colon transit time, and histological changes to the gut [3,4] as well as slower protein digestion [5]. Classic methods to determine protein digestibility in humans are very invasive and not applicable to vulnerable population. Hence, little data address the effect of aging on protein digestibility; but, it was reported to be reduced in old rats compared to young rats, especially for the low digestibility protein sources [6]. In contrast to digestion, muscle anabolism during aging is well documented in both animal models and humans. For instance, a lower postprandial protein synthesis rate was observed in older rats compared to adult rats [7,8] and similar results were obtained in human trial [9] and cohort study [10].

Data associating body composition to protein digestion are scarce. However, studies related to impact of overweight or obesity on protein metabolism and the resulting gut adaptations have been conducted in animal models and humans. It was notably shown that diet-induced obese mice underwent major intestinal adaptations, such as an increased intestinal permeability, a delayed transit time, and an increased amino acid absorption alongside an

upregulation of some amino acid transporters [11]. Additionally, diet-induced obese mice were shown to synthesize less skeletal muscle protein in response to nutrient ingestion [12] and this result was confirm in humans as a decline in muscle protein synthesis in response to a high protein meal was found in overweight and obese adults compared to lean controls [13].

Results of the aforementioned experiments suggest distinct effects of age and obesity on protein digestion and metabolism, but studies linking both factors are rare and mostly focus on their effect on muscle protein synthesis [14]. We hypothesized that age may decrease protein digestibility and that both age and adiposity may reduce protein utilization and synthesis. The aim of this study was to thus evaluate the combined effects of age and adiposity on casein protein and amino acid true digestibility, nitrogen postprandial utilization and fractional synthesis rates in different organs, after consumption of a ^{15}N-labeled casein meal in a model of young and middle-aged rats with varying levels of adiposity.

Materials and methods

Milk protein labeling

The cow milk was labeled by Ingredia (Arras, France). Briefly, two cows were administered 50 g of ^{15}N ammonium sulfate [99%, $(^{15}NH_4)_2SO_4$] via drinking water twice a day for 9 consecutive days. The milk was collected every day starting from the fourth day. An isotopic enrichment of 1.4 atom percent in the milk was obtained. ^{15}N native micellar casein was then obtain by filtration (Prodiet® 85B, Ingredia, Arras, France).

Experimental protocol

The study was performed in agreement with the European Union directive 2010/63/EU for animal experiments and approved by the ethics committee in animal experiments of INRAE Jouy-en-Josas (Comethea, registration number: 18-14) and the French Ministry of Higher Education and Research (APAFIS n°15907-2018061823133762 v1). Thirty male Wistar rats aged 1 month (n = 15) and 10 months (n = 15) at their arrival were purchased from Envigo (Horst, The Netherlands). They were housed in individual cages with wire bottoms to prevent coprophagia. Temperature was controlled and rats were subjected to a normal 12h/12h dark-light cycle, then gradually switched to a reversed dark-light cycle (dark period 07:00 to 19:00) during the fourth week of the experiment. Rats had unlimited access to water and chow for 7 days after arrival, before their respective experimental diets were administered (**Table 1**). Rats were allowed *ad libitum* access to feed for twenty-eight days (**Figure 1**) with either a standard diet or a Western diet in order to obtain rats of normal and high adiposity levels, within each age. Four groups were constituted (n=7/8): 2 months/normal adiposity (2m-na), 2 months/high adiposity (2m-ha), 11 months/normal adiposity (11m-na) and 11 months/high adiposity (11m-ha). Middle-aged rats were chosen in order to evaluate the effect of age without confounding effects of old age.

Starting from the sixth week (**Figure 1**), all groups were fed the same diet that corresponds to the standard diet composition but with casein (Prodiet® 85B, Ingredia, Arras, France) as the sole source of protein. This 1-week diet standardization was to limit any potential effect of the previous diet on postprandial tests. Rats were placed under a special protocol for a period of one week to accustom them to consuming a calibrated meal at the beginning of the dark phase, as described previously [15].

The day of euthanasia, rats consumed a calibrated meal of 4 g containing the ^{15}N-labeled casein protein. Blood was collected from the tail vein before meal ingestion and 1 h and 3 h after, and plasma was then stored at -20°C. Thirty minutes before euthanasia, rats were injected in the lateral tail vein with a flooding dose (150 µmol/100 g of body weight) of [1-^{13}C] valine (Eurisotop, Saint Aubin, France) under gaseous anesthesia. Rats were euthanized 6 h after meal ingestion, after cardiac puncture under gaseous anesthesia. The luminal contents of the different segments of the gastro-intestinal tract segments were rinsed with NaCl (0.9%), collected, and stored at -20°C until freeze-drying, as described previously [15]. Feces were also collected under the cages during the postprandial period. The mucosae of the duodenum, jejunum and ileum were scraped and muscle, liver, kidney, and a skin sample were collected, frozen in liquid nitrogen and stored at -80°C. Urine was retrieved during the postprandial period by placing an absorbent paper under the cages and by puncturing the bladder after euthanasia. The absorbent paper was rinsed with distilled water, and the urine eluates were stored at -20°C. Whole body composition was determined by dissection and weighing of the main organs and tissues.

Analytical methods

Protein and amino acid true digestibility was assessed by respectively following the ^{15}N recovery and the ^{15}N-amino acid recovery in the digestive contents, as described previously [15]. *In vivo* protein synthesis rates in the gastrocnemius muscle, liver, kidney and skin were estimated with the [^{13}C]-valine flooding dose method as described previously [16]. Total urea was determined in urine and plasma by the Urease-Berthelot method (Urea assay, Randox, Crumlin, UK). Plasma separation of the nitrogen fraction (protein, free amino acid and urea)

131 was achieved as described previously [17]. ^{15}N enrichment in plasma fraction and in urine
132 was measured as described previously [18].

133 The RNA expression of hormones [cholecystokinin (CCK), secretin] and the main peptide and
134 amino acid transporters in the intestinal mucosa was determined as described previously
135 [19]. The genes used are presented in **Supplementary Table 1**.

136

137 **Calculations**

138 Estimation of dietary nitrogen or amino acids (mmol) in the different digestive contents was
139 determined using the following formula:

140 $$N_{\text{diet digesta}} = N_{\text{tot}} \times \frac{APE_{\text{digesta}}}{APE_{\text{meal}}}$$

141 Where N_{tot} represents the total amount of nitrogen in the digesta sample (mmol) and APE is
142 the enrichment excess of ^{15}N in the digesta and meal. APE is defined as the enrichment of
143 the sample in atom percent (AP) minus the natural abundance. Orocaecal digestibility was
144 determined as a proxy of ileal digestibility in rats [15,20] in order to have a sufficient amount
145 of digesta for isotopic enrichment determination. Collection of digesta 6 h after meal intake
146 allows for near complete digestion and minimal duration of fermentation of digesta in the
147 caecum. True orocaecal nitrogen digestibility (%) was calculated as follows:

148 $$\text{True nitrogen orocaecal digestibility} = 100 \times \frac{N_{\text{ing}} - (N_{\text{diet ileum}} + N_{\text{diet caecum}})}{N_{\text{ing}}}$$

149 Where N_{ing} is the amount of nitrogen ingested by the rats (mmol). Because dietary nitrogen
150 was recovered in the stomach and thus did not enter into the digestive process, in all the
151 calculations, N_{ing} excluded this stomach residual amount. True orofecal nitrogen digestibility

was also determined considering nitrogen losses in the colon and feces, in addition to losses in the ileum and caecum. Similarly to true orocaecal nitrogen digestibility, true orocaecal digestibility for each individual amino acid was determined by estimating the dietary amino acid not absorbed in the intestinal tract and recovered in the digesta, as described previously [15].

The dietary nitrogen recovered in the urea body pool (mmol) was calculated assuming that urea was uniformly distributed throughout the total body water, as described previously [18]. The dietary nitrogen recovered in the urinary urea (mmol) was calculated assuming that the urinary ^{15}N-enrichment is a proxy of the urinary urea ^{15}N-enrichment, as described previously [18]. The total amount of dietary nitrogen transferred to urea ($N_{diet\ urea\ total}$, mmol) is the sum of the dietary nitrogen excreted in urinary urea and the dietary nitrogen recovered in the body urea pool. The dietary nitrogen recovered in proteins of organs or plasma (mmol) was calculated as previously described [21]. In plasma proteins, it was calculated assuming that plasma represented 3.5% of body weight [22]. The postprandial fractional protein synthesis rate (FSR, expressed in %/day) in the organs and tissues was calculated as previously described [21].

Statistical analyses

A power calculation was performed to determine the sample size required to detect significant differences. According to Gilani study evaluating true protein digestibility in young and old rats [23], an effect of age was observed for difference in digestibility > 3% for highly digestible protein sources. In studies using ^{15}N-labeled protein to determine digestibility, interindividual variability ranged from 0.7 to 2.3%, with a mean around 1.5% [15,18,21,24].

Hence, according to these data and with a power set at 0.90 and α set at 0.05, the sample size group was calculated to be 7 (G∗Power 3.1).

All results are expressed as mean ± SD and analyses were performed on GraphPad Prism 8.2.1. According to Quantile vs Quantile Plots and Shapiro Wilk tests [25], the true ileal digestibility data were assumed to be normally distributed. Unpaired T-tests were performed to evaluate differences in body composition between groups of the same age [26]. Differences between groups were tested using a two-way ANOVA with age and adiposity as factors and post hoc Bonferroni tests were applied for pairwise comparisons [27]. Kinetic of incorporation of dietary nitrogen in plasma proteins was analyzed using a mixed model with a compound symmetry covariance matrix, and with the group as a fixed effect and time as a repeated effect [28]. Differences were considered statistically significant with a P-value < 0.05.

Results

Body weight and body composition

The mean daily consumption of rats consuming the western diet was significantly higher during the 4 weeks of diet in comparison to rats communing the standard diet for both 2-month ($P < 0.0001$; 60 ± 3 kJ/d and 73 ± 5 kJ/d) and 11-month ($P = 0.0063$; 72 ± 6 kJ/d and 101 ± 21 kJ/d) groups. During the 4 weeks of standard or Western diet, the 2-month rats gained 172.0 ± 10.4 g and 184.8 ± 14.9 g for the normal and high adiposity groups, respectively (**Supplementary Figure 1**). The 11-month rats gained 59.9 ± 17.2 g and 95.1 ± 38.6 g for the normal and high adiposity groups, respectively. A difference in weight gain was

noted between 11-month rats (P = 0.0333) and a trend for the 2-month rats (P = 0.0791). No difference in final body weight between groups of the same age was observed, but the diets administered resulted in differences in body composition (**Table 2**). A difference in adiposity was noted between groups of the same age, with an increase of 45% in total adiposity in the 2-month rats (P = 0.0008) and 50% in the 11-month rats (P = 0.0004).

Dietary nitrogen recovery and true digestibility of nitrogen and amino acids

Rats consumed 3.8 ± 0.3 g of the test meal containing ^{15}N-labeled casein protein, which represents 4.8 ± 0.3 mmol of dietary nitrogen. A majority of the dietary nitrogen was found in the stomach and caecum, with a minimal amount in the small intestine, ileum and colon (**Table 3**). More dietary nitrogen was recovered in the stomach and the small intestine in the middle-aged compared to younger rat groups (P = 0.0188 and P = 0.0401, respectively). Based on nitrogen recovery in the different segments of the gastrointestinal tract, we calculated true nitrogen digestibility. True nitrogen digestibility of casein was high in all the groups (> 94%) and an effect of adiposity was found on both orofecal (P = 0.0402) and orocaecal digestibility (P = 0.0491) with the higher adiposity groups expressing higher digestibility values. Indeed, for true nitrogen digestibility a difference of ~1% was observed between groups of the same age. Similarly, higher adiposity levels were associated with an increase of the mean true digestibility of individual amino acids (P = 0.0370; Table 3). Hence, true orocaecal digestibility differences were observed for some amino acids, such as lysine (P = 0.0426), serine (P = 0.0313), aspartate/asparagine (P = 0.0400) and glutamate/glutamine (P = 0.0393).

220 **Postprandial distribution of dietary nitrogen**

221 **Table 4** presents the percentage of dietary nitrogen retained in some organs and transferred
222 to body and urinary urea (deamination pool). Dietary nitrogen recovered in the body urea
223 pool as well as total deamination were increased in middle-aged rats with normal adiposity
224 in comparison to their young counterparts ($P = 0.0183$), whereas values were comparable in
225 high adiposity young and middle-aged rats. There was no effect of age or adiposity on the
226 incorporation of ^{15}N in the tissues and organs, except in the liver where a significant effect of
227 age was observed ($P = 0.0359$) with higher incorporation of dietary nitrogen in older rats.
228 When calculated with respect to body weight **(Supplementary Table 2)**, a significant effect
229 of age was observed only in kidney ($P = 0.0007$) and skin ($P = 0.0066$), with lower
230 incorporation of dietary nitrogen in the kidney and skin of middle-aged rats.

231 **Figure 2** represents the incorporation of dietary nitrogen into plasma proteins over the
232 whole postprandial period according to the different groups. Overall, effects of group, time
233 and their interaction were observed ($P < 0.0001$). Three hours after the meal, middle-aged
234 rats with both normal and high adiposity presented a higher incorporation of dietary
235 nitrogen into plasma proteins compared to the young rats ($P < 0.05$). These differences
236 continued 6 h after the meal and at that time, the middle-aged rats with high adiposity also
237 presented higher incorporation of dietary nitrogen into plasma proteins then the middle-
238 aged normal adiposity rats ($P < 0.0001$). On the contrary, at this time point, the young rats
239 with high adiposity presented lower incorporation of dietary nitrogen than their lean
240 counterparts ($P = 0.0426$).

241

242 **Protein anabolism**

The postprandial FSR in the liver, gastrocnemius muscle, kidney and skin are presented in **Figure 3**. No effect of age or adiposity was observed on the FSR per hour in the liver and kidney. In the gastrocnemius muscle, age decreased the FSR ($P = 0.0063$) whereas adiposity increased it ($P = 0.0018$). Hence, muscle FSR of the middle-aged group with normal adiposity was significantly reduced compared to the young groups ($P = 0.0297$ and $P = 0.0005$ for comparison with 2-month rats with normal and high adiposity, respectively) whereas muscle FSR of the middle-aged group with high adiposity was not different from the young groups. Furthermore, an effect of age on protein synthesis in the skin was noted ($P = 0.0066$) with higher FSR per hour in the younger rat groups.

Expression of hormones and peptide and amino acid transporters in the intestinal mucosae

For hormones, the expression of CCK was higher in older rats ($P = 0.0047$), with an increase of 60 to 90% compared to young rats (**Table 5**). No effect of age, adiposity or their interaction was observed in the expression of transporters in the duodenum and the jejunum. In the ileum, an effect of adiposity was demonstrated for the expression of Slc6a19 ($P = 0.0339$), Slc38a2 ($P = 0.0075$) and Slc1a5 ($P = 0.0271$), with higher expression of these transporters in normal adiposity groups compared to their higher adiposity counterparts.

Discussion

A model of middle-aged rats with moderately high adiposity

This study aimed to evaluate the effects of age and adiposity on the protein digestibility and postprandial utilization of ^{15}N-labeled native micellar casein in rats. Rat is an animal model that has been extensively used to understand physiological and metabolic disorders associated with obesity and/or aging [29]. In the context of protein digestibility studies, rat is a suitable model [30] and good correlation exists between rats and humans digestibility values [31]. Since evaluation of amino acid bioavailability in humans is currently very invasive, expensive and complicated [32], rat model is instrumental in the investigation of the effect of aging and adiposity on protein digestibility and metabolism. The rats included in our study were young (2 month) and middle-aged (11 months) rats. To obtain different levels of adiposity, half of the rats consumed a high fat/high sucrose diet (Western diet) during 4 weeks. Despite no difference in final body weight, these rats presented significantly higher adiposity compared to their lean counterparts of the same age. The relatively short length of the nutritional intervention may explain the comparable final body weight within age groups. Standard thresholds for obesity have not been developed in rats [33]. However, adiposity levels varied from 8 to 32% in diet-induced obesity models in the literature [16,34-37] suggesting that the 9.4% and 20.0% of fat mass in the young and middle-aged high adiposity groups correspond to moderately high level of adiposity and thus, intermediate obesity .

Effect of age and adiposity on casein nitrogen and amino acid digestibility

In our study, depending on the group, the true orocaecal digestibility of micellar casein ranged between 94.6% and 96.3%, and the mean true amino acid digestibility from 95.1% to 96.6%, as reported previously (96.5% for protein and amino acid digestibility [18,38]). We

observed no effect of age on protein or amino acid digestibility of casein, in contrast with the study by Gilani & Sepehr [23]. They showed that 20-month-old rats presented a lower fecal digestibility for many protein sources, including casein, compared to young rats. This result suggests that in contrast to old rats, middle-aged rats may not present declined digestibility. Nevertheless, in our study, gastric emptying rate seemed to be modified with age since significant amount of dietary nitrogen was still in the stomach of middle-aged rats 6 h after meal. Studies conducted on young, adult, and old rats have demonstrated a delayed gastric emptying that appeared in adult rats and increased gradually in old rats [39,40]. In men, older age was also associated with a slower protein digestion [5]. The mechanisms are not well elucidated but it might be due to reduced gastric contractile forces [4]. Interestingly, we found a higher expression of CCK in the duodenum of the middle-aged rats, a hormone that is known to slow down gastric emptying [41]. Whereas age did not alter digestibility, we observed a significant effect of adiposity that increased true protein and amino acid digestibility by around 1%. As usually observed for highly digestible protein, the difference between groups was small and the effect of adiposity on digestibility has thus to be confirmed with a low digestibility protein, for which higher differences may be observed [32]. In a study performed on diet-induced obese mice, a higher nitrogen apparent digestibility was reported together with higher expression of the Slc6a20a and Slc36a1 amino acid transporters in the small intestine [11]. We observed a slight decrease in gene expression of some amino acid transporters in the ileum of the high adiposity rats and, while the majority of peptide and amino acid absorption occurs in the upper small intestine, no difference was observed in the duodenum or jejunum. Peptides and amino acids are mainly absorbed via transporters, but they can also be absorbed through other mechanisms, such as paracellular pathways [42]. Thus, the slightly improved digestibility found in the higher

311 adiposity groups might have been related to an increase in these transportation pathways
312 [43].

313

314 **Effect of age and adiposity on postprandial metabolic fate of casein nitrogen**

315 The use of ^{15}N-labelled casein allowed us to evaluate the postprandial metabolic fate of
316 dietary nitrogen. We first found that postprandial total deamination 6 h after meal intake
317 was increased in middle-aged rats with normal adiposity, suggesting an increased protein
318 catabolism in older rats. We also measured dietary nitrogen incorporation into proteins and
319 found an increased uptake of dietary nitrogen in the liver and a higher incorporation of
320 dietary nitrogen in plasma proteins (that are mainly composed of exported liver proteins) in
321 middle-aged rats compared to young rats. This is in line with higher splanchnic sequestration
322 observed in the elderly [44,45], but may also be partly due to higher body (and liver) weight
323 of these older rats as this effect is not observed after adjustment for weight. The absence of
324 any effect on the liver anabolic rate despite higher splanchnic sequestration is not surprising
325 since the 11-month-rats are heavier that the 2-month-rats. Nevertheless, as all organs are
326 heavier in the middle-aged groups, our results show a differential postprandial dietary
327 nitrogen disposal in the tissues. The first-pass splanchnic metabolism, including deamination
328 and incorporation of dietary nitrogen into proteins, resulted in a proportionally smaller
329 amount of dietary nitrogen available for protein synthesis in peripheral tissues. Accordingly,
330 we reported a decrease in postprandial protein synthesis rates in the skin and muscle of
331 middle-aged rats. A gradual age-associated decline in protein synthesis rate in the skin (2-
332 fold decrease from 2-month to 6 or 10-month old rats) has been previously observed, due to
333 a general decrease in protein synthesis rate with age but also to the high protein turnover

and accretion in skin during rat growth [46,47]. Furthermore, the effect of age on postprandial muscle protein synthesis has already been well demonstrated in rats [7,48-50] and humans [9,10,51]. Taken together, we found that middle-aged rats of 11 months displayed metabolic adaptations usually reported in aged rats, such as higher postprandial deamination, increased splanchnic retention and decreased peripheral protein synthesis rate. Interestingly, these alterations of protein metabolism were not all observed in the middle-aged rats with high adiposity levels.

In addition to a slightly increased true protein and amino acid digestibility, middle-aged rats with high adiposity levels had a postprandial deamination of casein comparable to younger rats. However, dietary nitrogen retention in the liver and incorporation in plasma proteins were comparable or even higher in middle-aged rats with high adiposity in comparison to normal adiposity. These results suggest that high adiposity middle-aged rats may have age-related higher nitrogen splanchnic sequestration, but that it was also accompanied by reduced losses in dietary amino acids through deamination. Therefore, more dietary nitrogen and amino acids may have been available for peripheral metabolism in these rats. As the amount of substrate is a key factor in regulating protein synthesis, it could explain why the protein synthesis rate in the muscle was higher in middle-aged rats with high adiposity compared to normal adiposity. The impact of adiposity on muscle protein synthesis has been previously observed in a study where muscle protein synthesis was increased at an intermediate stage of obesity [37]. Moreover, the role of insulin, together with a high availability of plasma amino acids, in muscle protein anabolism is now well established [52]. While insulin levels were not monitored in our study, we could suppose that a higher adiposity was accompanied by higher insulin levels [53] which could have stimulated muscle protein synthesis. Taken collectively, the moderately high levels of adiposity in our middle-

aged rats seemed to have counteracted the effect of aging on postprandial protein metabolism such as the age-related increase in amino acid postprandial deamination and decrease in muscle protein synthesis. The increased digestibility associated with higher adiposity may have partly participated in counterbalancing this effect by means of a higher absorption of dietary amino acids available for metabolic utilization. Due to the invasiveness of the classical method to evaluate protein digestibility in humans, no data are available for obese and/or aged people. However, a new minimally invasive method has been recently developed [54]. It will thus make it possible to determine amino acid digestibility in vulnerable people and extrapolate our results to humans.

Conclusion

In conclusion, this study provided the first data on the combined effects of adiposity levels and age on micellar casein true protein and amino acid digestibility and postprandial utilization in rats. A strength of our study is that we used ^{15}N labeled proteins that allowed us to track dietary nitrogen in the gastrointestinal tract, urea pools and in peripheral organ and tissues. A higher level of adiposity resulted in a slight increase in protein and individual amino acid digestibility values and seemed to compensate for the metabolic protein alteration linked with aging, including reduced muscle anabolic response. Although our results need to be confirmed in humans [55], our data suggest that moderate adiposity may contribute to healthy aging in the context of protein metabolism.

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Authorship

The authors' responsibilities were as follows: C.G., J.C., A.Ba. and A.Bo. contributed to the conception and design of the study; N.A., J.C. conducted the research; N.A., J.C., N.K., C.C., J.P., and M.C. contributed to data acquisition; N.A. and J.C. analyzed the data; N.A. wrote the original draft of the paper; J.C., C.G., D.A.M, A.Ba., and A.Bo. reviewed and edited the manuscript; C.G. administered the project; and all authors read and approved the final manuscript.

Statements and Declaration

Competing Interests and funding:

C.G., D.A.M., N.K., C.C., J.P., M.C. and J.C. declare that they have no conflict of interest. N.A., A.Ba., and A.Bo. are employed by Ingredia.

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Ethics approval: All procedures involving animals were in compliance with the European Union directive 2010/63/EU for animal experiments and approved by the ethics committee in animal experiments of INRAe Jouy-en-Josas (Comethea, registration number: 18-14) and the French Ministry of Higher Education and Research (APAFIS n°15907-2018061823133762 v1)

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Figure legends

Fig. 1 Experimental protocol of the study. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

Fig. 2 Incorporation of dietary nitrogen to plasma proteins over the 6-h postprandial period. Values are mean ± SD, n = 7 - 8 rats. $^{£}$ indicating a significant difference ($P < 0.05$) between the 2m (-na and -ha) and 11m (-na and -ha) groups and $^{\$}$ indicating a significant difference ($P < 0.05$) between every groups. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

Fig. 3 Fractional synthesis rate (FSR) in the gastrocnemius muscle (A), skin (B), liver (C), kidney (D) 6 h after meal ingestion. Values are mean ± SD, n = 7 - 8 rats. The effects of age and adiposity and their interaction were tested with a two-way ANOVA model. Values with different letters within a graph are statistically different. n.s, not significant. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

591 **Table 1** Composition of the experimental diets

	Standard diet (g/kg)	**Western diet (g/kg)**	**Casein diet (g/kg)**
Protein[1]	140	160	120
Starch	622	291	642
Sucrose	100	291	100
Soya bean oil	40	40	40
Lard	0	120	0
Mineral mix	35	35	35
Vitamin mix	10	10	10
Cellulose	50	50	50
Choline	3	3	3
Protein (%Energy)	14	14	14
Carbohydrates (%Energy)	75	51	75
Lipids (%Energy)	11	35	11
Energy (kJ/g)	15.0	18.1	15.5

592 [1] Total milk protein for Standard and Western diet and casein Prodiet® 85B (Ingredia, Arras, France) for Casein
593 diet
594

Table 2 Body weight and body composition in rats according to age and adiposity levels

Age	2 months			11 months		
Group	**2m-na**	**2m-ha**	***T-test***	**11m-na**	**11m-ha**	***T-test***
Body weight (g)	315.5 ± 18.9	323.2 ± 12.1	*n.s.*	536.0 ± 32.1	574.1 ± 52.0	*n.s.*
Total adiposity (%)	6.5 ± 0.9	9.4 ± 1.5 *	*0.0008*	13.3 ± 3.9	20.0 ± 1.2 *	*0.0004*
Total lean mass (%)	73.3 ± 2.3	72.5 ± 1.5	*n.s.*	70.5 ± 7.3	67.1 ± 1.0	*n.s.*
Subcutaneous AT (%)	3.5 ± 0.6	3.8 ± 0.4	*n.s.*	5.8 ± 2.6	9.5 ± 1.1 *	*0.0025*
Epididymal AT (%)	1.6 ± 0.4	1.7 ± 0.3	*n.s.*	2.4 ± 0.5	4.0 ± 0.8 *	*0.0005*
Retroperitoneal AT (%)	1.6 ± 0.3	1.8 ± 0.4	*n.s.*	2.7 ± 0.8	4.5 ± 0.6 *	*0.0003*
Mesenteric AT (%)	1.3 ± 0.1	1.8 ± 1.2	*n.s.*	1.9 ± 0.6	2.4 ± 0.6 *	*0.0002*
Brown AT (%)	0.2 ± 0.02	0.2 ± 0.03	*n.s.*	0.1 ± 0.03	0.2 ± 0.03 *	*0.0001*
Liver (g)	8.3 ± 0.7	8.5 ± 0.6	*n.s.*	10.9 ± 0.9	12.1 ± 1.4	*n.s.*
Gastrocnemius muscle (g)	1.6 ± 0.3	1.6 ± 0.1	*n.s.*	2.3 ± 0.2	2.5 ± 0.4	*n.s.*
Kidney (g)	1.7 ± 0.3	1.9 ± 0.1	*n.s.*	2.2 ± 0.1	2.3 ± 0.3	*n.s.*

Values are mean ± SD, n = 7 - 8 rats. Body weight and body composition were determined at the end of experiment. The differences in body composition were tested by an unpaired student t-test between groups of the same age. * Significantly different from normal adiposity group within same age. AT, adipose tissue. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

Table 3 Dietary nitrogen recovered in gastrointestinal segment 6 h after ingestion of the test meal containing ^{15}N-labelled casein protein and true digestibility of nitrogen and individual amino acid according to age and adiposity levels

Age	2 months		11 months		*P-value*		
Group	**2m-na**	**2m-ha**	**11m-na**	**11m-ha**	***Age effect***	***Adiposity effect***	***Age x Adiposity***
Dietary nitrogen recovery (% of ingested)							
Stomach	2.1 ± 5.5^{a}	5.5 ± 7.9^{ab}	7.3 ± 8.1^{ab}	14.8 ± 9.3^{b}	*0.0188*	*n.s.*	*n.s.*
Proximal intestine	0.6 ± 0.7	0.5 ± 0.3	0.9 ± 0.6	1.1 ± 0.7	*0.0401*	*n.s.*	*n.s.*
Ileum	0.06 ± 0.03	0.03 ± 0.02	0.03 ± 0.03	0.03 ± 0.01	*n.s.*	*n.s.*	*n.s.*
Caecum	3.9 ± 1.3	3.4 ± 1.3	4.1 ± 1.4	2.5 ± 0.4	*n.s.*	*0.0261*	*n.s.*
Colon + feces	0.7 ± 1.1	0.5 ± 0.4	0.4 ± 0.3	0.4 ± 0.3	*n.s.*	*n.s.*	*n.s.*
True nitrogen digestibility (% of ingested)							
Orofecal digestibility	94.1 ± 1.1	95.2 ± 1.7	94.5 ± 2.2	95.8 ± 0.7	*n.s.*	*0.0402*	*n.s.*
Orocaecal digestibility	94.8 ± 1.6	95.7 ± 1.5	95.0 ± 2.2	96.3 ± 0.4	*n.s.*	*0.0491*	*n.s.*
True amino acid orocaecal digestibility (% of ingested)							
Alanine	95.7 ± 1.3	95.7 ± 1.5	94.8 ± 1.4	96.0 ± 1.0	*n.s.*	*n.s.*	*n.s.*
Aspartate + asparagine	95.5 ± 1.4	95.8 ± 1.4	94.6 ± 1.7	96.4 ± 0.9	*n.s.*	*0.0400*	*n.s.*
Glycine	95.7 ± 1.4	96.0 ± 1.3	94.5 ± 1.6	96.1 ± 1.2	*n.s.*	*n.s.*	*n.s.*
Glutamate + glutamine	94.2 ± 1.7	94.9 ± 1.6	93.2 ± 2.1	95.3 ± 1.2	*n.s.*	*0.0393*	*n.s.*
Isoleucine	93.1 ± 2.1	93.6 ± 2.3	91.3 ± 2.7	93.6 ± 1.4	*n.s.*	*n.s.*	*n.s.*
Leucine	98.2 ± 0.5	98.3 ± 0.5	98.0 ± 0.5	98.6 ± 0.4	*n.s.*	*n.s.*	*n.s.*
Lysine	98.2 ± 0.6	98.3 ± 0.6	98.0 ± 0.6	98.7 ± 0.3	*n.s.*	*0.0426*	*n.s.*
Methionine	96.5 ± 1.1	96.4 ± 1.4	95.8 ± 1.5	97.0 ± 0.8	*n.s.*	*n.s.*	*n.s.*
Phenylalanine	99.2 ± 0.3	99.2 ± 0.2	99.1 ± 0.3	99.4 ± 0.3	*n.s.*	*n.s.*	*n.s.*
Proline	98.5 ± 0.4	98.5 ± 0.5	98.3 ± 0.5	98.8 ± 0.3	*n.s.*	*n.s.*	*n.s.*
Serine	87.1 ± 3.8	88.4 ± 3.5	83.8 ± 5.8	89.9 ± 3.8	*n.s.*	*0.0313*	*n.s.*
Threonine	96.6 ± 1.0	96.0 ± 2.6	96.0 ± 2.6	96.8 ± 1.0	*n.s.*	*n.s.*	*n.s.*
Valine	95.7 ± 1.3	95.8 ± 1.3	94.8 ± 1.5	96.4 ± 0.9	*n.s.*	*n.s.*	*n.s.*
Mean all AA	95.8 ± 1.1	96.2 ± 1.1	95.1 ± 1.4	96.6 ± 0.8	*n.s.*	*0.0370*	*n.s.*

Values are mean ± SD, n = 7 - 8 rats. The effect of the group was tested with a two-way ANOVA model. Values with different letters within the same row are statistically different. AA, amino acid; n.s, not significant. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

608 **Table 4** Transfer of dietary nitrogen to the urea pool and to the liver, gastrocnemius muscle, kidney,

609 and skin 6 h after ingestion of the experimental meal (expressed as % of ingested nitrogen)

Age	**2 months**		**11 months**		***P-value***		
Group	**2m-na**	**2m-ha**	**11m-na**	**11m-ha**	***Age effect***	***Adiposity effect***	***Age x Adiposity***
Body urea pool	3.9 ± 2.1[a]	5.3 ± 2.2[ab]	8.3 ± 1.9[b]	5.4 ± 3.1[ab]	*0.0221*	*n.s.*	*0.0296*
Urinary urea pool	2.4 ± 1.4	4.0 ± 2.1	4.1 ± 2.3	3.6 ± 2.1	*n.s.*	*n.s.*	*n.s.*
Total deamination	6.3 ± 3.1[a]	9.3 ± 3.8[ab]	12.4 ± 3.5[b]	9.0 ± 2.4[ab]	*0.0303*	*n.s.*	*0.0170*
Liver	8.6 ± 2.7	9.6 ± 3.8	11.9 ± 6.7	15.3 ± 7.3	*0.0359*	*n.s.*	*n.s.*
Muscle	0.8 ± 0.5	0.7 ± 0.7	1.0 ± 0.8	0.8 ± 0.8	*n.s.*	*n.s.*	*n.s.*
Kidney	1.7 ± 0.7	1.7 ± 0.4	2.0 ± 0.3	1.8 ± 0.3	*n.s.*	*n.s.*	*n.s.*
Skin	12.4 ± 3.8	14.0 ± 5.0	15.2 ± 3.7	16.8 ± 4.3	*n.s.*	*n.s.*	*n.s.*

610 Values are mean ± SD, n = 5 - 7 rats. The effects of age and adiposity and their interaction were tested with a
611 two-way ANOVA model. Values with different letters within the same row are statistically different. n.s, not
612 significant. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal
613 adiposity; 11m-ha: 11 months/high adiposity.

614

615 **Table 5** Expression of the main peptides and amino acids transporters and hormones in the mucosa of

616 the different segments of the small intestine according to age and adiposity levels

Age	2 months		11 months		*P-value*		
Group	**2m-na**	**2m-ha**	**11m-na**	**11m-ha**	***Age effect***	***Adiposity effect***	***Age x Adiposity***
Duodenum							
PEPT1	4.4 ± 3.9	4.5 ± 3.1	6.3 ± 3.5	4.4 ± 2.6	*n.s.*	*n.s.*	*n.s.*
CCK	2.2 ± 1.1	2.2 ± 1.1	3.5 ± 1.5	4.2 ± 1.6	*0.0047*	*n.s.*	*n.s.*
Secretin	4.7 ± 3.2	6.6 ± 5.4	5.0 ± 1.1	6.9 ± 6.9	*n.s.*	*n.s.*	*n.s.*
Jejunum							
PEPT1	1.2 ± 0.4	0.9 ± 0.3	1.0 ± 0.7	0.9 ± 0.3	*n.s.*	*n.s.*	*n.s.*
Slc6a19	2.5 ± 1.8	1.8 ± 0.5	1.7 ± 0.7	1.4 ± 1	*n.s.*	*n.s.*	*n.s.*
Slc38a2	1.2 ± 0.6	1.1 ± 0.3	1.5 ± 0.6	0.8 ± 0.2	*n.s.*	*n.s.*	*n.s.*
Slc1a1	2.7 ± 3.7	1.1 ± 0.7	1.5 ± 0.7	1.2 ± 1.4	*n.s.*	*n.s.*	*n.s.*
Slc6a14	3.8 ± 2.6	4.3 ± 3.4	7.0 ± 3.2	3.5 ± 2.0	*n.s.*	*n.s.*	*n.s.*
Slc36a1	2.2 ± 0.7	1.7 ± 0.4	2.1 ± 1.2	1.8 ± 0.7	*n.s.*	*n.s.*	*n.s.*
Slca31	3.0 ± 1.3	2.4 ± 2.7	3.4 ± 1.4	2.8 ± 1.8	*n.s.*	*n.s.*	*n.s.*
Slc1a5	1.1 ± 0.8	1.0 ± 0.5	1.4 ± 0.9	0.8 ± 0.2	*n.s.*	*n.s.*	*n.s.*
Ileum							
PEPT1	3.7 ± 1.4	4.0 ± 1.1	4.4 ± 1.6	3.4 ± 0.8	*n.s.*	*n.s.*	*n.s.*
Slc6a19	1.5 ± 1.0	1.0 ± 0.2	1.4 ± 0.5	1.0 ± 0.3	*n.s.*	*0.0339*	*n.s.*
Slc38a2	5.9 ± 1.7^{a}	4.5 ± 0.9^{ab}	6.9 ± 4.2^{ab}	4.0 ± 1.1^{b}	*n.s.*	*0.0075*	*n.s.*
Slc1a1	1.8 ± 0.6	1.4 ± 0.4	1.6 ± 0.6	1.4 ± 0.4	*n.s.*	*n.s.*	*n.s.*
Slc6a14	6.3 ± 2.8	5.4 ± 1.9	7.6 ± 4.7	7.8 ± 5.8	*n.s.*	*n.s.*	*n.s.*
Slc36a1	4.0 ± 0.5	3.8 ± 0.7	5.8 ± 3.4	4.3 ± 0.8	*n.s.*	*n.s.*	*n.s.*
Slca31	8.5 ± 2.9	8.2 ± 2.3	9.4 ± 4.1	7.1 ± 1.3	*n.s.*	*n.s.*	*n.s.*
Slc1a5	5.1 ± 2.9	4.1 ± 1.9	8.2 ± 5.0	3.7 ± 1.5	*n.s.*	*0.0271*	*n.s.*

617 Values are mean ± SD, n = 7 - 8 rats. The results are expressed in arbitrary units. The effects of age and adiposity
618 and their interaction were tested with a two-way ANOVA model. Values with different letters within the same
619 row are statistically different. CCK, cholecystokinin; PEPT1, Peptide transporter 1; Slc, solute carrier; n.s, not
620 significant. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal
621 adiposity; 11m-ha: 11 months/high adiposity.

FIGURE 1

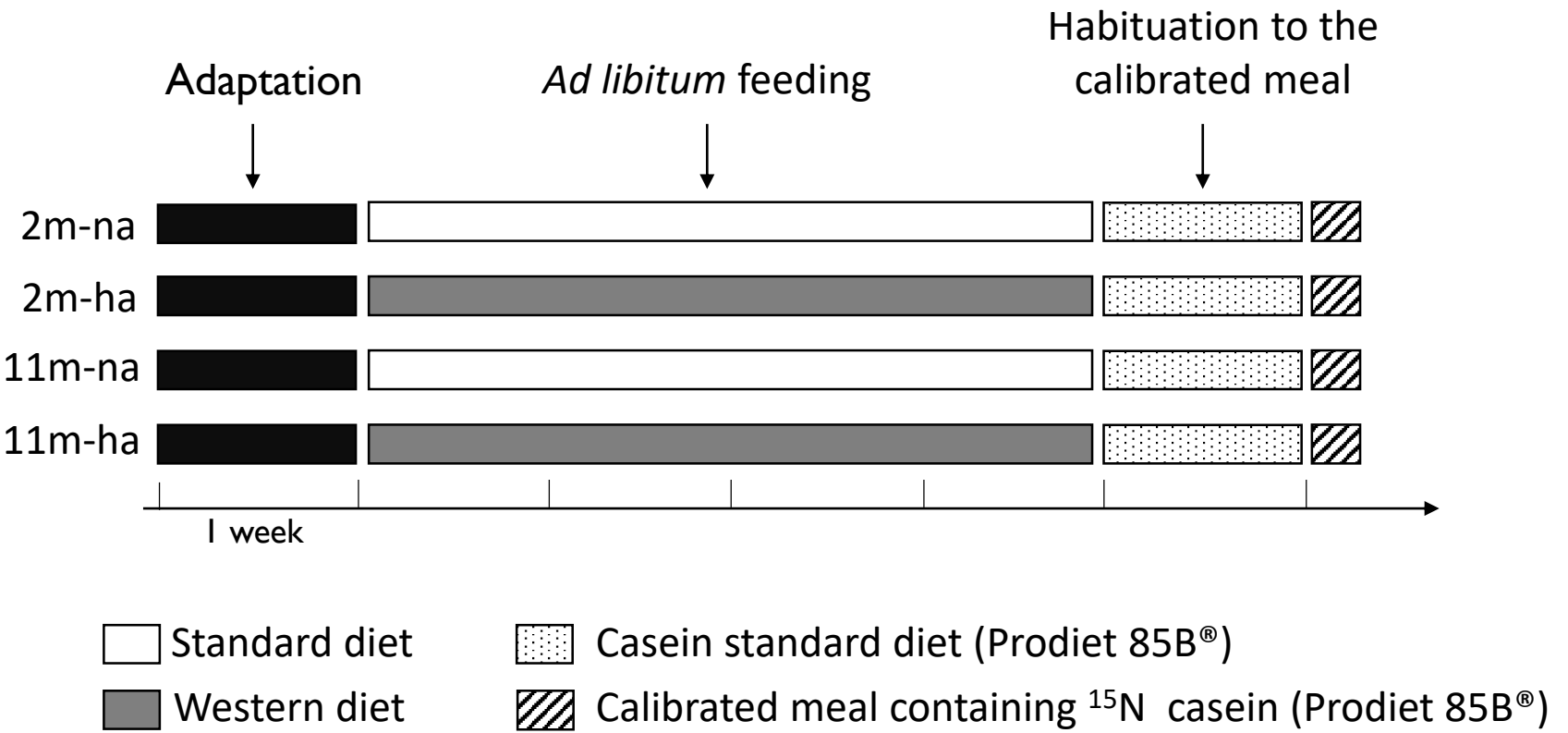

Adaptation
Ad libitum feeding
Habituation to the calibrated meal
2m-na
2m-ha
11m-na
11m-ha
1 week
Standard diet
Western diet
Casein standard diet (Prodiet 85B®)
Calibrated meal containing ^{15}N casein (Prodiet 85B®)

Figure 2

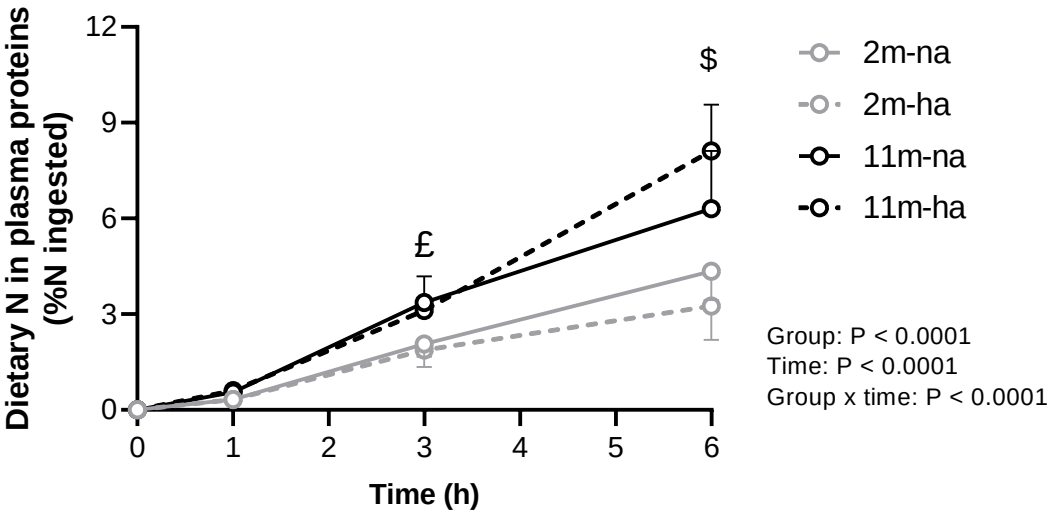
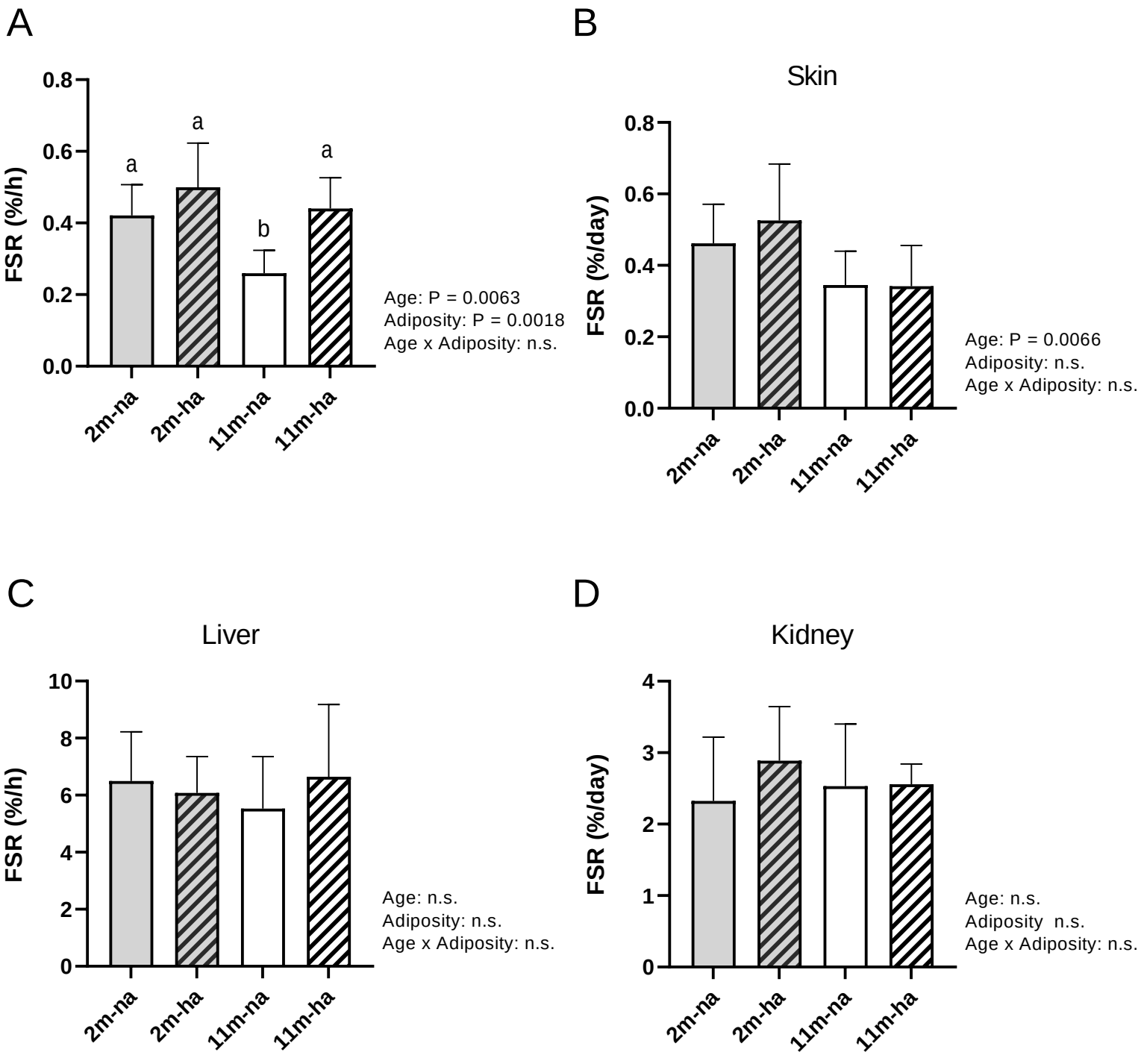

Dietary N in plasma proteins (%N ingested)
12
9
6
3
0
0
1
2
3
4
5
6
Time (h)
£
$
2m-na
2m-ha
11m-na
11m-ha
Group: P < 0.0001
Time: P < 0.0001
Group x time: P < 0.0001

Figure 3


A
FSR (%/h)
0.8
0.6
0.4
0.2
0.0
a
a
b
a
2m-na
2m-ha
11m-na
11m-ha
Age: P = 0.0063
Adiposity: P = 0.0018
Age x Adiposity: n.s.
B
Skin
FSR (%/day)
0.8
0.6
0.4
0.2
0.0
2m-na
2m-ha
11m-na
11m-ha
Age: P = 0.0066
Adiposity: n.s.
Age x Adiposity: n.s.
C
Liver
FSR (%/h)
10
8
6
4
2
0
2m-na
2m-ha
11m-na
11m-ha
Age: n.s.
Adiposity: n.s.
Age x Adiposity: n.s.
D
Kidney
FSR (%/day)
4
3
2
1
0
2m-na
2m-ha
11m-na
11m-ha
Age: n.s.
Adiposity n.s.
Age x Adiposity: n.s.

Moderate adiposity levels counteract protein metabolism modifications associated with aging in rats

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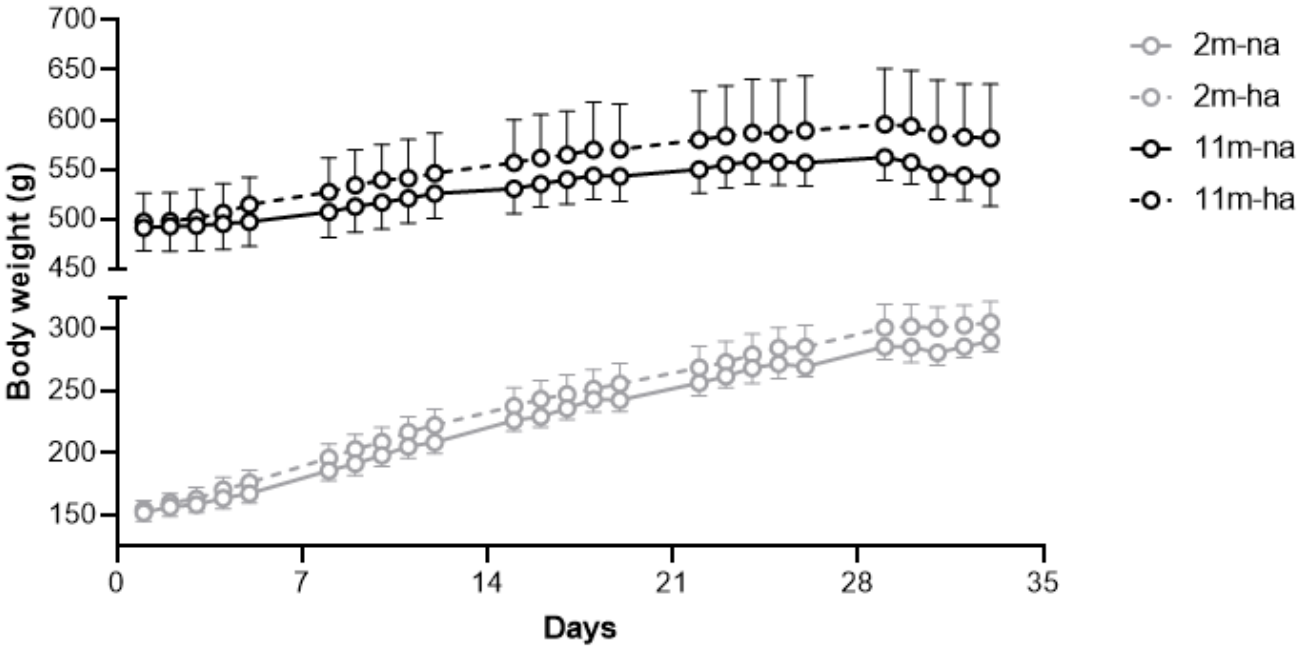
Supplemental data



Supplementary Figure 1. Body weight of the 2-month and 11-month rats from their arrival to the sixth week of experiment. Values are expressed as mean ± SD (n = 7 - 8). Within each age group, the two-way ANOVA revealed a significant effect of time and interaction of time and group ($P < 0.001$), without any group effect. No post hoc difference was observed between the different adiposity level groups in young or middle-aged rats. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.

Supplementary Table 1. Primer sequences designed with Prime Express software and used for duodenum, jejunum and ileum mRNA analysis

Gene	Froward Primer (5' to 3')	Reverse primer (3' to 5')
β-actin	GCTTCTTTGCAGCTCCTTCGT	AGCGCAGCGATATCGTCAT
CCK	CAGGTCCGCAAAGCTCCTT	TCCAGGCTCTGCAGGTTCT
Seceretin	TGCTGCTCTCAAGTTCTTTCGT	CGTCCCGTCCGAGTGTCT
PEPT1	GGCTGAGGCAGGCCACTT	AGCAAGGAGGCGAACAGAAC
Slc6a19	CCGAGCGCTTTGATGACTGT	GGCAGGTCGAACCCATTG
Slc38a2	TCTGCAGGCGGACATTAACC	ACGCGGCAGGCAGATG
Slc1a1	GTCCTGAGTGGGCTTGCAAT	CCGCACGACTATGAAATAGATCAGT
Slc6a14	TCTGTGTGACTCAGGCTGGAA	CCCATCCAGCACAGAAGTGA
Slc36a1	TTCTGCTGCGTCTACTTTGTGTTT	TGGCTGCCTCTATCACCTGTT
Slca31	GCGGTCCATGACAAAGGTTTAA	TCGATTGGAACCAAGGATGTTT
Slc1a5	TCCAATCTGGTGTCTGCTTCTG	TGGTCCATGGTTGCATTGC

Supplementary Table 2. Transfer of dietary nitrogen to the liver, gastrocnemius muscle, kidney, and skin 6 h after ingestion of the experimental meal (expressed as % of ingested nitrogen per 100 g of body weight)

Age	**2 months**		**11 months**		***P-value***		
Group	**2m-na**	**2m-ha**	**11m-na**	**11m-ha**	***Age effect***	***Adiposity effect***	***Age x Adiposity***
Liver	2.7 ± 0.8	3.0 ± 1.2	2.2 ± 1.3	2.6 ± 1.2	*n.s.*	*n.s.*	*n.s.*
Muscle	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	*n.s.*	*n.s.*	*n.s.*
Kidney	0.6 ± 0.2[a]	0.5 ± 0.1[a]	0.4 ± 0.1[ab]	0.3 ± 0.1[b]	*0.0007*	*n.s.*	*n.s.*
Skin	3.9 ± 1.2	4.4 ± 1.6	2.8 ± 0.7	3.0 ± 0.8	*0.0066*	*n.s.*	*n.s.*

Values are mean ± SD, n = 7 - 8 rats. The effects of age and adiposity and their interaction were tested with a two-way ANOVA model. Values with different letters within the same row are statistically different. *n.s*, not significant. 2m-na, 2 months/normal adiposity; 2m-ha, 2 months/high adiposity; 11m-na, 11 months/normal adiposity; 11m-ha: 11 months/high adiposity.