This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This Accepted Manuscript will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Stable isotope analysis of dietary arginine accrual and disposal efficiency in male rats fed diets of different protein content.

Floriana Rotondo\textsuperscript{1}, Tania Sanz\textsuperscript{1}, José-Antonio Fernández-López\textsuperscript{1,2,3}, Marià Alemany\textsuperscript{1,2,3}, Xavier Remesar\textsuperscript{1,2,3*}

\textsuperscript{1}\textit{Department of Biochemistry and Molecular Medicine, Faculty of Biology, University of Barcelona, Barcelona, Spain,}\textsuperscript{2}\textit{Institute of Biomedicine, University of Barcelona, Barcelona, Spain,}\textsuperscript{3}\textit{CIBER-OBN. Barcelona, Spain}

*: Corresponding Author

Prof. Xavier Remesar

Department of Biochemistry and Molecular Medicine, Faculty of Biology, University of Barcelona

Av. Diagonal 643; 08023 Barcelona, Spain

Tel: +34 934021518; Fax: +34 934037064; e-mail: xremesar@ub.edu

Shortened title: Arginine accrual is dependent of diet

Key words: $^{15}$N-arginine; cafeteria diet; high-protein diet; arginine accrual; non accounted nitrogen
Abstract

The administration of diets with different protein/energy ratios induce variable but distinctive responses in rats; an excessive protein content tends to decrease fat accumulation, but reversion of this ratio tends to increase adipose tissue mass. The fate of N derived from amino acid metabolism is not only dependent on energy and dietary protein; the increased excretion of urea elicited by high-protein diets contrasts with the lower urea excretion (despite excess dietary protein and energy) in rats fed a cafeteria diet. After one month of exposure to high-protein (HPD) or cafeteria (CD) diets, we administered a gavage of $^{15}$N-arginine to undisturbed adult male rats, in order to trace the utilization of this not-recyclable-N amino acid under diets with different protein/energy relationships. Rats fed high-protein diet excreted higher amounts of N in urine and showed much lower gastrointestinal content of label. The CD rats decreased the excretion of urine N. Both groups’ N balance showed a significant proportion of N not-accounted-for (but excreted nevertheless), the proportion being especially large in the HPD group. In conclusion, the process of disposal of amino acid N through the so far unknown pathway for "non-accounted-for N" is, thus essentially dependent on excess amino acid availability; independently of urea cycle operation and diet energy content.

Introduction

The considerable worldwide extension of metabolic syndrome, and the widely extended therapeutic failure in the treatment of one of its main and patent symptoms, obesity, has spurned a renewed interest in the use of modified-composition diets for its treatment. Evidently, low- and very low-energy diets significantly affect the body weight of the patients, often for a time, but the changes are seldom permanent. Further to that, morbid obesity has been found to be practically impervious to dietary treatments.

After the growing disenchantment with most hypocaloric diets for obesity treatment, alone or combined with drugs and exercise, ketogenic, dissociate, and high-protein diets have been tested, and then widely used with limited success, if any. High-protein diets, are widely used by sportsmen and body builders, in the assumption that excess dietary protein increases body muscle mass and helps eliminate fat tissue. Most of the subjects consuming these diets also limit the intake of lipid and carbohydrate, and often combine the dietary manipulation with strenuous exercise training and the consumption of anabolic hormones and dietary supplements. The extreme dangers that these cocktails may produce have been repeatedly analysed, exposed and denounced. In any case, the aura of high-protein diets remain as a —possibly— last ditch for the dietary treatment of obesity.
The problem is, however, that our knowledge of body amino acid metabolism, and the fate of dietary amino acids, and even the pathways used, has been neglected in the last decades. High-protein diets have been used for the treatment of obesity without actually knowing how the dietary modification may alter the energy homoeostasis of the body and, especially, ignoring the elaborated mechanisms that prevent the loss of "precious" 2-amino N, especially that of essential amino acids. This well-known evolutionary trend maintains alive perhaps, the most disfavoured half of the World’s population, but we do not know yet (in their full extent) the mechanisms and regulative paths that determine this biological trait.

The results obtained so far with high-protein diets are a widely discordant mixed bag, since there is no uniform pattern of results obtained from different experimental designs, and more often than not the effects observed fall well within the wide range of variation of "normal" diets. In many experiments done on rodents, the additional dietary N load often consists of purified, high quality protein. This fact makes even more complicated the evaluation of the results, since they combine the alteration of diet protein proportion with the higher essential amino acid availability, and modifications in energy density and the proportions of carbohydrate and lipids. The case of cafeteria diets is paradigmatic, they are hyperlipidic, but their protein content is usually in the higher range of normalcy. However, urea production, i.e. amino acid oxidation, is decreased, and the difference is not compensated by a parallel body protein accrual.

In animal studies, dietary supplementation with arginine improves muscle protein deposition and intramuscular lipid concentrations, while reducing fat mass. These studies suggest that arginine regulates lipid and protein content in a tissue-specific way. Thus, the lipogenic responses of adipocytes to arginine depend on the cell type and differentiation stage. From these data, it seems that any arginine surplus is oxidized. However, an “arginine paradox” has been observed: the more arginine enters the body, more is destroyed, affecting metabolic processes, such as the production of nitric oxide.

In spite of the technical complexities inherent to the use in vivo of stable isotopes, there are multiple advantages of using guanido group-labelled arginine as a tracer for dietary protein fate. Arginine is relatively abundant in proteins and is a key intermediate of the urea cycle. Most of the waste N goes through the urea cycle and is consequently incorporated to the guanido group of arginine, which is practically not recycled, thus, it cannot intervene (and be diluted) in transamination processes, a main reason why it has been used often in N tracer studies, including analyses of protein turnover. These metabolic patterns make arginine an excellent candidate to follow amino acid utilization by different tissues after an oral load and determine the differences of amino acid metabolic use, and regulation, between cafeteria and high-protein diet.
Experimental Methods

Animals and experimental setup

All animal handling procedures and the experimental setup were carried out in accordance with the animal handling guidelines of the European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used (DAAM 6911). This limited keeping the animals isolated in metabolic cages to a maximum of 24 h to prevent unacceptable levels of stress.

Nine-week-old male Wistar rats (Janvier, Le Genest-Saint-Israel, France) were used. The animals were randomly divided into three groups (N = 6 each) and were fed ad libitum, for 30 days, either standard rat chow (SD) (Teklad 2014, Teklad diets, Madison WI, USA) (initial rat weight 356±5 g), a simplified cafeteria diet (CD) (initial rat weight 350±6 g), or high-protein diet (HPD) (initial rat weight 354±6 g). All animals had free access to water. They were housed in 2-rat solid-bottom cages, and were kept in a controlled environment (lights on from 08:00 to 20:00, temperature 21.5-22.5ºC and 50-60% humidity). Body weight and food consumption were recorded daily. Calculation of food ingested was done as previously described by weighing the differences in food offered and debris left.

Diets

In the standard diet (Teklad 2014) (Table 1), 20% of digestible energy was derived from protein, 13% from lipids, and 67% from carbohydrates (including 10% from low MW oligosaccharides). The main components of standard diet were (as specified by manufacturer): wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil and mineral and vitamin supplements.

The cafeteria diet (CD) was formed by plain cookies spread with liver pâté, bacon, standard chow pellets, water and milk supplemented with 300 g/L sucrose plus 10 g/L of a mineral and vitamin supplement (Meritene, Nestlé, Esplugues, Spain). All components were kept fresh (i.e. renewed daily). From the analysis of diet components and the ingested items, we calculated that, in CD, a mean 41% of energy was derived from lipids, 12% of energy was derived from protein, and 47% of energy was derived from carbohydrates (20% from oligosaccharides). The analysis of food consumption of rats on the cafeteria diet showed that the ingestion of the different food choices was fairly constant in type and quantity consumed per rat and day as previously observed. The computed nutrient consumption along the period studied, expressed as a percentage of total energy...
ingested was 20.3% for pellet, 26.1% for cookies, 17.9% for bacon, 16.52% for pâté and 19.2% for sugar-enriched milk.

The high-protein diet (HPD) was prepared by the addition of high quality protein (cow milk casein) (J.Escuder, Rubí, Spain) and low quality protein (fish gelatin) (J.Escuder), to ground standard chow. The mix (16.5 g of standard chow + 2.35 g of casein + 2.05 g of gelatin + 0.2 mL of sunflower oil + 17 mL of water) was thoroughly mixed to a paste and then extruded from syringes to form pellets, dried at 40°C for 24 hours. Aversion tests to this diet gave negative results. The energy derived from proteins for the HPD was 41%, that from lipids was 12% and that derived from carbohydrates was 47%. Nitrogen content of all diet components was measured with a semi-automatic Kjeldahl procedure using a ProNitro S system (JP Selecta, Abrera, Spain). Lipid content was measured with the Folch et al. method.31

Supplemental Table 2 shows the mean fatty acid composition of the diets. These data were calculated for each food item according to a previous study using a standard method for fatty acid analysis.33 The cafeteria diet showed higher levels of most fatty acids than controls, as expected, except for linoleic acid, which was present in higher quantities in standard diet.

Arginine-\textsuperscript{15}N tracer gavage and tissue sampling

After 29-days of dietary treatment, the rats were given a gavage of 1 mL of 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO USA) in water, containing 60 pmol of L-arginine-[guanidineimino-\textsuperscript{15}N]-hydrochloride (98% atom \textsuperscript{15}N) (Sigma Aldrich, Munich, Germany), using a polyethylene intra-gastric cannula.

After the gavage, the rats were transferred for 24 h to individual plastic metabolic cages (Techniplast Gazzada, Buggugiate, Italy), maintaining the environmental and dietary conditions described, but recovering urine and faeces separately.

On day 30, the rats were anesthetized with isoflurane and then killed by exsanguination with a dry-heparinized syringe, through the exposed aorta. Tissue samples (liver, kidneys, three white adipose tissue (WAT) locations, interscapular brown adipose tissue (IBAT), hind leg muscle and intestines) were dissected, cleaned (gut) and rapidly frozen in liquid nitrogen. They were maintained at -70°C until processed for analyses. Plasma and erythrocyte fractions were obtained by centrifugation of the blood. The remaining carcass, blood and dissection debris were sealed in polyethylene bags, which were subsequently autoclaved at 120°C for 2 h, the bag contents were weighed and then minced to a smooth paste with a blender (obtaining a total rat homogenate).

An additional control group of six control diet-fed animals was used to obtain the basal values.
of N content. These rats (360±8 g) were reared in parallel to the other experimental groups, but they were not given the $^{15}$N-Arg gavage. No differences in weight, and food consumption were observed when comparing this group and the SD.

**Metabolite analyses**

Urine and plasma parameters were measured using commercial kits (BioSystems, Barcelona, Spain): urea (#11537), glucose (#11504), total cholesterol (#11505) and triacylglycerols (#11528). Total proteins were measured in tissue homogenates, total blood and plasma using the Lowry et al. method. Plasma samples were deproteinized with acetone and the supernatants were used for amino acid analysis as previously described.

**$^{15}$N Analysis**

Aliquots of frozen tissues (about 200 mg) were homogenized in 2 mL of pure water (resistivity 18 MΩ) using a cell disruptor (IKA, Staufen, Germany), and then sonicated (Sonics Vibracell VC130PB, Sonics & Materials, Newtown, CT USA) to ensure complete cell disruption. The homogenates were then used directly for analysis. Aliquots of 5-10 µL of homogenized samples (equivalent to 0.5-1 mg of fresh tissue) were introduced in tin capsules (3.3 x 5 mm; Cromlab, Barcelona, Spain), filled with Al$_2$O$_3$ as adsorbent. The microcapsules were sealed, and used for $^{15}$N-enrichment measurement. The analyses were done with a Delta C gas chromatography-combustion-isotopic ratio mass spectrometer (GC-C-IRMS) (Finnigan MAT, Bremen, Germany) coupled to an elemental analyser (Flash 1112; Thermo Fisher, Waltham MA USA).

The $^{15}$N/$^{14}$N isotope ratios were expressed on a relative scale as deviation, referred as $\partial$ units with the notation $\%$ (parts of thousand), relative to the isotope ratio content of international standards. The corresponding $\partial$ values for the samples were determined from the equation:

$$\partial = ([R_{sa}/R_{at}] - 1) \times 1000$$

Where $R_{sa}$ is the $^{15}$N/$^{14}$N ratio obtained for each sample and $R_{at}$ correspond to the $^{15}$N/$^{14}$N ratio of the standards.

The values of $\partial$ were expressed as atom percentage (at%) using the formula:

$$Nat\% = 100 \times (\frac{^{15}N}{^{15}N + ^{14}N})$$

The net enrichment (atom percentage excess) in $^{15}$N was calculated from the difference between the
atom percentages of samples and their corresponding blanks (i.e. the results obtained from the same tissues of control rats, which did not receive the labelled arginine gavage):

\[
\text{Atom percentage excess} = \text{at}\% \text{ sample} - \text{at}\% \text{ blank}
\]

Finally, using the values of atom percentage excess, arginine molecular weight and Avogadro’s number, we computed the results to express the proportion of isotopic marker in relation to the total amount of arginine ingested (i.e. the sum of diet and gavage).

**Statistical analysis**

Statistical comparisons were carried out using one- or two-way ANOVA analyses with the Prism 5 program (GraphPad Prism, Palo Alto CA, USA).

**Results**

Table 2 shows the rat size and energy intake during the 30-day dietary treatment. The cafeteria-fed group showed the highest weight increase during treatment, and also the highest energy intake and growth rate, whereas the HPD group showed the lowest values for all these parameters. The energy cost of growth was higher in CD and HPD groups than in SD. The mean nitrogen intake was higher in HPD group than either SD and CD groups.

Cafeteria-fed rats experienced marked increases in the weights of three different white adipose tissue depots (alone or combined weight) in contrast with the lowest values of the HPD group. The HPD rats showed, also, lower small intestine weights. These data are shown in detail in Supplemental Table 1.

Plasma values for metabolites are presented in Table 3. The HPD group had the highest plasma urea concentrations. Arginine levels were higher in the CD and in the HPD groups compared with SD whereas ornithine was lower in the CD group. By contrast, the HPD rats showed the lowest citrulline levels. The diet treatment did not influence significantly the plasma levels of glucose, cholesterol and total protein main excretion fractions.

Figure 1 shows the proportions of ingested $^{15}$N label for all three dietary groups, expressed as accrued and excreted fractions. There were no significant differences between the groups in the proportion of $^{15}$N accrual. However, when the values of non-accrued (i.e. excreted) $^{15}$N atoms were sorted in their main excretion fractions, the HPD group showed an almost nil presence of $^{15}$N in stool and gastrointestinal content, this way partly compensated by the highest values of urine-excreted label, and a large proportion of label lost (not-accounted for). Compared with the control SD rats, the CD group only showed a lower urine-excreted label and, a higher proportion of the not-
accounted-for N fraction.

Analyses of urine urea levels showed that in all groups, urine N was justified in proportions higher than 98% by urea, making the values for urea N and urine N practically interchangeable.

Table 4 depicts the distribution of total $^{15}$N atoms in tissues. When expressed as percentage of absorbed N, the values were highest for kidney in the HPD group and for epidydimal and retroperitoneal WAT in CD rats. However, when the data were expressed per g of protein, the cafeteria-fed rats showed higher values in epidydimal WAT and plasma, but lower in the IBAT than in SD group. The low levels of $^{15}$N in the IBAT of the cafeteria group were also observed in the HPD group.

Figure 2 shows the relationship between $^{15}$N atoms and total diet arginine ingestion and with respect to whole-body arginine. The HPD group showed the lowest values for the number of $^{15}$N atoms retained in relation to the arginine ingested. Both, the HPD and CD animals showed lower values for total $^{15}$N content than the SD controls. Finally, the HPD rats showed the lowest arginine (in fact, guanido group) specific activity (i.e., the $^{15}$N / total-Arg ratio). Nevertheless, despite the differences exposed, no statistically significant differences in arginine accrual were found between the three dietary treatments used.

Discussion

The golden rule of survival for mammals establishes that glycaemia has to be maintained, and amino acids, especially the essential ones, preserved. In our experimental model, the animals had no problems of amino acid availability; the only plausible question was the need to dispose of their excess. There was, neither, any deficit in dietary energy availability; at least theoretically, since the energy density of the food of HPD diet was the same than that of SD (that of CD was higher), their differences rested only in the proportion of nutrients offered. However, this factor dramatically modified the handling of ingested protein. In the SD group, the accrual of labelled arginine was higher than in the other groups, which resulted in the highest labelled arginine specific activity (and, in contrast that of HPD group, lowest). The label in the intestinal content and stool contained a large proportion of that ingested in both SD and CD groups, but was almost absent in HPD animals, which is in agreement with a speedier and more efficient digestion of protein in diets with high protein content. This seems to be clearly an adaptive modulation of the gut function, which ability to process foods is modified according to the composition of the diet within a relatively short period.
Most of the absorbed label was found in the total homogenate, largely because skeletal muscle
the main quantitative receptor because of its size (about 45% of the body lean mass) and high
protein content, liver concentrated a significant amount of the label, probably because of its
critical role in amino acid homoeostasis and disposal, a task shared (for dietary protein) with the
intestine.

The changes observed in urea excretion can be traced, too, to the primary energy partitioning
role of intestine. High availability of carbohydrate in enterocytes raises the activity of the pentose-P
pathway, raising the levels of NADPH, which modulates the disposal of amino N by activation of
the synthesis of ornithine and citrulline, precursors of (and fuel to sustain) the liver urea cycle.
This situation explains the case of SD and HPD groups, but not that of CD, since there was
sufficient dietary carbohydrate in the gut but the urea cycle was not activated as expected. In fact,
all the substrate relationships described respond to corollaries of the golden rules of homoeostasis
for survival, in short: high lipid saves glucose. This is so because lipids were present only in limited
amount and/or sporadically in the diet of our long line of ancestors (and in the diet of present-day
rodents). When lipid is present in the bloodstream, the metabolic meaning is clear: there are no
dietary sources of energy and we are consuming reserves (our own lipid); then we must save energy,
and save glucose, preserve amino acids, etc. High carbohydrate availability translates into a large
amount of dietary energy that allows us to save amino acids, the extent of salvage being related to
their own relative availability. High dietary protein spurns amino acid oxidation for use as energy,
since there is no place where excess N can be stored, and protein is a fair source of energy too.
Nevertheless, low dietary protein is a red line that requires its preservation for survival. HPD rats
oxidize dietary protein as energy source to a high level (resulting in high urea excretion), because
the relative amount of carbohydrate was lower than under standard conditions. However, not all
excess amino acids go through the canonical urea cycle pathway, since a large proportion of N is
eliminated through alternative unknown pathways (N gap), as previously reported and repeated here.

The results obtained for CD rats agree with a number of previously published papers, i.e.
cafeteria diet increases body size, the mass of adipose tissue and the accrual of protein, increases
appetite and generates a N-gap, higher than that of SD, in their nitrogen balance.
CD rats showed a marked decrease in urea excretion, despite having a protein intake similar
to that of the SD group. Clearly, the high presence of dietary lipid (and excess energy availability)
blocked the oxidation of amino acids, and inhibited the urea cycle, as previously described.
The HPD diet elicits a clear correlation between higher dietary 2-amino N intake and
increased urea excretion. The rats fed the HPD were slightly smaller, with less adipose tissue than
the SD-fed rats, and their energy intake was lower. This may be, at least in part, a consequence of the higher satiating effect of protein;\textsuperscript{52} but protein intake induces, also, a higher thermic response to food than other nutrients,\textsuperscript{53} thus decreasing the efficiency of growth, as observed here. In any case the most important differences between dietary groups may lie, in the cost of live tissue deposition since, contrarily to what we expected, the labelled arginine that found its way into body protein was maximal in SD group and minimal in HPD group. This counterintuitive result implies that the use of amino acids for energy takes its toll, lowering considerably the overall energy efficiency of the animal.

In all groups, the proportion of N excreted with respect to that ingested, and the distribution of label between organs and tissues was similar, but the extent of the "unaccounted for N" (in any case, excreted) was higher in both CD and HPD rats than in rats fed the SD. Thus, the explanation of urea cycle inhibition as justification for the activation of other (so far unknown) pathways than the urea cycle for N disposal, used to explain the existence of the wide N balance gap in CD-fed rats could not be sustained.\textsuperscript{49} The HPD rats had an apparently fully functional urea cycle, as proven by their high plasma urea, and high daily urinary N excretion, again as urea, but they showed an N-gap that doubled that of CD rats. This unexplained form of N excretion has been proven in a number of different settings,\textsuperscript{54} and has been found to be directly related to the diet.\textsuperscript{22} It has been attributed both to high energy and/or lipid intake, but the results presented here seem to point further away, to the existence of a regular mechanism for the disposal of excess available amino N from amino acids. This is a difficult task because evolution has fine-tuned our highly effective mechanisms to preserve amino N, leaving almost no space for disposal of eventual (rare in Nature) excesses.

In the case of cafeteria diet, previous studies have shown that the excess of lipids hampers the utilization of glucose because of insulin resistance.\textsuperscript{55} The unused glucose tends to accumulate and create a problem by itself, which usually ends in the form of additional lipid stores and often results in type 2 diabetes.\textsuperscript{56} The flow of dietary protein-derived amino acids cannot be processed at the required speed through the urea cycle because the excess of glucose and energy just prevents it. However, the increasing excess of amino N could not be stored, and, consequently is eliminated in significant proportions through the postulated N-gap-related pathway.\textsuperscript{22,49} This partial parallelism in N disposal between CD and HPD groups suggests that the N-gap pathway must be a well-established mechanism, regulated in some way by its substrates’ excess. The data presented here clearly show this high capacity and gross modulation. The activation of this postulated (albeit unknown) process is not dependent on the excess of available energy. It is related, instead to the excess of amino acids available built-up by the combination of high dietary supply and because the regular oxidative pathways are blocked by eons-old preservation schemes.\textsuperscript{57} The HPD rats have a
fully active urea cycle, uninhibited by high glucose or lipid, which would make the alternate
pathway unnecessary unless it were "regulated" by other parameters such as an excess of amino
acid N. This elusive pathway seems conceived more as an emergency safety mechanism rather than
as a developed efficient energy-providing pathway. The high cost of deposition of energy observed
in the HPD and CD rats may suggest that the mechanism is not energetically efficient.\textsuperscript{58}

In sum, in the present study, developed using arginine marked with the stable isotope \textsuperscript{15}N, we
have observed that the intestine and liver exert the initial triage of dietary nutrients, that in the case
of amino acids is controlled by the availability of 2-amino N in relation to carbohydrate, but also by
the amount (and eventual excess) of amino acids. The main regular mechanism of N disposal is the
urea cycle, but a repeatedly detected and yet unknown N gap-generating pathway seems to take care
of the surplus of amino acid N that apparently could not be processed via urea cycle. The
parallelism with cafeteria diet, which urea synthesis pathway is inhibited by excess lipid (and
carbohydrate) energy proves that this unknown mechanism is widely extended and not only
dependent on excess energy but on an excess of amino acids.

Acknowledgements

We want to appreciate the help and counsel in the analysis of isotopes of Dr. P. Teixidor from
the Mass Spectrometry Unit of Scientific and Technological Services of University of Barcelona.

Financial support

This study was done with the partial support of grant of the Plan Nacional de Ciencia y
Tecnología de los Alimentos (AGL-2011-23635), as well as of the CIBER-OBN research web. F.
Rotondo was the recipient of a Leonardo-Unipharma program (Italy) fellowship, and later a
postdoctoral fellowship from the Government of Catalonia.

Conflict of interest

None.

Autorship
X.R, M.A and J.A.F.L designed the study; F.R. and T.S. carried out the experiments; X.R. calculated the data and carried out statistical analyses; X.R. and M.A. wrote the manuscript. All authors discussed the text and contributed to the final version.

References


421 59

422

423
<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD</th>
<th>CD</th>
<th>HPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>480</td>
<td>545</td>
<td>334</td>
</tr>
<tr>
<td>Fibre</td>
<td>22</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>Ashes</td>
<td>47</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td>Lipid</td>
<td>40</td>
<td>211</td>
<td>38</td>
</tr>
<tr>
<td>Protein</td>
<td>143</td>
<td>138</td>
<td>286</td>
</tr>
<tr>
<td>Metabolizable energy *</td>
<td>12</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Amino acid content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>9.1</td>
<td>9.6</td>
<td>17</td>
</tr>
<tr>
<td>Glx</td>
<td>29</td>
<td>23</td>
<td>48</td>
</tr>
<tr>
<td>Ala</td>
<td>9.0</td>
<td>4.9</td>
<td>17</td>
</tr>
<tr>
<td>Gly</td>
<td>7.1</td>
<td>6.8</td>
<td>28</td>
</tr>
<tr>
<td>Thr</td>
<td>5.1</td>
<td>5.3</td>
<td>9.2</td>
</tr>
<tr>
<td>Pro</td>
<td>12</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Ser</td>
<td>7.2</td>
<td>5.6</td>
<td>13</td>
</tr>
<tr>
<td>Leu</td>
<td>14</td>
<td>9.6</td>
<td>21</td>
</tr>
<tr>
<td>Ile</td>
<td>6.1</td>
<td>5.7</td>
<td>11</td>
</tr>
<tr>
<td>Val</td>
<td>6.9</td>
<td>6.2</td>
<td>13</td>
</tr>
<tr>
<td>Phe</td>
<td>7.0</td>
<td>5.4</td>
<td>12</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.2</td>
<td>2.9</td>
<td>8.3</td>
</tr>
<tr>
<td>Met</td>
<td>3.1</td>
<td>2.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Cys</td>
<td>3.0</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Lys</td>
<td>7.1</td>
<td>7.2</td>
<td>15</td>
</tr>
<tr>
<td>His</td>
<td>4.1</td>
<td>3.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Arg</td>
<td>8.1</td>
<td>6.7</td>
<td>15</td>
</tr>
<tr>
<td>Trp</td>
<td>2.0</td>
<td>1.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The data are presented in g·kg$^{-1}$, except for metabolizable energy (*) expressed in MJ·kg$^{-1}$. The data corresponding to CD were calculated from the composition of the mean diet (i.e. mix of foods) intake.
Table 2  Weight, and intake (energy, nitrogen) of male rats subjected to diets with different protein content

<table>
<thead>
<tr>
<th>parameter</th>
<th>units</th>
<th>diets</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>CD</td>
</tr>
<tr>
<td>Weight (final)</td>
<td>g</td>
<td>491 ± 11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>532 ± 16&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Weight increase</td>
<td>g</td>
<td>127 ± 8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>147 ± 11&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Energy intake</td>
<td>kJ d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>370 ± 4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>556 ± 13&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nitrogen intake</td>
<td>mg d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>696 ± 7&lt;sup&gt;A&lt;/sup&gt;</td>
<td>720 ± 18&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cost of accrual</td>
<td>kJ g&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>88.1 ± 5.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>116 ± 8&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way ANOVA; post-hoc test (Tuckey test): groups with different superscript letters are statistically different (P<0.05).
Table 3  Plama metabolite levels of rats subjected to different diets.

<table>
<thead>
<tr>
<th>parameter</th>
<th>units</th>
<th>SD</th>
<th>CD</th>
<th>HPD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>mM</td>
<td>10.3 ± 0.7A</td>
<td>10.1 ± 0.6A</td>
<td>10.2 ± 1.6A</td>
<td>NS</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>mM</td>
<td>0.99 ± 0.08A</td>
<td>1.32 ± 0.15A</td>
<td>0.90 ± 0.06B</td>
<td>0.0292</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mM</td>
<td>2.35 ± 0.62A</td>
<td>1.05 ± 0.29A</td>
<td>0.91 ± 0.23A</td>
<td>NS</td>
</tr>
<tr>
<td>Urea</td>
<td>mM</td>
<td>5.38 ± 0.34A</td>
<td>5.41 ± 0.39A</td>
<td>6.73 ± 0.15B</td>
<td>0.0112</td>
</tr>
<tr>
<td>Total proteins</td>
<td>g L⁻¹</td>
<td>57.1 ± 2.7A</td>
<td>55.4 ± 1.3A</td>
<td>52.9 ± 1.5A</td>
<td>NS</td>
</tr>
<tr>
<td>Arginine</td>
<td>µM</td>
<td>107 ± 15A</td>
<td>164 ± 8.3B</td>
<td>146 ± 10AB</td>
<td>0.0032</td>
</tr>
<tr>
<td>Ornithine</td>
<td>µM</td>
<td>228 ± 17A</td>
<td>66.1 ± 8.1B</td>
<td>241 ± 22A</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Citrulline</td>
<td>µM</td>
<td>40.2 ± 2.2A</td>
<td>51.3 ± 4.2A</td>
<td>24.1 ± 3.2B</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way ANOVA; post-hoc test (Tuckey test): groups with different superscript letters are statistically different (P<0.05); NS = not statistically significant.
Table 4. Total $^{15}\text{N}$ atoms in tissues (expressed as a percentage of absorbed nitrogen) and total $^{15}\text{N}$ atoms in tissues x $10^{19}$ (per gram of protein) in rats subjected to different diets

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD</th>
<th>CD</th>
<th>HPD</th>
<th>P</th>
<th>SD</th>
<th>CD</th>
<th>HPD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6.95 ± 1.17$^\text{A}$</td>
<td>5.06 ± 1.50$^\text{A}$</td>
<td>7.11 ± 1.15$^\text{A}$</td>
<td>NS</td>
<td>3.25 ± 0.52$^\text{A}$</td>
<td>2.15 ± 0.46$^\text{A}$</td>
<td>2.44 ± 0.21$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.90 ± 0.37$^\text{AB}$</td>
<td>1.28 ± 0.32$^\text{A}$</td>
<td>2.83 ± 0.50$^\text{B}$</td>
<td>0.0484</td>
<td>9.16 ± 2.45$^\text{A}$</td>
<td>4.82 ± 1.06$^\text{A}$</td>
<td>5.89 ± 0.51$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Epididymal WAT</td>
<td>0.31 ± 0.04$^\text{A}$</td>
<td>1.99 ± 0.62$^\text{B}$</td>
<td>0.68 ± 0.24$^\text{AB}$</td>
<td>0.0185</td>
<td>2.77 ± 0.49$^\text{A}$</td>
<td>11.4 ± 2.47$^\text{B}$</td>
<td>3.70 ± 0.37$^\text{A}$</td>
<td>0.0015</td>
</tr>
<tr>
<td>Retroperitoneal WAT</td>
<td>0.24 ± 0.02$^\text{A}$</td>
<td>0.89 ± 0.29$^\text{B}$</td>
<td>0.38 ± 0.07$^\text{AB}$</td>
<td>NS</td>
<td>2.82 ± 0.62$^\text{A}$</td>
<td>1.82 ± 0.14$^\text{A}$</td>
<td>3.00 ± 0.23$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Mesenteric WAT</td>
<td>0.39 ± 0.06$^\text{A}$</td>
<td>0.63 ± 0.31$^\text{A}$</td>
<td>0.73 ± 0.23$^\text{A}$</td>
<td>NS</td>
<td>3.56 ± 0.76$^\text{A}$</td>
<td>2.81 ± 0.74$^\text{A}$</td>
<td>3.97 ± 0.76$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>IBAT</td>
<td>0.10 ± 0.04$^\text{A}$</td>
<td>0.09 ± 0.02$^\text{A}$</td>
<td>0.27 ± 0.10$^\text{A}$</td>
<td>NS</td>
<td>7.22 ± 1.11$^\text{A}$</td>
<td>3.35 ± 0.87$^\text{B}$</td>
<td>3.09 ± 0.47$^\text{B}$</td>
<td>0.0062</td>
</tr>
<tr>
<td>Small intestine</td>
<td>12.3 ± 2.0$^\text{A}$</td>
<td>12.3 ± 2.1$^\text{A}$</td>
<td>15.1 ± 3.1$^\text{A}$</td>
<td>NS</td>
<td>25.7 ± 5.7$^\text{A}$</td>
<td>14.6 ± 3.9$^\text{A}$</td>
<td>20.1 ± 3.2$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>2.02 ± 0.52$^\text{A}$</td>
<td>3.35 ± 0.75$^\text{A}$</td>
<td>3.46 ± 1.24$^\text{A}$</td>
<td>NS</td>
<td>3.93 ± 0.46$^\text{A}$</td>
<td>8.37 ± 1.54$^\text{B}$</td>
<td>3.91 ± 0.57$^\text{A}$</td>
<td>0.0079</td>
</tr>
<tr>
<td>Blood cells</td>
<td>1.82 ± 0.32$^\text{A}$</td>
<td>3.32 ± 1.04$^\text{A}$</td>
<td>3.46 ± 1.04$^\text{A}$</td>
<td>NS</td>
<td>0.87 ± 0.24$^\text{A}$</td>
<td>0.86 ± 0.20$^\text{A}$</td>
<td>0.76 ± 0.21$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Skeletal muscle *</td>
<td>28.5 ± 1.4$^\text{A}$</td>
<td>33.8 ± 9.1$^\text{A}$</td>
<td>23.9 ± 2.8$^\text{A}$</td>
<td>NS</td>
<td>2.16 ± 0.74$^\text{A}$</td>
<td>1.85 ± 0.59$^\text{A}$</td>
<td>1.06 ± 0.18$^\text{A}$</td>
<td>NS</td>
</tr>
<tr>
<td>Rest of tissues (homogenate)</td>
<td>44.1 ± 1.9$^\text{A}$</td>
<td>37.8 ± 3.6$^\text{A}$</td>
<td>43.3 ± 2.0$^\text{A}$</td>
<td>NS</td>
<td>1.85 ± 0.48$^\text{A}$</td>
<td>1.55 ± 0.26$^\text{A}$</td>
<td>1.29 ± 0.12$^\text{A}$</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Striated muscle mass was estimated using previous reports (27). The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way ANOVA; post-hoc test (Tuckey test): groups with different superscript letters are statistically different (P<0.05); NS = not statistically significant.
LEGEND TO FIGURES

Figure 1. Distribution of $^{15}$N atoms in different fractions of rats fed standard, cafeteria and high-protein diets.

A. Total atoms (expressed as a percentage of ingested label) were distributed in two large pools: accrued (white columns) and excreted (stacked black columns).

The data are the mean ± SE of six different animals. Statistical comparisons between groups: Two-way ANOVA: Accrued (P=0.0756), Excreted (P=0.5359).

B. Total atoms excreted (expressed as a percentage of ingested label): gastrointestinal content plus faeces (brown columns) and urine and other ways of excretion (stacked yellow columns).

The data are the mean ± SE of six different animals. Statistical comparisons between groups: Two-way ANOVA: Gastrointestinal content plus faeces (P=0.0053), urine and other ways of excretion (P=0.0031). Post-hoc Tuckey test: * = P < 0.05 vs SD group; • = P < 0.05 of HPD vs CD group.

C. Total $^{15}$N atoms excreted in other ways: urine (yellow columns) and non-accounted for N fractions (stacked pink columns).

The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way ANOVA for the different fractions: Urine (P=0.0053), Non-accounted for N (P=0.0189). Post-hoc Tuckey test: * = P < 0.05 vs SD group; • = P < 0.05 of HPD vs CD group.

Figure 2. Number of $^{15}$N atoms ingested, specific activity of $^{15}$N atoms in relation to arginine ingestion and arginine accrued in 24 hours.

Total body arginine content was estimated using previously published data (61).

The data are the mean ± SE of six different animals. White columns: SD group, black columns: CD group, striped columns: HPD group.

The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way ANOVA; post-hoc test (Tuckey test): * = P < 0.05 vs SD group; + = P < 0.05 of HPD vs CD group.
\[
\begin{align*}
\text{Accrued} & \quad \text{Excreted} \\
\text{Urine} & \quad \text{Non-accounted for} \\
\text{Gut content + faeces} & \\
\text{Urine and others} & \\
\end{align*}
\]