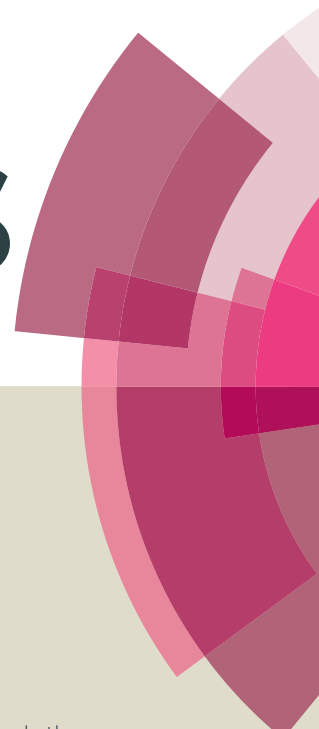


# RSC Advances



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2

3 **Stable isotope analysis of dietary arginine accrual and disposal efficiency in male rats fed diets**  
4 **of different protein content.**

5 Floriana Rotondo<sup>1</sup>, Tania Sanz<sup>1</sup>, José-Antonio Fernández-López<sup>1,2,3</sup>, Marià Alemany<sup>1,2,3</sup>, Xavier  
6 Remesar<sup>1,2,3</sup> \*

7

8 <sup>1</sup>Department of Biochemistry and Molecular Medicine, Faculty of Biology, University of  
9 Barcelona, Barcelona, Spain, <sup>2</sup>Institute of Biomedicine, University of Barcelona, Barcelona, Spain,  
10 and <sup>3</sup>CIBER-OBN. Barcelona, Spain

11

12 \*: Corresponding Author

13 Prof. Xavier Remesar

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

15 Av. Diagonal 643; 08023 Barcelona, Spain

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

17

18 Shortened title: Arginine accrual is dependent of diet

19 Key words: <sup>15</sup>N-arginine; cafeteria diet; high-protein diet; arginine accrual; non accounted nitrogen

20

## 21 Abstract

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

37

## 38 Introduction

39 The considerable worldwide extension of metabolic syndrome, and the widely extended  
40 therapeutic failure in the treatment of one of its main and patent symptoms, obesity,<sup>1</sup> has spurred a  
41 renewed interest in the use of modified-composition diets for its treatment<sup>2</sup>. Evidently, low- and  
42 very low-energy diets significantly affect the body weight of the patients, often for a time, but the  
43 changes are seldom permanent.<sup>3,4</sup> Further to that, morbid obesity has been found to be practically  
44 impervious to dietary treatments.<sup>5</sup>

45 After the growing disenchantment with most hypocaloric diets for obesity treatment,<sup>6</sup> alone  
46 or combined with drugs and exercise, ketogenic, dissociate, and high-protein diets have been tested,  
47 and then widely used with limited success, if any.<sup>7,8,9</sup> High-protein diets, are widely used by  
48 sportsmen and body builders, in the assumption that excess dietary protein increases body muscle  
49 mass and helps eliminate fat tissue.<sup>10</sup> Most of the subjects consuming these diets also limit the  
50 intake of lipid and carbohydrate, and often combine the dietary manipulation with strenuous  
51 exercise training and the consumption of anabolic hormones and dietary supplements.<sup>11</sup> The  
52 extreme dangers that these cocktails may produce have been repeatedly analysed, exposed and  
53 denounced.<sup>12</sup> In any case, the aura of high-protein diets remain as a –possibly— last ditch for the  
54 dietary treatment of obesity.<sup>13</sup>

55 The problem is, however, that our knowledge of body amino acid metabolism, and the fate of  
56 dietary amino acids, and even the pathways used, has been neglected in the last decades.<sup>14</sup> High-  
57 protein diets have been used for the treatment of obesity without actually knowing how the dietary  
58 modification may alter the energy homeostasis of the body and, especially, ignoring the elaborated  
59 mechanisms that prevent the loss of "precious" 2-amino N, especially that of essential amino  
60 acids.<sup>15</sup> This well-known evolutionary trend maintains alive perhaps, the most disfavoured half of  
61 the World's population, but we do not know yet (in their full extent) the mechanisms and regulative  
62 paths that determine this biological trait.<sup>16</sup>

63 The results obtained so far with high-protein diets are a widely discordant mixed bag, since  
64 there is no uniform pattern of results obtained from different experimental designs, and more often  
65 than not the effects observed fall well within the wide range of variation of "normal" diets.<sup>17</sup> In  
66 many experiments done on rodents, the additional dietary N load often consists of purified, high  
67 quality protein. This fact makes even more complicated the evaluation of the results, since they  
68 combine the alteration of diet protein proportion with the higher essential amino acid availability,  
69 and modifications in energy density and the proportions of carbohydrate and lipids.<sup>18,19</sup> The case of  
70 cafeteria diets is paradigmatic, they are hyperlipidic,<sup>20</sup> but their protein content is usually in the  
71 higher range of normalcy.<sup>21</sup> However, urea production, i.e. amino acid oxidation, is decreased, and  
72 the difference is not compensated by a parallel body protein accrual.<sup>22</sup>

73 In animal studies, dietary supplementation with arginine improves muscle protein deposition  
74 and intramuscular lipid concentrations, while reducing fat mass.<sup>23</sup> These studies suggest that  
75 arginine regulates lipid and protein content in a tissue-specific way.<sup>24</sup> Thus, the lipogenic responses  
76 of adipocytes to arginine depend on the cell type and differentiation stage.<sup>25</sup> From these data, it  
77 seems that any arginine surplus is oxidized.<sup>26</sup> However, an "arginine paradox" has been observed:  
78 the more arginine enters the body, more is destroyed, affecting metabolic processes, such as the  
79 production of nitric oxide<sup>27</sup>.

80 In spite of the technical complexities inherent to the use *in vivo* of stable isotopes, there are  
81 multiple advantages of using guanido group-labelled arginine as a tracer for dietary protein fate.  
82 Arginine is relatively abundant in proteins,<sup>27</sup> and is a key intermediate of the urea cycle. Most of the  
83 waste N goes through the urea cycle and is consequently incorporated to the guanido group of  
84 arginine, which is practically not recycled,<sup>28</sup> thus, it cannot intervene (and be diluted) in  
85 transamination processes, a main reason why it has been used often in N tracer studies, including  
86 analyses of protein turnover.<sup>29</sup> These metabolic patterns make arginine an excellent candidate to  
87 follow amino acid utilization by different tissues after an oral load and determine the differences of  
88 amino acid metabolic use, and regulation, between cafeteria and high-protein diet.

89

90

91 **Experimental Methods**92 *Animals and experimental setup*

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

98 Nine-week-old male Wistar rats (Janvier, Le Genest-Saint-Isle, France) were used. The  
99 animals were randomly divided into three groups (N = 6 each) and were fed *ad libitum*, for 30 days,  
100 either standard rat chow (SD) (Teklad 2014, Teklad diets, Madison WI, USA) (initial rat weight  
101 356±5 g), a simplified cafeteria diet (CD)<sup>21</sup> (initial rat weight 350±6 g), or high-protein diet (HPD)  
102 (initial rat weight 354±6 g). All animals had free access to water. They were housed in 2-rat solid-  
103 bottom cages, and were kept in a controlled environment (lights on from 08:00 to 20:00,  
104 temperature 21.5-22.5°C and 50-60% humidity). Body weight and food consumption were recorded  
105 daily. Calculation of food ingested was done as previously described by weighing the differences in  
106 food offered and debris left.<sup>30</sup>

107 *Diets*

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

113 The cafeteria diet (CD) was formed by plain cookies spread with liver pâté, bacon, standard  
114 chow pellets, water and milk supplemented with 300 g/L sucrose plus 10 g/L of a mineral and  
115 vitamin supplement (Meritene, Nestlé, Esplugues, Spain). All components were kept fresh (i.e.  
116 renewed daily). From the analysis of diet components and the ingested items, we calculated that, in  
117 CD, a mean 41% of energy was derived from lipids, 12% of energy was derived from protein, and  
118 47% of energy was derived from carbohydrates (20% from oligosaccharides). The analysis of food  
119 consumption of rats on the cafeteria diet showed that the ingestion of the different food choices was  
120 fairly constant in type and quantity consumed per rat and day as previously observed<sup>21,22</sup>. The  
121 computed nutrient consumption along the period studied, expressed as a percentage of total energy

122 ingested was 20.3% for pellet, 26.1 % for cookies, 17.9% for bacon, 16.52% for pâté and 19.2% for  
123 sugar-enriched milk.

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

133 Supplemental Table 2 shows the mean fatty acid composition of the diets. These data were  
134 calculated for each food item according to a previous study<sup>32</sup> using a standard method for fatty acid  
135 analysis<sup>33</sup>. The cafeteria diet showed higher levels of most fatty acids than controls, as expected,  
136 except for linoleic acid, which was present in higher quantities in standard diet.

137

### 138 *Arginine-<sup>15</sup>N tracer gavage and tissue sampling*

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

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

146 On day 30, the rats were anesthetized with isoflurane and then killed by exsanguination with a  
147 dry-heparinized syringe, through the exposed aorta. Tissue samples (liver, kidneys, three white  
148 adipose tissue (WAT) locations, interscapular brown adipose tissue (IBAT), hind leg muscle and  
149 intestines) were dissected, cleaned (gut) and rapidly frozen in liquid nitrogen. They were  
150 maintained at -70°C until processed for analyses. Plasma and erythrocyte fractions were obtained by  
151 centrifugation of the blood. The remaining carcass, blood and dissection debris were sealed in  
152 polyethylene bags, which were subsequently autoclaved at 120°C for 2 h,<sup>22</sup> the bag contents were  
153 weighed and then minced to a smooth paste with a blender (obtaining a total rat homogenate).

154 An additional control group of six control diet-fed animals was used to obtain the basal values

155 of N content. These rats (360±8 g) were reared in parallel to the other experimental groups, but they  
156 were not given the <sup>15</sup>N-Arg gavage. No differences in weight, and food consumption were observed  
157 when comparing this group and the SD.

158

### 159 *Metabolite analyses*

160 Urine and plasma parameters were measured using commercial kits (BioSystems, Barcelona,  
161 Spain): urea (#11537), glucose (#11504), total cholesterol (#11505) and triacylglycerols (#11528).  
162 Total proteins were measured in tissue homogenates, total blood and plasma using the Lowry *et al.*  
163 method.<sup>34</sup> Plasma samples were deproteinized with acetone<sup>35</sup> and the supernatants were used for  
164 amino acid analysis as previously described.<sup>21</sup>

165

### 166 <sup>15</sup>N Analysis

167 Aliquots of frozen tissues (about 200 mg) were homogenized in 2 mL of pure water  
168 (resistivity 18 MΩ) using a cell disruptor (IKA, Staufen, Germany), and then sonicated (Sonics  
169 Vibracell VC130PB, Sonics & Materials, Newtown, CT USA) to ensure complete cell disruption.  
170 The homogenates were then used directly for analysis. Aliquots of 5-10 μL of homogenized samples  
171 (equivalent to 0.5-1 mg of fresh tissue) were introduced in tin capsules (3.3 x 5 mm; Cromlab,  
172 Barcelona, Spain), filled with Al<sub>2</sub>O<sub>3</sub> as adsorbent. The microcapsules were sealed, and used for <sup>15</sup>N-  
173 enrichment measurement. The analyses were done with a Delta C gas chromatography-combustion-  
174 isotopic ratio mass spectrometer (GC-C-IRMS) (Finnigan MAT, Bremen, Germany) coupled to an  
175 elemental analyser (Flash 1112; Thermo Fisher, Waltham MA USA).

176 The <sup>15</sup>N/<sup>14</sup>N isotope ratios were expressed on a relative scale as deviation, referred as  $\delta$  units  
177 with the notation ‰ (parts of thousand), relative to the isotope ratio content of international  
178 standards.<sup>36</sup>

179 The corresponding  $\delta$  values for the samples were determined from the equation:

$$180 \quad \delta = ([R_{aa}/R_{at}]-1) \times 1000$$

181 Where  $R_{aa}$  is the <sup>15</sup>N/<sup>14</sup>N ratio obtained for each sample and  $R_{at}$  correspond to the <sup>15</sup>N/<sup>14</sup>N ratio of  
182 the standards.

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

$$184 \quad \text{Nat}\% = 100 \times ({}^{15}\text{N}/[{}^{15}\text{N} + {}^{14}\text{N}])$$

185 The net enrichment (atom percentage excess) in <sup>15</sup>N was calculated from the difference between the



186 atom percentages of samples and their corresponding blanks (i.e. the results obtained from the same  
187 tissues of control rats, which did not receive the labelled arginine gavage):

188 
$$\text{Atom percentage excess} = \text{at\% sample} - \text{at\% blank.}$$

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

### 192 *Statistical analysis*

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

195

## 196 **Results**

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

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

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

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



218 accounted-for N fraction.

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

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

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

234

## 235 Discussion

236

237 The golden rule of survival for mammals establishes that glycaemia has to be maintained, and  
238 amino acids, especially the essential ones, preserved.<sup>37</sup> In our experimental model, the animals had  
239 no problems of amino acid availability; the only plausible question was the need to dispose of their  
240 excess. There was, neither, any deficit in dietary energy availability; at least theoretically, since the  
241 energy density of the food of HPD diet was the same than that of SD (that of CD was higher), their  
242 differences rested only in the proportion of nutrients offered. However, this factor dramatically  
243 modified the handling of ingested protein. In the SD group, the accrual of labelled arginine was  
244 higher than in the other groups, which resulted in the highest labelled arginine specific activity (and,  
245 in contrast that of HPD group, lowest). The label in the intestinal content and stool contained a large  
246 proportion of that ingested in both SD and CD groups, but was almost absent in HPD animals,  
247 which is in agreement with a speedier and more efficient digestion of protein in diets with high  
248 protein content.<sup>38</sup> This seems to be clearly an adaptive modulation of the gut function, which ability  
249 to process foods is modified according to the composition of the diet within a relatively short  
250 period.<sup>39</sup>

251 Most of the absorbed label was found in the total homogenate, largely because skeletal muscle  
252 the main quantitative receptor because of its size (about 45 % of the body lean mass) and high  
253 protein content,<sup>40</sup> liver concentrated a significant amount of the label, probably because of its  
254 critical role in amino acid homoeostasis and disposal,<sup>41</sup> a task shared (for dietary protein) with the  
255 intestine.

256 The changes observed in urea excretion can be traced, too, to the primary energy partitioning  
257 role of intestine. High availability of carbohydrate in enterocytes raises the activity of the pentose-P  
258 pathway, raising the levels of NADPH, which modulates the disposal of amino N by activation of  
259 the synthesis of ornithine and citrulline,<sup>42</sup> precursors of (and fuel to sustain) the liver urea cycle.<sup>43</sup>  
260 This situation explains the case of SD and HPD groups, but not that of CD, since there was  
261 sufficient dietary carbohydrate in the gut but the urea cycle was not activated as expected. In fact,  
262 all the substrate relationships described respond to corollaries of the golden rules of homoeostasis  
263 for survival, in short: high lipid saves glucose. This is so because lipids were present only in limited  
264 amount and/or sporadically in the diet of our long line of ancestors (and in the diet of present-day  
265 rodents).<sup>44</sup> When lipid is present in the bloodstream, the metabolic meaning is clear: there are no  
266 dietary sources of energy and we are consuming reserves (our own lipid); then we must save energy,  
267 and save glucose, preserve amino acids, etc.<sup>45</sup> High carbohydrate availability translates into a large  
268 amount of dietary energy that allows us to save amino acids, the extent of salvage being related to  
269 their own relative availability.<sup>46</sup> High dietary protein spurns amino acid oxidation for use as energy,  
270 since there is no place where excess N can be stored, and protein is a fair source of energy too.<sup>47</sup>  
271 Nevertheless, low dietary protein is a red line that requires its preservation for survival.<sup>48</sup> HPD rats  
272 oxidize dietary protein as energy source to a high level (resulting in high urea excretion), because  
273 the *relative* amount of carbohydrate was lower than under standard conditions. However, not all  
274 excess amino acids go through the canonical urea cycle pathway, since a large proportion of N is  
275 eliminated through alternative unknown pathways (N gap), as previously reported<sup>49</sup> and repeated  
276 here.

277 The results obtained for CD rats agree with a number of previously published papers, i.e.  
278 cafeteria diet increases body size, the mass of adipose tissue and the accrual of protein, increases  
279 appetite and generates a N-gap, higher than that of SD, in their nitrogen balance.<sup>21,22</sup>

280 CD rats showed a marked decrease in urea excretion, despite having a protein intake similar  
281 to that of the SD group. Clearly, the high presence of dietary lipid (and excess energy availability)  
282 blocked the oxidation of amino acids, and inhibited the urea cycle, as previously described.<sup>48</sup>

283 The HPD diet elicits a clear correlation between higher dietary 2-amino N intake and  
284 increased urea excretion.<sup>51</sup> The rats fed the HPD were slightly smaller, with less adipose tissue than

285 the SD-fed rats, and their energy intake was lower. This may be, at least in part, a consequence of  
286 the higher satiating effect of protein;<sup>52</sup> but protein intake induces, also, a higher thermic response to  
287 food than other nutrients,<sup>53</sup> thus decreasing the efficiency of growth, as observed here. In any case  
288 the most important differences between dietary groups may lie, in the cost of live tissue deposition  
289 since, contrarily to what we expected, the labelled arginine that found its way into body protein was  
290 maximal in SD group and minimal in HPD group. This counterintuitive result implies that the use  
291 of amino acids for energy takes its toll, lowering considerably the overall energy efficiency of the  
292 animal.

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

306 In the case of cafeteria diet, previous studies have shown that the excess of lipids hampers the  
307 utilization of glucose because of insulin resistance.<sup>55</sup> The unused glucose tends to accumulate and  
308 create a problem by itself, which usually ends in the form of additional lipid stores and often results  
309 in type 2 diabetes.<sup>56</sup> The flow of dietary protein-derived amino acids cannot be processed at the  
310 required speed through the urea cycle because the excess of glucose and energy just prevents it.  
311 However, the increasing excess of amino N could not be stored, and, consequently is eliminated in  
312 significant proportions through the postulated N-gap-related pathway.<sup>22,49</sup> This partial parallelism in  
313 N disposal between CD and HPD groups suggests that the N-gap pathway must be a well-  
314 established mechanism, regulated in some way by its substrates' excess. The data presented here  
315 clearly show this high capacity and gross modulation. The activation of this postulated (albeit  
316 unknown) process is not dependent on the excess of available energy. It is related, instead to the  
317 excess of amino acids available built-up by the combination of high dietary supply and because the  
318 regular oxidative pathways are blocked by eons-old preservation schemes.<sup>57</sup> The HPD rats have a

319 fully active urea cycle, uninhibited by high glucose or lipid, which would make the alternate  
320 pathway unnecessary unless it were "regulated" by other parameters such as an excess of amino  
321 acid N. This elusive pathway seems conceived more as an emergency safety mechanism rather than  
322 as a developed efficient energy-providing pathway. The high cost of deposition of energy observed  
323 in the HPD and CD rats may suggest that the mechanism is not energetically efficient.<sup>58</sup>

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

333

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343

### 344 **Conflict of interest**

345 None.

346

### 347 **Authorship**

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

351

## 352 References

- 353 1 World Health Organization, *Obesity and overweight. Factsheet number 311* (updated January  
354 2015).
- 355 2 A. Makris and G.D. Foster, *Psychiatr. Clin. North. Am.*, 2011, **34**, 813–827.
- 356 3 T.Hu, K.T. Mills, L. Yao, K.Demanleis, M.Eloustaz, W.S.Yancy Jr, T.N. Kelly, J.HE and L.A.  
357 Bazzano, *Am. J. Epidemiol.*, 2012, **176**, suppl. 7, S44-S54.
- 358 4 R.C.Z. Asher, T.L. Burrows and C.E. Collins, *Nutr. Dietet.*, 2013, **70**, 101–112.
- 359 5 E.M. Piché, A. Auclair, J. Harvey, S. Marceau and P. Porier, *Can. J. Cardiol.*, 2015, **31**, 153-166.
- 360 6 R.L. Williams, L.G. Wood, C.E. Collins and R. Callister, *Obesity Rev.*, 2015, **16**, 171–186.
- 361 7 A.M. Johnstone, *Proc. Nutr. Soc.*, 2012, **71**, 339–349.
- 362 8 A. Paoli, A. Rubini, J.S. Volek and K.A. Grimaldi, *Eur. J. Clin. Nutr.*, 2013, **67**, 789–796.
- 363 9 K.J. Petzke, A. Freudenberg and S. Klaus, *Int. J. Mol. Sci.*, 2014, **15**, 1374-1391.
- 364 10 D.H. Pesta and V.T. Samuel, *Nutr. Metabol.*, 2014, **11**, 53.
- 365 11 C.P. Lambert, L.L. Frank and W.J. *Sports Med.*, 2004, **34**, 317-327.
- 366 12 K.A. Gudzone, R.S. Doshi, A.K. Mehta, Z.W. Chaudhry, D.K. Jacobs, R.M. Vakil, C.J. Lee,  
367 S.N. Bleich and J.M. Clark, *Ann. Intern. Med.*, 2015, **162**, 501-512.
- 368 13 S.M. Pasiakos, *J. Food Sci.*, 2015, 80, suppl 1, A2-A7.
- 369 14 M. Alemany M, *Nutr. Res. Rev.*, 2012, 25, 18-28.
- 370 15 G. Wu, *Amino Acids*, 2009, **37**, 1-17.
- 371 16 C.D. Morrison and T. Laeger, *Trends Endocrinol. Metab.*, 2015, 26, 256-262.
- 372 17 F.M. Sacks, G.A. Bray, V.J. Carey *N. Engl. J. Med.*, 2009, 360, 859–873.
- 373 18 J.A. Gilbert, N.T. Bendsen, A. Tremblay, and A.Astrup, *Nutr. Metabol. Cardiovasc. Dis.*, 2011,  
374 **21**, suppl. 2, B16-B31.
- 375 19 M. Cuenca-Sánchez, D. Navas-Carrillo and E. Orenes-Piñero, *Adv. Nutr.*, 2015, **15**, 260-266.
- 376 20 N.J. Rothwell and M.J. Stock, *J. Nutr.*, 1988, **118**, 925-928.

- 377 21 I. Rafecas, M. Esteve, J.A. Fernández-López, X. Remesar and M. Alemany, *Mol. Cell.*  
378 *Biochem.*, 1993, **121**, 45-58.
- 379 22 M. Esteve, I. Rafecas, X. Remesar and M. Alemany, *Int. J. Obesity*, 1992, **16**, 237-244.
- 380 23 W. Jobgen, C.J. Meininger, S.C. Jobgen, P.LI, M.-J. Lee, S.B.Smith, T.E.Spencer, S.K.Fried  
381 and G.Wu, *J. Nutr.*, 2009, **139**, 230–237.
- 382 24 B. Tan, X. Li, Y. Yin, Z.Wu, C.Liu, C.D.Tekwe and G.Wu, *Front. Biosci.*, 2012, **17**, 2237-  
383 2246.
- 384 25 J. Appleton, *Alternative Med. Rev.*, 2012, **7**, 512-522.
- 385 26 F.S. Dioguardi, *J. Nutrigenet. Nutrigenom.*, 2011, **4**, 90–98.
- 386 27 T.J. Maher, B.S. Glaesser and R.J. Wurtman, *Am. J. Clin. Nutr.*, 1984, **39**, 722-729.
- 387 28 J.A. Nettleton and D.M. Hegsted, *J. Nutr.*, 1974, **104**, 916-921.
- 388 29 V.R. Young and A. Ajami, *Proc. Nutr. Soc.*, 1999, **58**, 15-32.
- 389 30 E. Prats, M. Monfar, J. Castellà, R. Iglesias and M.Alemany, *Physiol. Behav.*, 1989, **45**, 263-  
390 272.
- 391 31 J. Folch, M. Lees and G.H. Sloane Stanley, *J. Biol. Chem.*, 1957, **226**, 497-509.
- 392 32 X.Remesar, A.Antelo, C.Llivina, E.Albà, L.Berdié, S.Agnelli, S.Arriarán, J.A.Fernández-  
393 López, M.Alemany, *Peer J*, 2015, **3**, e1083.
- 394 33 W.W.Christie, *Advan. Lipid Methodol.*, 1993, **2**: 69-111.
- 395 34 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J.Randall, *J. Biol. Chem.*, 1951, **193**, 265-275.
- 396 35 L. Arola, E. Herrera and M. Alemany, *Anal. Biochem.*, 1977, **82**, 236-239.
- 397 36 O. Felip, A. Ibarz, J. Fernández-Borrás, M.Beltrán, M.Martín-Pérez, J.V.Planas and J.Blasco,  
398 *Br. J. Nutr.*, 2012, **107**, 834-844.
- 399 37 M. Stumvoll, *Diabetologia*, 2004, **47**, 770–781.
- 400 38 M. Dangin, Y. Boire, C. Guillet and B.Beaufrière, *J. Nutr.*, 2002, **132**, 3228S-3233S.
- 401 39 W.H. Karasov, D.H. Solberg and J. M. Diamond, *Am. J. Physiol.*, 1987, **252**, G614-G625.
- 402 40 L. Arola, E. Herrera and M. Alemany, *Rev. Esp. Fisiol.*, 1979, **35**, 215-218.
- 403 41 D.F. Goldspink and F.J. Kelly, *Biochem. J.*, 1984, **217**, 507–516.
- 404 42 R.F. Bertolo and D.G. Burrin, *J. Nutr.*, 2008, **138**, 2032S-2039S.
- 405 43 C. Breuillard, L. Cynober and C. Moina, *Amino Acids*, 2015, **47**, 685-691.

- 406 44 S.B. Eaton, *Eur. J. Nutr.*, 2000, **39**, 67–70.
- 407 45 H. N. Munro, *J. Parenteral Enteral Nutr.*, 1982, **6**, 271-279.
- 408 46 B.E. Metzger and N. Freinkel, *Biol. Neonate*, 1987, **51**, 78-85.
- 409 47 R.W. Swick and N.J. Benevenga, *J. Dairy. Sci.*, 1977, **60**, 505-515.
- 410 48 M.F. McCarthy and J.J. DiNicolantonio, *Age*, 2015, **37**, doi: 10.1007/s11357-015-9823-8.
- 411 49 M. Esteve, I. Rafecas, X.Remesar and M. Alemany, *Biochem. Int.*, 1992, **26**, 687-694.
- 412 50 T. Barber, J.R. Viña, J. Viña and J. Cabo, *Biochem. J.*, 1985, **15**, 675-81.
- 413 51 D.R. Rémésy, C. Demigné and P. Faornoux P, *Nutr. Res.*, 1985, **5**, 1093-1102.
- 414 52 J.D. Radcliffe and A.J.F. Webster, *Br. J. Nutr.*, 1978, **39**, 483-492.
- 415 53 C. Jean, S. Rome, V. Mathé, J.-F. Huneau, N. Aattouri, G. Fromentin, C.L. Achagiotis and D.  
416 Tomé, *J. Nutr.*, 2001, **131**, 91-98.
- 417 54 G. Costa, L. Ullrich, F. Kantor and J.F. Holland, *Nature*, 1968, **218**, 546–551.
- 418 55 E.J. Schaefer, J.A. Gleason and M.L. Dansinger, *J. Nutr.*, 2009, **139**, 1257S-1262S.
- 419 56 M.F. Chong, B.A. Fielding and K.N. Frayn, *Proc. Nutr. Soc.*, 2007, **66**, 52-59.
- 420 57 S.B. Eaton and S.B. Eaton III, *Eur. J. Nutr.*, 2000, **39**, 67-70
- 421 58 M.S. Westerterp-Plantenga, *Regul. Peptides*, 2008, **149**, 67-69.
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- 423



424 TABLE 1 Composition of the diets used  
425

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

426  
427 The data are presented in  $\text{g}\cdot\text{kg}^{-1}$ , except for metabolizable energy (\*) expressed in  $\text{MJ}\cdot\text{kg}^{-1}$ . The data  
428 corresponding to CD were calculated from the composition of the mean diet (i.e. mix of foods)  
429 intake.

430

431

432

433  
434 Table 2 Weight, and intake (energy, nitrogen) of male rats subjected to diets with different  
435 protein content

parameter	units	diets			P
		SD	CD	HPD	
Weight (final)	g	491 ± 11 <sup>AB</sup>	532 ± 16 <sup>A</sup>	461 ± 17 <sup>B</sup>	0.0175
Weight increase	g	127 ± 8 <sup>A</sup>	147 ± 11 <sup>A</sup>	84 ± 9 <sup>B</sup>	0.0021
Energy intake	kJ d <sup>-1</sup>	370 ± 4 <sup>A</sup>	556 ± 13 <sup>B</sup>	336 ± 10 <sup>A</sup>	<0.0001
Nitrogen intake	mg d <sup>-1</sup>	696 ± 7 <sup>A</sup>	720 ± 18 <sup>A</sup>	1531 ± 41 <sup>B</sup>	<0.0001
Cost of accrual	kJ g <sup>-1</sup>	88.1 ± 5.0 <sup>A</sup>	116 ± 8 <sup>B</sup>	118 ± 5 <sup>B</sup>	0.0112

436  
437 The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-  
438 way ANOVA; post-hoc test (Tuckey test): groups with different superscript letters are statistically  
439 different (P<0.05).

440

441  
442 Table 3 Plasma metabolite levels of rats subjected to different diets.  
443

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

444  
445 The data are the mean ± SE of six different animals. Statistical comparisons between groups: One-way  
446 ANOVA; post-hoc test (Tuckey test): groups with different superscript letters are statistically different  
447 (P<0.05); NS = not statistically significant.  
448

Table 4. Total  $^{15}\text{N}$  atoms in tissues (expressed as a percentage of absorbed nitrogen) and total  $^{15}\text{N}$  atoms in tissues  $\times 10^{19}$  (per gram of protein) in rats subjected to different diets

Parameter	$^{15}\text{N}$ accrued as % of absorbed N				$^{15}\text{N} \times 10^{19}$ atoms $\text{g}^{-1}$ of tissue protein			
	SD	CD	HPD	P	SD	CD	HPD	P
Liver	$6.95 \pm 1.17^{\text{A}}$	$5.06 \pm 1.50^{\text{A}}$	$7.11 \pm 1.15^{\text{A}}$	NS	$3.25 \pm 0.52^{\text{A}}$	$2.15 \pm 0.46^{\text{A}}$	$2.44 \pm 0.21^{\text{A}}$	NS
Kidney	$1.90 \pm 0.37^{\text{AB}}$	$1.28 \pm 0.32^{\text{A}}$	$2.83 \pm 0.50^{\text{B}}$	0.0484	$9.16 \pm 2.45^{\text{A}}$	$4.82 \pm 1.06^{\text{A}}$	$5.89 \pm 0.51^{\text{A}}$	NS
Epididymal WAT	$0.31 \pm 0.04^{\text{A}}$	$1.99 \pm 0.62^{\text{B}}$	$0.68 \pm 0.24^{\text{AB}}$	0.0185	$2.77 \pm 0.49^{\text{A}}$	$11.4 \pm 2.47^{\text{B}}$	$3.70 \pm 0.37^{\text{A}}$	0.0015
Retroperitoneal WAT	$0.24 \pm 0.02^{\text{A}}$	$0.89 \pm 0.29^{\text{B}}$	$0.38 \pm 0.07^{\text{AB}}$	NS	$2.82 \pm 0.62^{\text{A}}$	$1.82 \pm 0.14^{\text{A}}$	$3.00 \pm 0.23^{\text{A}}$	NS
Mesenteric WAT	$0.39 \pm 0.06^{\text{A}}$	$0.63 \pm 0.31^{\text{A}}$	$0.73 \pm 0.23^{\text{A}}$	NS	$3.56 \pm 0.76^{\text{A}}$	$2.81 \pm 0.74^{\text{A}}$	$3.97 \pm 0.76^{\text{A}}$	NS
IBAT	$0.10 \pm 0.04^{\text{A}}$	$0.09 \pm 0.02^{\text{A}}$	$0.27 \pm 0.10^{\text{A}}$	NS	$7.22 \pm 1.11^{\text{A}}$	$3.35 \pm 0.87^{\text{B}}$	$3.09 \pm 0.47^{\text{B}}$	0.0062
Small intestine	$12.3 \pm 2.0^{\text{A}}$	$12.3 \pm 2.1^{\text{A}}$	$15.1 \pm 3.1^{\text{A}}$	NS	$25.7 \pm 5.7^{\text{A}}$	$14.6 \pm 3.9^{\text{A}}$	$20.1 \pm 3.2^{\text{A}}$	NS
Blood plasma	$2.02 \pm 0.52^{\text{A}}$	$3.35 \pm 0.75^{\text{A}}$	$3.46 \pm 1.24^{\text{A}}$	NS	$3.93 \pm 0.46^{\text{A}}$	$8.37 \pm 1.54^{\text{B}}$	$3.91 \pm 0.57^{\text{A}}$	0.0079
Blood cells	$1.82 \pm 0.32^{\text{A}}$	$3.32 \pm 1.04^{\text{A}}$	$3.46 \pm 1.04^{\text{A}}$	NS	$0.87 \pm 0.24^{\text{A}}$	$0.86 \pm 0.20^{\text{A}}$	$0.76 \pm 0.21^{\text{A}}$	NS
Skeletal muscle *	$28.5 \pm 1.4^{\text{A}}$	$33.8 \pm 9.1^{\text{A}}$	$23.9 \pm 2.8^{\text{A}}$	NS	$2.16 \pm 0.74^{\text{A}}$	$1.85 \pm 0.59^{\text{A}}$	$1.06 \pm 0.18^{\text{A}}$	NS
Rest of tissues (homogenate)	$44.1 \pm 1.9^{\text{A}}$	$37.8 \pm 3.6^{\text{A}}$	$43.3 \pm 2.0^{\text{A}}$	NS	$1.85 \pm 0.48^{\text{A}}$	$1.55 \pm 0.26^{\text{A}}$	$1.29 \pm 0.12^{\text{A}}$	NS

\*Striated muscle mass was estimated using previous reports (27). The data are the mean  $\pm$  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  $^{15}\text{N}$  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  $\pm$  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  $\pm$  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  $^{15}\text{N}$  atoms excreted in other ways: urine (yellow columns) and non-accounted for N fractions (stacked pink columns).

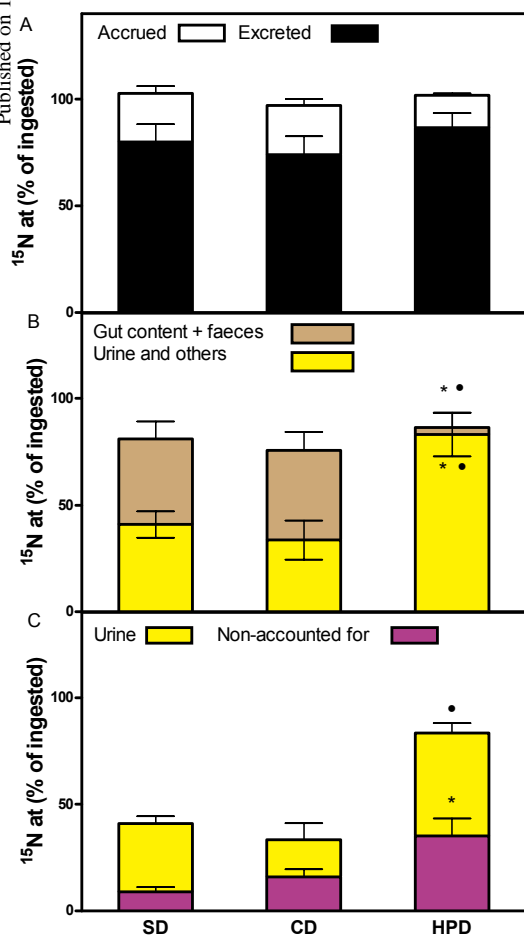
The data are the mean  $\pm$  SE of six different animals. Statistical comparisons between groups: One-way ANOVA for the different fractions: Urine ( $P=0.0053$ ), Non-accounted for N ( $P=0.0189$ ). Post-hoc Tuckey test: \* =  $P < 0.05$  vs SD group; • =  $P < 0.05$  of HPD vs CD group.

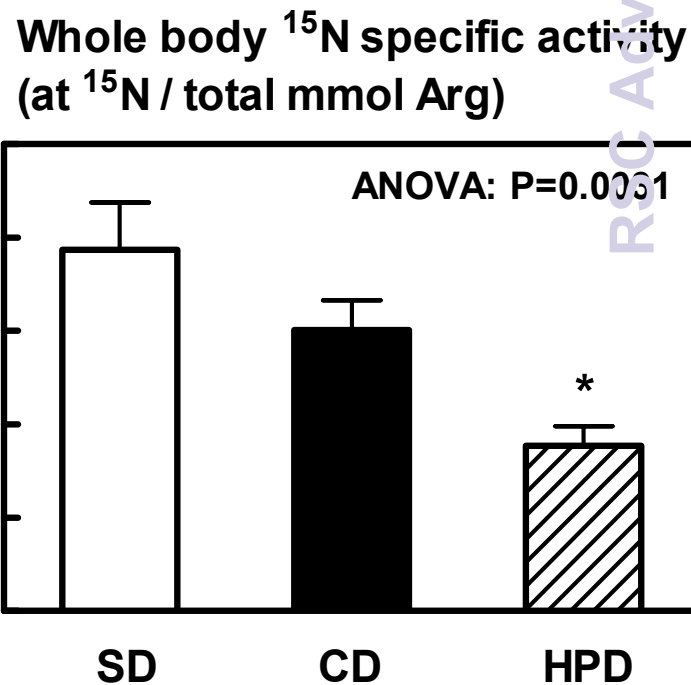
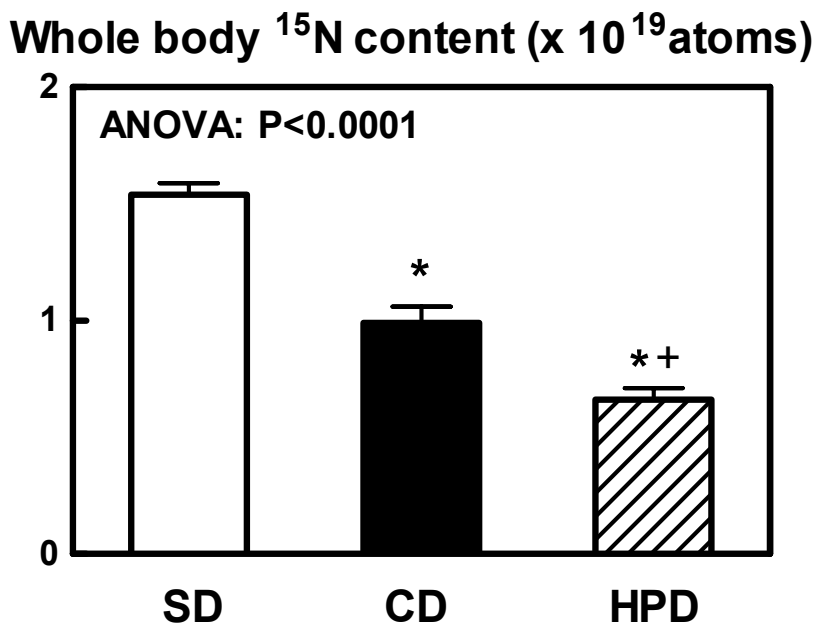
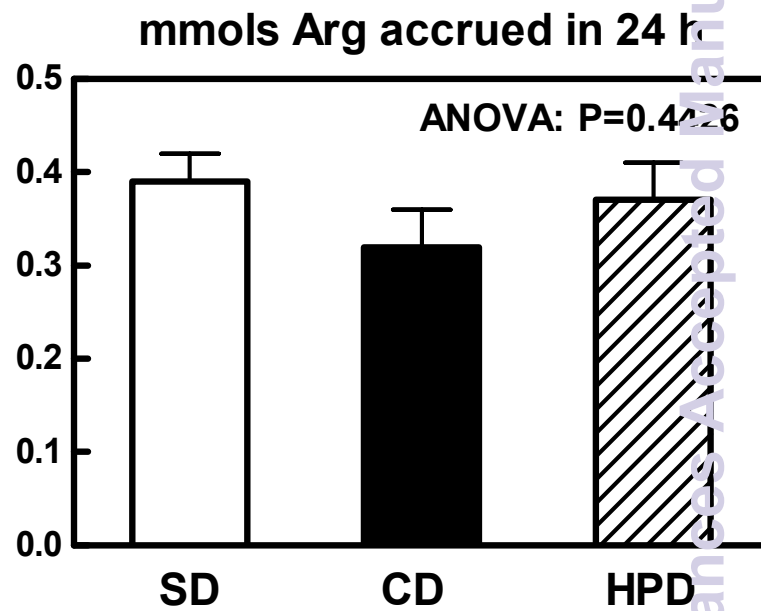
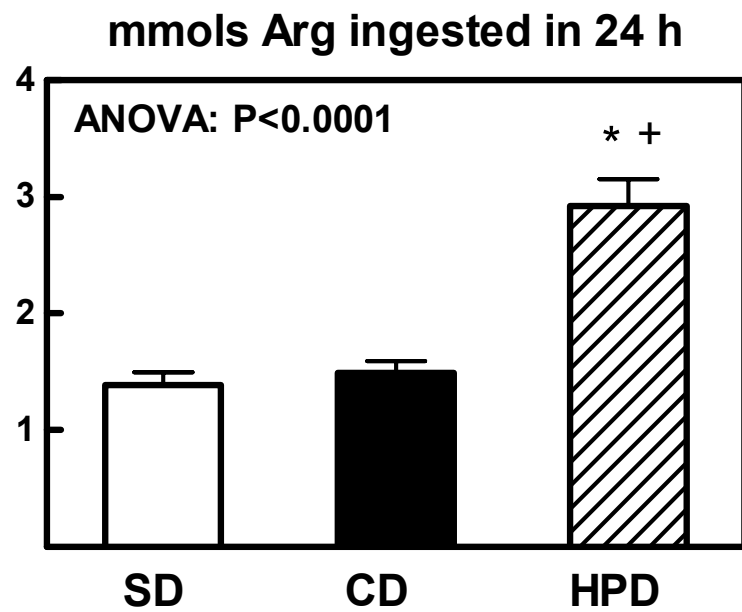
Figure 2. Number of  $^{15}\text{N}$  atoms ingested, specific activity of  $^{15}\text{N}$  atoms in relation to arginine ingestion and arginine accrued in 24 hours.

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

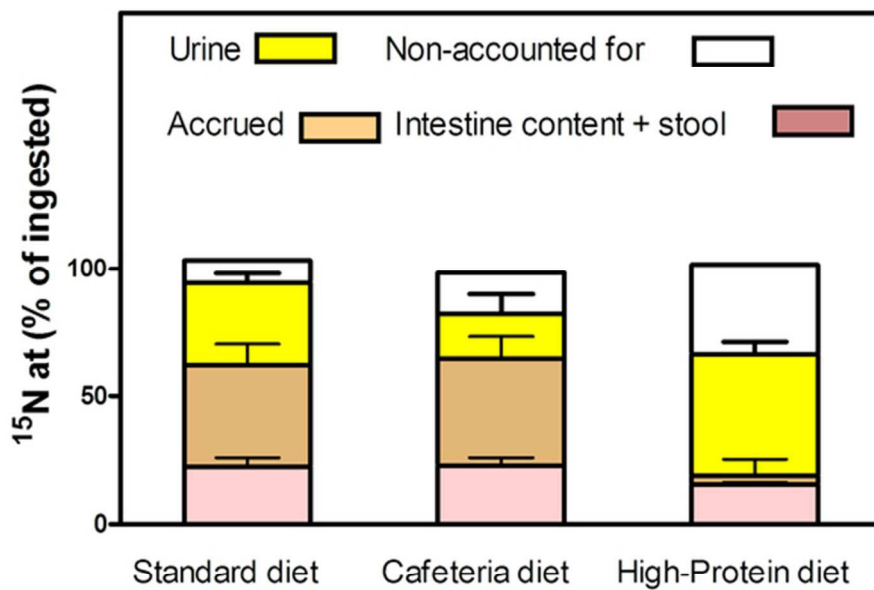
The data are the mean  $\pm$  SE of six different animals. White columns: SD group, black columns: CD group, striped columns: HPD group.

The data are the mean  $\pm$  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.









56x39mm (300 x 300 DPI)