

1 **The isotopic nitrogen turnover rate as a proxy to evaluate in the long-term the protein**
2 **turnover in growing ruminants**

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14 **Abstract**

15 Protein turnover is an energy-consuming process essential for ensuring the maintenance of
16 living organisms. Gold standard methods for protein turnover measurement have inherent
17 drawbacks precluding their generalization for large farm animals and during long periods. We
18 proposed here a non-invasive proxy of the whole-body fractional protein degradation rate
19 (**WBPT**; whole-body protein turnover for a growing animal) in the long term and in a large
20 number of beef cattle. The proxy is based on the rate at which urine-N and plasma proteins
21 are progressively depleted in ^{15}N after a slight decrease in the isotopic N composition of diet
22 (i.e. diet-switch). We aimed to test the ability of this proxy to adequately discriminate the
23 WBPT of 36 growing-fattening young bulls assigned to different dietary treatments known to
24 impact the protein turnover rate: the protein content (Normal vs High) and amino acid profile
25 (balanced vs unbalanced in methionine). The ^{15}N depletion rate found in plasma proteins
26 represented their fractional synthesis rate, whereas the slowest depletion rate found in urines
27 was interpreted as a proxy of the WBPT. The proxy here tested in urines suggested different
28 WBPT values between Normal vs High protein diets but not between balanced vs unbalanced
29 methionine diets. In contrast, the proxy tested in plasma indicated that both dietary conditions
30 affected the fractional synthesis rate of plasma proteins. We consider that the rate at which
31 urines are progressively ^{15}N -depleted following an isotopic diet-switch could be proposed as a
32 non-invasive proxy of the whole-body protein turnover rate in large farm animals.

33

34 **Keywords:** protein turnover, isotope, ^{15}N , ruminant, beef cattle

35

36 **Introduction**

37 Protein turnover refers to the renewal of proteins in a cell, tissue or organism and
38 involves the continuous degradation and synthesis of proteins not leading to net changes in

39 protein mass. The protein accretion occurring in the growing animal is the result of a greater
40 intensity of protein synthesis over protein degradation. In the growing state, the protein
41 turnover rate (amount of replaced proteins per unit of time) equals to total protein degradation
42 or alternatively to the amount of freshly synthesized protein necessary to replace the degraded
43 protein. The protein turnover in growing ruminants is huge compared to the net protein
44 deposition with as high as 94% of the whole body protein synthesis only serving to
45 counterbalance the protein degradation occurring in fattening young bulls (Lobley, 2003).
46 Besides, this huge turnover is accompanied by a high-energy cost for the ruminant, with only
47 protein synthesis accounting for 23% of total energy expenditure in ruminants (Caton et al.,
48 2000). Nevertheless, this dynamic state represents an essential mechanism for life by enabling
49 maintenance services such as metabolic regulation, cellular repair and rapid adaptation against
50 environmental changes, among other functions. This double faceted role of protein turnover
51 (“service” function for maintenance vs energy-consuming mechanism) explain why this
52 animal trait has been of interest for animal nutritionist and physiologist in the last decades.

53 Standard methods for whole-body protein turnover assessment are based on either
54 precursor or end-product methods (Waterlow, 1984) consisting in continuous or single dose
55 intravenous infusions of stable isotope labelled amino acids (AA) and the quantification of the
56 labelling in either the free AA precursor pool (plasma) or catabolic end-products (urine),
57 respectively. The main drawbacks associated to the evaluation of protein turnover rate
58 through these standard methods are i) short-term evaluation (from few hours [precursor
59 method] to few days [end-product method]), ii) invasive procedure and usually non-
60 physiological conditions, iii) the requirement of a controlled environment with confined
61 animals and usually steady-state conditions [precursor method] iv) expensive methods which
62 precludes applying it to a big number of large farm animals such as beef cattle. Alternative,
63 less expensive and less invasive methods have been explored, such as total urinary 3-methyl-

64 histidine excretion, to evaluate the whole-body protein degradation. However, 3-methyl-
65 histidine urinary excretion is also based on relatively short term evaluation (i.e. usually
66 between 4 and 6 days), restricted to myofibrillar muscle protein (myosin-actin) turnover, not
67 applicable for sheep or pigs (Nishizawa et al., 1979) and subjected to an accurate estimation
68 of the muscle mass in the body (Castro-Bulle et al., 2007).

69 A new isotopic approach was originally developed in animal ecophysiology (Fry and
70 Arnold, 1982; Tieszen et al., 1983) to model the rate at which animal's tissues incorporate the
71 isotopic signature of a new diet in view of accurate food web assessment. It is currently being
72 applied mainly in marine animal species (Abimorad et al., 2014; Mohan et al., 2016) to
73 evaluate the metabolic turnover rate of animal's tissues. This isotopic method consists in
74 following the dynamic of isotopic change (i.e. enrichment or depletion) of animal's tissues
75 immediately after a change in the natural isotopic composition of the diet (i.e. diet-switch).
76 The rate at which animal's tissues will incorporate the isotopic signature of the new diet
77 seems to be mostly driven by their protein turnover rate (Carleton and Martinez del Rio, 2005;
78 MacAvoy et al., 2005; Braun et al., 2013) and some authors suggest that protein turnover rate
79 might be predicted from isotopic incorporation studies (Carleton et al., 2008). This approach
80 has already been explored in ruminant's tissues (Harrison et al., 2011; Bahar et al., 2014) but,
81 as its original purpose, just for assessing the time span needed for muscles to reflect the
82 isotopic signature of the diet in view of meat authentication (i.e isotopic dietary
83 reconstruction). However, the isotopic approach used in those studies in ruminant and marine
84 animal species did not involve repeated measures from the same animal, but only one tissue
85 sample per animal, and individuals slaughtered at different times. Thus, these studies only
86 informed about the turnover of specific sampled tissues and not of the whole animal body.
87 Because most of the nitrogen from animal proteins is excreted through urines, once degraded
88 and their AA catabolized, our hypothesis is that the urinary isotopic N turnover rate assessed

89 from multi-point sampling from the same animal is a non-invasive proxy of the long term
90 whole-body fractional protein degradation rate (**WBPT**; whole-body protein turnover in a
91 growing animal).

92 Thus, the aim of this study was to adapt the tissue isotopic turnover approach
93 (MacAvoy et al., 2005; Abimorad et al., 2014) to the ruminant's urine and test it as a non-
94 invasive, less disturbing proxy of the long term WBPT in a relatively large number of
95 growing-fattening beef cattle. In addition, we also measured the isotopic turnover of plasma
96 proteins as an indicator of the fractional protein synthesis rate (**FSR**) of plasma proteins.
97 Because no gold standard method is currently available for measuring the WBPT in the long
98 term (several months) we evaluated this proxy by assessing its ability to detect differences
99 across two dietary factors known to impact the whole-body protein turnover in a different
100 extent: i) the protein content and intake as a strong and well-known effect (Waterlow, 2006)
101 and ii) the dietary amino acid profile as a less important modulator of protein turnover rate in
102 cattle (Wessels et al., 1997). For this purpose, we measured the kinetics of nitrogen isotopic
103 ($\delta^{15}\text{N}$) depletion in urines and plasma proteins over a 5-month period following a slight
104 decrease in the $\delta^{15}\text{N}$ of the diet in 36 fattening young bulls fed diets formulated at two
105 different protein and methionine contents. Preliminary results has been published as an
106 abstract (Cantalapiedra-Hijar et al., 2017).

107 **Material and methods**

108 The experiment was conducted at Herbipôle (Inra, UE 1414, Theix, France) in compliance
109 with the National Legislation on Animal Care. The C2EA-02 animal research ethics
110 committee (Auvergne, France) prospectively approved this research and thereafter the
111 Ministry of Agriculture (France) validated it with the approval number #7180-
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113 *Animals, diets and performance test*

114 Thirty-six growing-fattening Charolais bulls (320±33kg and 266±22d) were assigned to one
115 of the four experimental diets (n=9/treatment) resulting from a factorial 2 × 2 design: two
116 dietary metabolizable protein levels (100% [Normal] vs 120% [High] of requirements; INRA,
117 2018) crossed with two dietary methionine content (unbalanced diet [2.0 g Met/100 g
118 metabolizable protein] vs balanced diet [2.6 g Met/100 g metabolizable protein]). All diets
119 were iso-NE per kg DM and consisted of around 54% grass silage, 6% of wheat straw and
120 40% concentrate with amounts adjusted daily to ensure at least 10% of refusals.

121 *Dietary isotopic N switch*

122 The method here applied to assess the WBPT was based on the rate at which tissues and
123 animal N pools incorporate a new dietary ¹⁵N isotopic signature following a dietary isotopic
124 switch (Carleton and Martinez del Rio, 2005). However, because the range of natural ¹⁵N
125 abundance values ($\delta^{15}\text{N}$; ¹⁵N/¹⁴N ratio deviation from the international standard [atmospheric
126 N₂]) in ruminant's feed remains quite narrow (between 0 and 5‰ on average according to our
127 experience [Cantalapiedra-Hijar et al., 2015]) we decided to create an artificial dietary
128 isotopic switch by incorporating in the diet extrinsic ¹⁵N labelled urea as previously reported
129 (Bahar et al., 2014). The ¹⁵N-urea once degraded into ¹⁵N-ammonia and taken up by the
130 rumen microorganisms will enrich the animal's tissues through the absorbed microbial ¹⁵N-
131 AA. Results from a preliminary trial aiming to define the conditions for implementing this
132 method in ruminants showed that for a given animal the fraction of ¹⁵N-urea bypassing
133 directly to the urines, without being first incorporated into microbial proteins, was highly
134 variable across time and individuals and led to problems for fitting the progressive urinary
135 ¹⁵N-enrichment post-diet switch (data not shown). Thus, we decided to evaluate the animal
136 ¹⁵N turnover rates during a depletion (Abimorad et al., 2014) rather than enrichment (Bahar et
137 al., 2014) phase. For this, animals were progressively enriched in ¹⁵N over 35 days while
138 adapting to their respective diet; each animal received daily in the morning (0830) a capsule

139 (10 x 3mm) containing 20 mg of ^{15}N labelled urea (98% APE; Sigma–Aldrich, St. Louis,
140 USA). This amount of ^{15}N labelled urea and time was chosen for enhancing the urinary ^{15}N
141 between 10 and 15‰ above that of the basal diet according to results obtained from a
142 preliminary trial. The capsule was mixed with around 200 grams of concentrate (the one
143 assigned to the animal) in a small bucket within the feeder just before the only meal
144 distribution (0900). Twenty minutes after administration it was systematically checked that
145 the capsule had been swallowed by the animal. Less than 5% of capsules were administered
146 by hand directly in the mouth when animals did not swallow it within the first twenty
147 minutes. The rest of the diet was then distributed to the feeder. On day 36, animals no longer
148 received the ^{15}N labelled urea, and blood and urine were sampled from that day (d0) onwards.

149 *Sampling and ^{15}N analysis*

150 Blood was sampled at 0900 by venipuncture from the caudal vein of all animals on d0, d3, d7,
151 d11, d15, d21, d35, d49, d78 and d141 (n=10 per animal) after stopping the ^{15}N labelled urea
152 administration (d0 refers to 24h after the last ^{15}N -urea administration). Blood was collected
153 into 9 mL evacuated tubes (BD vacutainer, Plymouth, UK) containing lithium heparin as an
154 anticoagulant, centrifuged within the first hour at 2500 g for 15 min at 4°C and stored at -
155 20°C for determination of $\delta^{15}\text{N}$ values in total plasma proteins as previously described
156 (Cantalapiedra-Hijar et al., 2015). Urinary spot samples were obtained from all animals
157 between 0900 and 1000 on d0, d1, d2, d3, d4, d7, d9, d11, d14, d17, d35, d70, d142 (n = 13).
158 For urinary sampling, animals were head blocked during 1h while eating in the morning and a
159 bucket (30 x 30 x 15 cm) placed around the penis through two elastic ropes knotted on the
160 back of the animal. Most of animals urinated during the first hour and only few samplings
161 were conducted beyond the first hour and always within the first three hours. Twenty mL of
162 urine were then transferred into one tube containing 1 mL of 30% H_2SO_4 and filtered later in
163 the lab through a 30 μm standard filter paper to remove fine particles. Filtered and acidified

164 spot urine samples were stored at -20°C before determination of $\delta^{15}\text{N}$ values. Freeze-dried
165 plasma proteins and liquid urinary samples pipetted onto nitrogen-free absorbent
166 (chromosorb) were weighted in tin capsules and analyzed for N stable isotope composition
167 ($\delta^{15}\text{N}$) by using an isotope-ratio mass spectrometer (Isoprime, VG Instruments, Manchester,
168 UK) coupled to an elemental analyzer (EA Vario Micro Cube, Elementar, Germany).
169 Tyrosine was used as internal standard and included in every run to correct for possible
170 variations in the raw values determined by the mass spectrometer. Results were expressed
171 using the delta notation.

172 *Isotopic turnover rate modelling and statistical analysis*

173 The post-diet switch $\delta^{15}\text{N}$ kinetics measured in each pool (plasma protein and urine) were
174 carefully analyzed according to Martinez del Rio and Carleton (2012) by testing if they obey
175 to 1st-order or higher-order (2nd-order) kinetics according to the following mono- and bi-
176 exponential models, respectively:

177 Mono-exponential model: $\delta^{15}\text{N}(t) = \delta^{15}\text{N}_{\infty} + (\delta^{15}\text{N}_0 - \delta^{15}\text{N}_{\infty}) \times \exp^{-k \times t}$ (1)

178 Bi-exponential model: $\delta^{15}\text{N}(t) = \delta^{15}\text{N}_{\infty} + (\delta^{15}\text{N}_0 - \delta^{15}\text{N}_{\infty}) \times [p \times \exp^{-k_1 \times t} + (1-p) \times \exp^{-k_2 \times t}]$ (2)

179 where t (d) is the time since the ^{15}N diet-switch, $\delta^{15}\text{N}(t)$ (‰) is the pool $\delta^{15}\text{N}$ value at time t ,
180 $\delta^{15}\text{N}_0$ (‰) is the pool initial $\delta^{15}\text{N}$ value, and $\delta^{15}\text{N}_{\infty}$ (‰) is the asymptotic value of the pool
181 after the animal has reached isotopic steady state with its basal diet (without ^{15}N -urea
182 administration). In the mono-exponential model, k (d^{-1}) is the fractional isotopic turnover rate
183 of the pool, while in the bi-exponential model, k_1 and k_2 (d^{-1}) are two distinct fractional
184 isotopic turnover rates and p and $(1-p)$ their respective contributions to the whole isotopic
185 turnover.

186 To diagnose if a mono-exponential model was sufficient to adequately fit the $\delta^{15}\text{N}$ kinetics,
187 we used the reaction progress variable approach ((Martinez del Rio and Carleton, 2012;
188 Cerling et al., 2007) that it is based on the rearrangement of equation [1] to yield:

189 $\ln(1-F) = \ln [(\delta^{15}\text{N}(t) - \delta^{15}\text{N}_\infty) / (\delta^{15}\text{N}_0 - \delta^{15}\text{N}_\infty)] = -k \times t$ (3)

190 where (1-F) measures the remaining $\delta^{15}\text{N}$ distance to the new equilibrium as a proportion of
191 the total isotopic distance between the initial and the asymptotic $\delta^{15}\text{N}$ reached at equilibrium.
192 As illustrated in S1 Appendix, we decided what model was required on the basis of a visual
193 inspection of a plot of $\ln(1-F)$ against time, depending on whether $\ln(1-F)$ was a decreasing
194 linear function of time with slope equal to $-k$ (mono-exponential model) or a sequence of 2
195 lines of increasingly shallow slopes equal to $-k_1$ and $-k_2$ (bi-exponential model). This
196 graphical analysis was further supported by the Akaike Information criterion, where lower
197 values indicates a superiority of one model over another.

198 The model parameters (i.e., $\delta^{15}\text{N}_\infty$, $\delta^{15}\text{N}_0$ and k for the mono-exponential model, and
199 $\delta^{15}\text{N}_\infty$, $\delta^{15}\text{N}_0$, k_1 , k_2 and p for the bi-exponential model) were estimated using a non-linear
200 procedure and statistically analyzed through a non-linear mixed-effect model (nlme package;
201 Pinheiro and Bates, 2000) fitted by maximum likelihood in the R software with experimental
202 dietary factors (protein content, methionine content and their interaction) considered as fixed
203 effects and the animal as a random effect. To avoid over-parametrization in the most complex
204 model (bi-exponential) and avoid a failure for the model to converge, the random effect of
205 animal was only considered on those parameters where significant animal variability was
206 noted through graphical and statistical analysis (Pinheiro and Bates, 2000). Significant effects
207 were declared when the $P \leq 0.05$ and a trend was considered when $0.05 > P > 0.10$.

208 **Results**

209 No significant interaction ($P > 0.05$) between protein level and methionine concentration was
210 observed for any of the variables here analyzed. Thus, for the shake of clarity, results are
211 presented independently for each of these two experimental factors. During the length of the
212 applied approach (around 5 months) bulls fed High protein diets had on average higher ($P =$
213 0.005) daily gains (1.82 kg/d) compared to those fed Normal protein diets (1.63 kg/d on

214 average). On the other hand, methionine balanced diets tended ($P = 0.09$) on average to
215 promote higher daily gains (1.78 kg/d) than bulls fed unbalanced diets (1.67 kg/d).

216 *Urinary isotopic turnover rate*

217 For $\delta^{15}\text{N}$ kinetics in urines, the reaction progress approach clearly identified two independent
218 slopes with a cut-off between d4 and d7 (Insets in Figs 1 and 2). This demonstrated the
219 existence of two distinct rates, a rapid and a slow, of urinary $\delta^{15}\text{N}$ depletion after the diet-
220 switch, and justified the need of a bi-exponential model to adequately fit these data. The
221 individual $\delta^{15}\text{N}$ kinetics were indeed correctly fitted for almost all animals ($r^2 \geq 0.96$; $n=34$)
222 using a bi-exponential model, except for 2 animals that were badly fitted with no apparent
223 explanation and that were therefore excluded from the analyses. When all data were pooled
224 and analyzed through a mixed-effect bi-exponential model, only a significant effect of the
225 dietary protein content was observed on the fractional rates of ^{15}N -depletion in urines, where
226 animals fed High protein diets showed higher values during both the first rapid (89.9 vs. 70.0
227 %/d; $P = 0.008$) and the second slow (10.3 vs. 8.01%/d; $P < 0.001$) phases of $\delta^{15}\text{N}$ turnover
228 compared to animals fed Normal protein diets (Fig 1). No effect of methionine content was
229 observed on any model parameter ($P > 0.10$; Fig 2).

230 *Plasma isotopic turnover rate*

231 For $\delta^{15}\text{N}$ kinetics in plasma proteins, the reaction progress approach showed a single slope
232 (Insets in Figs 3 and 4). This demonstrated the existence of a single, homogenous rate of $\delta^{15}\text{N}$
233 depletion in plasma proteins after the diet-switch, and justified that a mono-exponential model
234 was sufficient to adequately fit these data. The individual $\delta^{15}\text{N}$ kinetics in plasma proteins
235 were indeed correctly fitted ($r^2 \geq 0.98$; $n=36$) through a mono-exponential asymptotic model.
236 When all data were pooled and analyzed through a mixed-effect asymptotic model, lower
237 plasma protein $\delta^{15}\text{N}$ values were observed in animals fed High compared to Normal protein
238 diets (Fig 3) on first day (d0; $P = 0.04$) and at equilibrium (d142; $P = 0.09$). In contrast, the

239 effect of balancing diets in terms of methionine content tended ($P = 0.09$) to have a higher
240 $\delta^{15}\text{N}$ value in plasma proteins on d0 (Figure 4) but not at equilibrium ($P = 0.47$). The
241 fractional ^{15}N -depletion rate in plasma proteins was higher in animals fed High compared to
242 Normal protein diets (4.42 vs 4.08%/d; $P = 0.02$) and in those fed diets balanced compared to
243 unbalanced in methionine (4.38 vs 4.10%/d; $P = 0.05$).

244 **Discussion**

245 Following a diet change, animal's tissues progressively assimilate the isotopic
246 signature of the new diet (Carleton and Martinez del Rio, 2005). The rate at which this occur
247 is known as the isotopic turnover rate and depends largely on how fast the metabolic tissue
248 replacement occurs (Tieszen et al., 1983; Arneson et al., 2006). We adapted the tissue isotopic
249 turnover approach, extensively explored by ecologists (Martinez del Rio and Carleton, 2012),
250 to the case of ruminant's urines and plasma proteins to respectively evaluate in the long term
251 (5 months) the WBPT (protein turnover in a growing animal) and the FSR of plasma protein
252 in 36 growing-fattening cattle. This isotopic approach is simple to set up, relatively cheap
253 (estimated to be less than 300 dollars per bull), allowing to obtain measurements over long
254 periods (several months) and without perturbing animals (unlike classic tracer methods with
255 invasive procedures under non-physiological conditions). Although the mechanistic
256 interpretation of isotopic turnover rates is always a complex issue (Martinez del Rio and
257 Anderson-Sprecher, 2008), it is proposed that the rates at which urines and plasma proteins
258 are progressively ^{15}N -depleted following an isotopic diet-switch represent non-invasive (or
259 less invasive) proxies of the long-term WBPT and plasma protein FSR, respectively. Such
260 proxies could be very useful for future studies on feed efficiency (protein turnover as an
261 energy-consuming process), animal robustness (protein turnover as a maintenance service)
262 and meat quality (in vivo protein turnover associated to the rate of post mortem proteolysis
263 and thus to meat tenderness) carried out on large number of animals.

264 *Biological meaning of isotopic turnover rates*

265 The simplicity of the method here described may contrast with the need to address
266 some methodological considerations for interpreting our results. The rate at which animal's
267 tissues will incorporate the isotopic signature of the new diet seems to be mostly driven by
268 their protein turnover rate according to many previous reports (Carleton and Martinez del Rio,
269 2005; MacAvoy et al., 2005; Braun et al., 2013). Moreover, this has been confirmed by
270 mechanistic models (Martinez del Rio and Carleton, 2012; Poupin et al., 2012), demonstrating
271 that the main driver determining the rate of assimilation of the new dietary $\delta^{15}\text{N}$ value into a
272 tissue after a diet-switch is the protein FSR of that tissue. Thus, the isotopic turnover rate we
273 found in the plasma protein pool (i.e., the k model parameter; Figs 3 and 4) represented its
274 own FSR (i.e., the sum of its fractional degradation rate and fractional growth rate) and may
275 be proposed as a way to indirectly evaluate the liver FSR of plasma proteins. In contrast, to
276 the best of our knowledge the isotopic turnover rate of urine has never been analyzed
277 mechanistically and, as further discussed, its biological meaning is mostly ascribed to the
278 WBPT. Some considerations and assumptions need, however, to be discussed to support our
279 biological interpretation.

280 First, the urinary $\delta^{15}\text{N}$ kinetics post diet-switch did not only reflect the N metabolism
281 of the animal but also that of its symbiotic rumen microbiota. Urines would theoretically also
282 drain the previously ^{15}N enriched microbiota as they renovate their own structural proteins
283 and N pools (amino acids but also purine derivatives). However this would have
284 quantitatively a short-term impact on urinary $\delta^{15}\text{N}$ kinetics due to the high protein turnover
285 rate of rumen microbiota (127 to 686%/d in rumen bacteria; Wallace and McPherson, 1987).
286 Second, because of the complex and extensive urea recycling in ruminants we should not
287 exclude the fact that the clearance rate of the previously administered ^{15}N labelled urea from
288 the whole body after stopping its administration might contribute in an undetermined extent to

289 the urinary ^{15}N depletion rate during the first hours. In this sense, it has been demonstrated in
290 beef cattle that most infused ^{15}N -labelled urea is excreted through urines within the first 48h
291 (Wessels et al., 1997), and so its impact on urinary ^{15}N depletion rate would be very short.
292 Third, we can not exclude that differences in the N absorption, and thus in the urinary N
293 excretion, expected across diets (Normal vs High protein diets) might contribute to the
294 observed differences in the urinary ^{15}N depletion rate. Indeed, the dietary N influx into the
295 plasma pool, while being much smaller than the endogenous AA influx by protein
296 degradation (Lobley, 2000), had the largest $\delta^{15}\text{N}$ difference with body AA at the beginning of
297 the diet-switch (dietary AA were largely ^{15}N depleted compared to the body AA previously
298 ^{15}N enriched during the administration of ^{15}N labelled urea). In consequence, the urinary $\delta^{15}\text{N}$
299 kinetics were probably mainly driven by the dietary AA influx during the very first days (k_1
300 phase), before being mostly driven by the endogenous AA influx released from the whole-
301 body protein degradation thereafter (k_2 phase). All these three factors (rumen microbiota
302 metabolism, isotopic labelled urea clearance rate and differences in N absorption across
303 treatments) would have thus a limited short impact on the urinary ^{15}N turnover rate only
304 during the first rapid phase (k_1). We think that the ^{15}N depletion rate in urines during the
305 second slow phase (k_2) is mostly driven by the release rate of endogenous ^{15}N labeled AA
306 from degradation of previously labeled body proteins. This progressive release of previously
307 labeled AA will translate first into the free plasma AA pool and then into the urea-N pool
308 resulting from their catabolism. Therefore, we assume that the urinary ^{15}N depletion rate
309 during the second slow phase (k_2) is mostly the consequence of the WBPT, whereas the rate
310 during the first rapid phase (k_1) is likely a mix from the previously evoked mechanisms
311 together with (in some undetermined extent) the protein degradation rate of some very fast
312 animal turning-over N pools.

313 A limitation to our interpretation could be that some N-containing compounds in urine
314 are metabolically unrelated to protein degradation and AA oxidation. This could represent a
315 potential issue particularly in ruminants where urinary urea-N excretion, the main urinary
316 nitrogenous component, may origin in a high extent from rumen ammonia production (INRA,
317 2018) and so from nitrogenous compounds unrelated to protein degradation and AA
318 oxidation. It can be acknowledged that our modelled k_2 likely integrates the outflow rate of
319 those minor urinary nitrogenous compounds non related to AA oxidation and with an
320 endogenous (animal) origin (e.g. creatine, creatinine, endogenous purine derivatives, 3-
321 methylhistidine and other free AA). Such endogenous nitrogenous compounds were likely
322 previously labelled during ^{15}N urea administration likewise body proteins and progressively
323 released from the animal body through the urines. However it should be stressed that i) its
324 contribution to total urinary N remains relatively modest in high productive animals (0.05 g
325 N/kg BW/d; INRA, 2018) and ii) for some of these nitrogenous compounds their release rate
326 is closely related to protein turnover rate (e.g. 3-methyl-histidine as an index of muscle
327 protein degradation and endogenous purine derivatives from nucleic acid turnover rate).

328 For those urinary nitrogenous compounds non related to AA oxidation and with a
329 ruminal origin (mostly purine derivatives [allantoin and uric acid], hippuric acid and rumen
330 ammonia mostly transformed into urea) it can be argued, as previously mentioned, that it
331 would have probably an impact on the first rapid phase of the urinary ^{15}N depletion rate (k_1)
332 but as long as the plasma absorption of such compounds remains relatively constant across
333 time they will not have an effect on the second slow phase of this depletion (k_2). Taking all
334 this together, we can consider that even if k_2 does not strictly correspond only to the WBPT,
335 it may reflect quantitatively this flux and can be proposed as a proxy to evaluate it.

336 Finally, we recognize that the proposed approach based on isotope decay rates may be
337 problematic for having a real and accurate estimation of WBPT because of the phenomenon

338 of amino acid reutilization, (i.e. the reincorporation of labelled amino acids released by
339 protein degradation during protein re-synthesis). Indeed, because overall rates of protein
340 turnover markedly exceed rates of dietary protein intake much of the amino acid substrates for
341 protein synthesis derive from protein degradation (Lobley, 2000). However, even if AA re-
342 utilization could impact the ^{15}N values at each time point of the isotopic decay we assume
343 that a higher protein turnover (associated also to a higher AA-reutilization) will always have a
344 higher k_2 than a low protein turnover. Thus, for comparing treatments (or even individuals) in
345 terms of WBPT this approach can still serve as a proxy.

346 To evaluate the aforementioned biological interpretations, we tested the ability of our
347 method to detect differences across two dietary factors, the protein content (Normal vs High)
348 and AA profile (diets balanced vs unbalanced in methionine), known to impact the whole-
349 body protein turnover rate in a different extent.

350 *Isotopic turnover rates across dietary factors impacting protein metabolism*

351 Dietary protein content determines the dietary AA flux to the organism and is the
352 major dietary determinant of the whole-body protein turnover rates in humans (Waterlow,
353 2006) and farm animals (Lobley, 2000) through the action of catabolic and anabolic
354 hormones. In the present study, the urinary ^{15}N -depletion rates during both the first rapid (k_1)
355 and second slow (k_2) phases increased on average around 28% when increasing the
356 metabolizable protein content by 20%. This finding is in line with the expected increases in
357 dietary AA influx and WBPT that have been well recorded in k_1 and k_2 , respectively. When a
358 similar diet-switch approach was applied on adult rats fed diets at or much above protein
359 requirements (10 vs 30 % CP), the urinary turnover rate of ^{15}N increased more than two folds,
360 in agreement with an expected higher protein turnover rate (Braun et al., 2013). Our values
361 for the WBPT (average $k_2 = 9.1\%/d$) are, however, higher than those reported in the few
362 studies using standard isotopic methods in growing ruminants, which themselves

363 encompasses a wide range (3.88-8.00%/d whole-body protein FSR in growing heifers [Lobley
364 et al., 1980]; 6.15 -7.63%/d WBPT in growing lambs [Liu et al., 1995]; calculated WBPT rate
365 of around 1.8%/d in growing steers [Wessels et al., 1997]) but still lower than other estimates
366 (19.7%/d WBPT rate in growing lambs [Davis et al., 1981]). Although the methods are not
367 comparable (few days vs several months) and our higher values compared to other cattle
368 studies (Wessels et al., 1997; Lobley et al., 1980) could be just the consequence of using late
369 maturing breeds with high growth rates (1.70 kg/d on average for the studied period) and
370 retaining between 35-45% of their energy as protein (Geay and Robelin, 1979), the obtained
371 values will need further confirmation and interpretation.

372 We also found that the plasma protein ¹⁵N-depletion rate (k) increased with the dietary
373 protein content, supporting the idea of a greater protein metabolism at all body levels as
374 revealed through the analysis of urines. Likewise, Tsahar et al. (2008) also found in birds that
375 the ¹⁵N turnover rate in plasma doubled (from around 11 to 20 %/d) when dietary protein
376 shifted from 7 to 16 % CP. The impact of dietary protein content on the protein turnover rate
377 of plasma proteins was demonstrated in the 50's through the elegant study by Steinbock and
378 Tarver (1954) who injected into rats plasma from donor rats in which the proteins were
379 previously labelled with [³⁵S] methionine. However this effect was not observed in ruminants
380 through standard isotopic methods, where the plasma proteins FSR remained unchanged in
381 dairy cows fed different dietary metabolizable protein content (Raggio et al., 2007) or sheep
382 undergoing a shift between the fasted to fed state (Connell et al., 1997).

383 On the other hand, no changes in the rate at which urines are progressively depleted in
384 ¹⁵N following an isotopic diet-switch were found between unbalanced vs balanced diets in
385 terms of methionine content. In contrast, plasma protein ¹⁵N-depletion rate was slightly
386 increased, indicating that protein FSR was enhanced for plasma proteins and likely also at
387 other different body levels. Our findings may indicate that the observed trend for Met

388 balanced diets to improve beef cattle performances observed in this experiment
389 (Cantalapiedra-Hijar et al., 2017) and others (Wessels et al., 1997) could stem from an
390 increased whole-body protein synthesis rather than a reduction in the whole-body protein
391 degradation.

392 **Conclusions**

393 We believe that the rate at which animal's urines are depleted in ^{15}N after stopping the
394 administration of ^{15}N labelled urea in the diet (i.e. isotopic turnover rate after diet switch) can
395 be proposed as a non-invasive and simple proxy to evaluate in the long term the whole-body
396 protein turnover rate in a high number of animals. Further studies are warranted to explore the
397 potential and limits of this new promising isotopic approach for evaluating protein turnover.
398 Specifically, future works should evaluate the impact of AA reutilization and urea-N
399 recycling on the N isotopic dynamics in urine and tissues after a diet-switch.

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404 **References**

405 Abimorad EG, Ducatti C, Castellani D, Jomori RK, Portella MC, Carneiro DJ. The use of
406 stable isotopes to investigate the effects of supplemental lysine and methionine on protein
407 turnover and amino acid utilization in pacu, *Piaractus mesopotamicus*,
408 juveniles. *Aquaculture*. 2014;433: 119-124.
409 Arneson LS, MacAvoy S, Basset E. Metabolic protein replacement drives tissue turnover in
410 adult mice. *Can J Zool*. 2006;84: 992-1002

- 411 Bahar B, Harrison SM, Moloney AP, Monahan FJ, Schmidt O. Isotopic turnover of carbon
412 and nitrogen in bovine blood fractions and inner organs. *Rapid Commun Mass*
413 *Spectrom.* 2014;28:1011-1018.
- 414 Braun A, Auerswald K, Vikari A, Schnyder H. Dietary protein content affects isotopic carbon
415 and nitrogen turnover. *Rapid Commun Mass Spectrom.* 2013;27: 2676-2684.
- 416 Cantalapiedra-Hijar G, Ortigues-Marty I, Sepchat B, Agabriel J, Huneau JF, Fouillet H. Diet-
417 animal fractionation of nitrogen stable isotopes reflects the efficiency of nitrogen
418 assimilation in ruminants. *Br J Nutr.* 2015;113: 1158-1169
- 419 Cantalapiedra-Hijar G, Bahloul L, Chantelauze C, Largeau V, Khodorova N, Fouillet H,
420 Ortigues-Marty I. Improved cattle performance by methionine-balanced diets does not
421 results from decreased protein degradation. *Proc Eur Assoc Anim Prod.* 2018; 476
422 (Abstract).
- 423 Carleton SA., Martinez del Rio C. The effect of cold-induced increased metabolic rate on the
424 rate of ¹³C and ¹⁵N incorporation in house sparrows (*Passer domesticus*). *Oecologia.*
425 2005;144: 226-232.
- 426 Carleton SA, Kelly L, Anderson-Sprecher R, Martinez del Rio C. Should we use one-, or
427 multi-compartment models to describe ¹³C incorporation into animal tissues? *Rapid*
428 *Commun Mass Spectrom.* 2008;22: 3008-3014.
- 429 Castro Bulle FCP, Paulino PV, Sanches AC, Sainz RD. Growth, carcass quality, and protein
430 and energy metabolism in beef cattle with different growth potentials and residual feed
431 intakes. *J Anim Sci.* 2007;85: 928-936.
- 432 Caton JS, Bauer ML, Hidari H. Metabolic components of energy expenditure in growing beef
433 cattle-review. *Asian-Australas J Anim Sci.* 2000;13: 702-710.

- 434 Cerling TE, Ayliffe LK, Dearing MD, Ehleringer JR, Passey BH, Podlesak DW et al.
435 Determining biological tissue turnover using stable isotopes: the reaction progress
436 variable. *Oecologia*. 2007;151: 175-189.
- 437 Connell A, Calder AG, Anderson SE, Lobley GE. Hepatic protein synthesis in the sheep:
438 effect of intake as by use of stable-isotope-labelled glycine, leucine and phenylalanine. *Br J*
439 *Nutr.* 1997;77: 255-271
- 440 Davis SR, Barry TN, Hughson GA. Protein synthesis in tissues of growing lambs. *Br J*
441 *Nutr.* 1981;46: 409-419.
- 442 Fry B, Arnold C. Rapid $^{13}\text{C}/^{12}\text{C}$ turnover during growth of brown shrimp (*Penaeus*
443 *aztecus*). *Oecologia*. 1982;54: 200-204.
- 444 Geay Y, Robelin J. Variation of meat production capacity in cattle due to genotype and level
445 of feeding: genotype-nutrition interaction. *Liv Prod Sci.* 1979;6: 263-276.
- 446 Harrison SM, Schmidt O, Moloney AP, Kelly SD, Rossmann A, Schellenberg A et al. Tissue
447 turnover in ovine muscles and lipids as recorded by multiple (H, C, O, S) stable isotope
448 ratios. *Food Chem.* 2011;124: 291-297.
- 449 INRA feeding system for ruminants. Wageningen Academic Publishers, Wageningen, the
450 Netherlands. 2018.
- 451 MacAvoy SE, Macko SA, Arneson LS. Growth versus metabolic tissue replacement in mouse
452 tissues determined by stable carbon and nitrogen isotope analysis. *Can J Zool.* 2005;83: 631-
453 641.
- 454 Martinez del Rio CMD, Anderson-Sprecher R. Beyond the reaction progress variable: the
455 meaning and significance of isotopic incorporation data. *Oecologia*. 2008;156: 765-772.
- 456 Martinez del Rio CMD, Carleton SA. How fast and how faithful: the dynamics of isotopic
457 incorporation into animal tissues. *J Mammal.* 2012;93: 353-359.

- 458 Mohan JA, Smith SD, Connelly TL, Attwood ET, McClelland JW, Herzka SZ et al. Tissue-
459 specific isotope turnover and discrimination factors are affected by diet quality and lipid
460 content in an omnivorous consumer. *J Exp Mar Biol Ecol.* 2016;479: 35-45.
- 461 Liu SM, Lobley GE, MacLeod NA, Kyle DJ, Chen XB, Ørskov ER. Effects of long-term
462 protein excess or deficiency on whole-body protein turnover in sheep nourished by
463 intragastric infusion of nutrients. *Br J Nutr.* 1995;73: 829-839.
- 464 Lobley GE. Protein turnover—what does it mean for animal production? *Can J Anim Sci.*
465 2003; 83: 327-340.
- 466 Lobley GE, Milne V, Lovie JM, Reeds PJ, Pennie K. Whole body and tissue protein synthesis
467 in cattle. *Br J Nutr.* 1980;43: 491-502.
- 468 Nishizawa N, Toyoda Y, Noguchi T, Hareyama S, Itabashi H, Funabiki R. N τ -
469 Methylhistidine content of organs and tissues of cattle and an attempt to estimate fractional
470 catabolic and synthetic rates of myofibrillar proteins of skeletal muscle during growth by
471 measuring urinary output of N τ -methylhistidine. *Br J Nutr.* 1979;42: 247-252.
- 472 Pinheiro JC, Bates DM. Linear mixed-effects models. In *Mixed-effects models in S and S-*
473 *Plus* (ed. J. Chambers, W. Eddy, Hardle, S. Sheather, L. Tierney). Springer, New York,
474 USA. 2000. pp. 3-56.
- 475 Poupin N, Huneau JF, Mariotti F, Tomé D, Bos C, Fouillet H. Isotopic and modeling
476 investigation of long-term protein turnover in rat tissues. *Amer J Physiol Regul Integr Comp*
477 *Physiol.* 2012;304: R218-R231.
- 478 Raggio G, Lobley GE, Berthiaume R, Pellerin D, Allard G, Dubreuil P et al. Effect of protein
479 supply on hepatic synthesis of plasma and constitutive proteins in lactating dairy cows. *J*
480 *Dairy Sci.* 2007;90: 352-359.
- 481 Steinbock HL, Tarver H. Plasma protein. 5. The effect of the protein content of the diet on
482 turnover. *J Biol Chem.* 1954;209: 127-132.

- 483 Tieszen LL, Boutton TW, Tesdahl KG, Slade NA. Fractionation and turnover of stable carbon
484 isotopes in animal tissues: implications for $\delta^{13}\text{C}$ analysis of diet. *Oecologia*. 1983;57: 32-
485 37.
- 486 Tsahar E, Wolf N, Izhaki I, Arad Z, Martinez del Rio C. Dietary protein influences the rate of
487 ^{15}N incorporation in blood cells and plasma of Yellow-vented bulbuls (*Pycnonotus*
488 *xanthopygos*). *J Exp Biol*. 2008;211: 459-465.
- 489 Wallace RJ., McPherson CA. Factors affecting the rate of breakdown of bacterial protein in
490 rumen fluid. *Br J Nutr*. 1987;58: 313-323.
- 491 Waterlow JC. Protein turnover with special reference to man. *Exp Physiol*. 1984; 69: 409-438.
- 492 Waterlow JC. 2006. Protein turnover. CAB International, Nosworthy Wag Wallingford,
493 Oxfordshire OX10 8DE, UK.
- 494 Wessels RH, Titgemeyer EC, St Jean G. Effect of amino acid supplementation on whole-body
495 protein turnover in Holstein steers. *J Anim Sci*. 1997;75: 3066-3073.

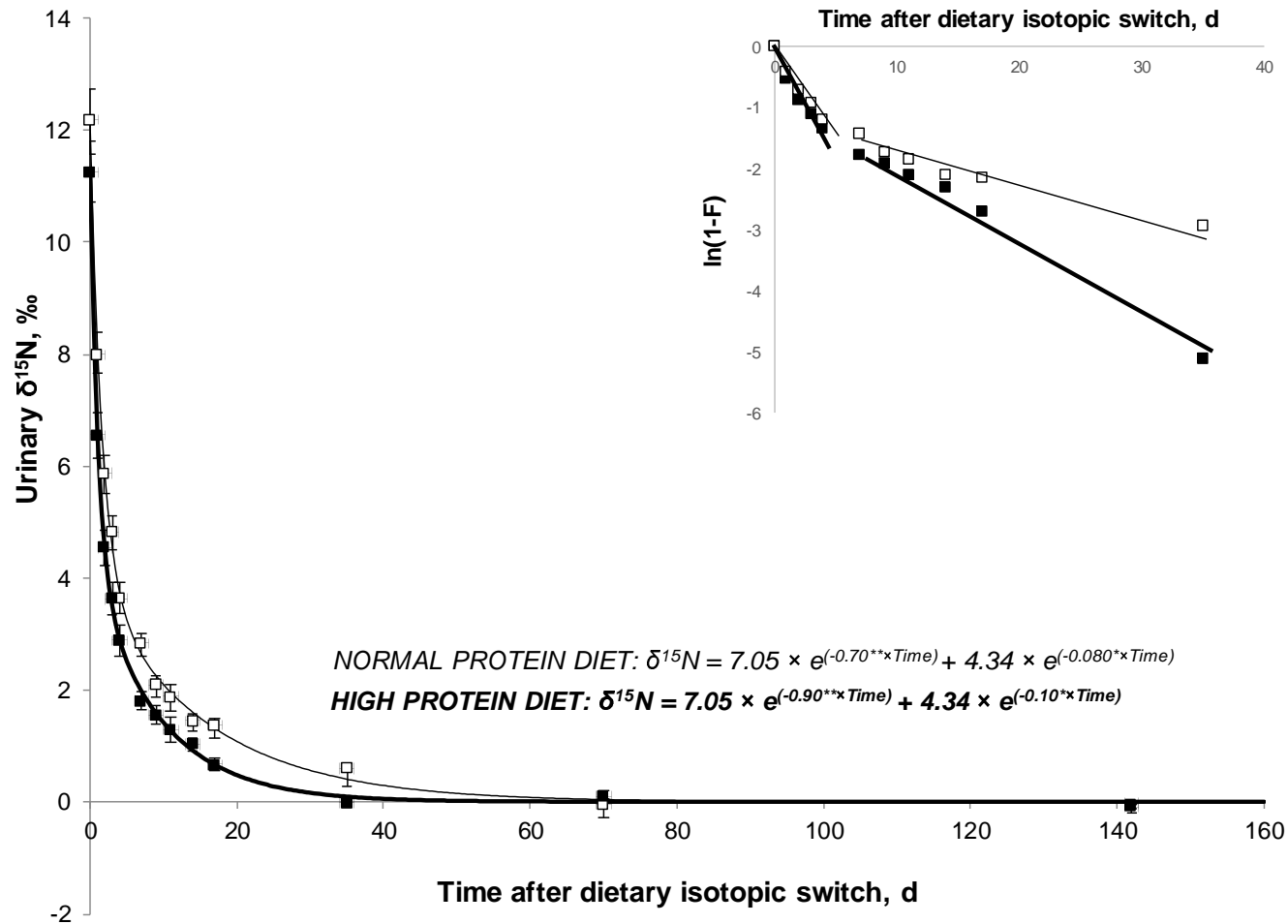


Figure 1. Kinetics of ^{15}N -depletion in urines after a ^{15}N diet-switch (see Material and Methods) in Charolais fattening bulls fed either Normal (thin line) or High (thick line) protein diets. Compared to animals fed Normal protein diet (n = 17), animals fed High protein diets (n = 17) showed higher fractional rates of urinary ^{15}N -depletion during both the first rapid (89.9 vs. 70.0%/d; P = 0.008) and the second slow (10.3

vs. 8.01 %/d; $P < 0.001$) phases. Inset represents the reaction-progress variable approach [$\ln(1-F)$; see Material and Methods] diagnosing that adequately fitting the ^{15}N -depletion kinetics in urine required a bi-exponential model with two rates for two - rapid and slow - phases (Martinez del Rio and Carleton, 2012). Significant differences in model parameters across treatments are depicted by stars (** $P < 0.01$; * $P < 0.05$).

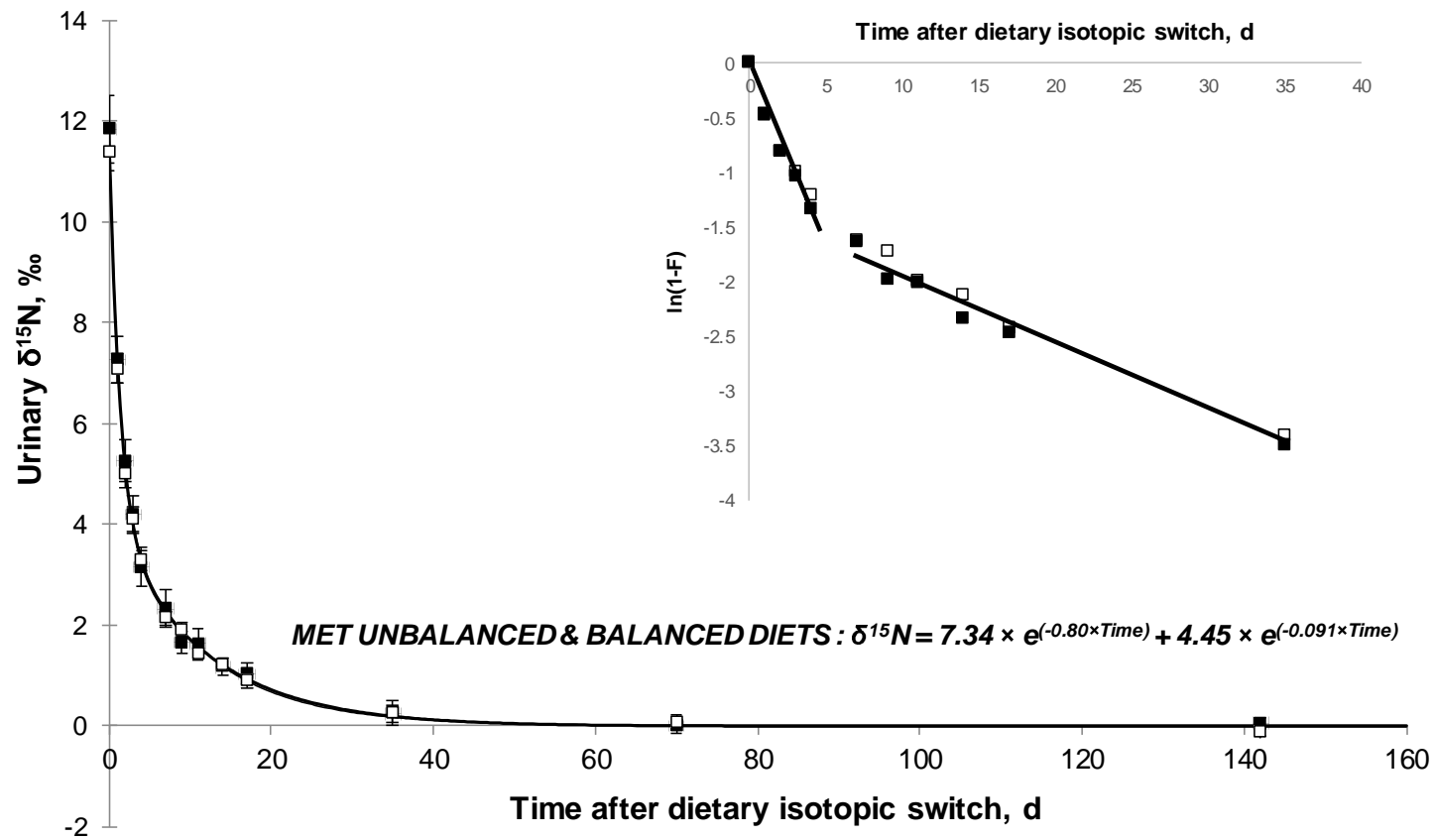


Figure 2. Kinetics of ^{15}N -depletion in urines after a ^{15}N diet-switch (see Material and Methods) in Charolais fattening bulls fed either balanced (thin line) or unbalanced (thick line) diets in terms of methionine content. Compared to animals fed diets unbalanced in methionine ($n = 17$), animals fed diets balanced in methionine ($n = 17$) showed similar fractional rates of urinary ^{15}N -depletion during both the first rapid (80.0%/d; $P = 0.92$) and the second slow (9.10%/d; $P = 0.80$) phases. Inset represents the reaction-progress variable approach [$\ln(1-F)$;

see Material and Methods] diagnosing that adequately fitting the ^{15}N depletion kinetics in urine required a bi-exponential model with two rates for two - rapid and slow - phases (Martinez del Rio and Carleton, 2012).

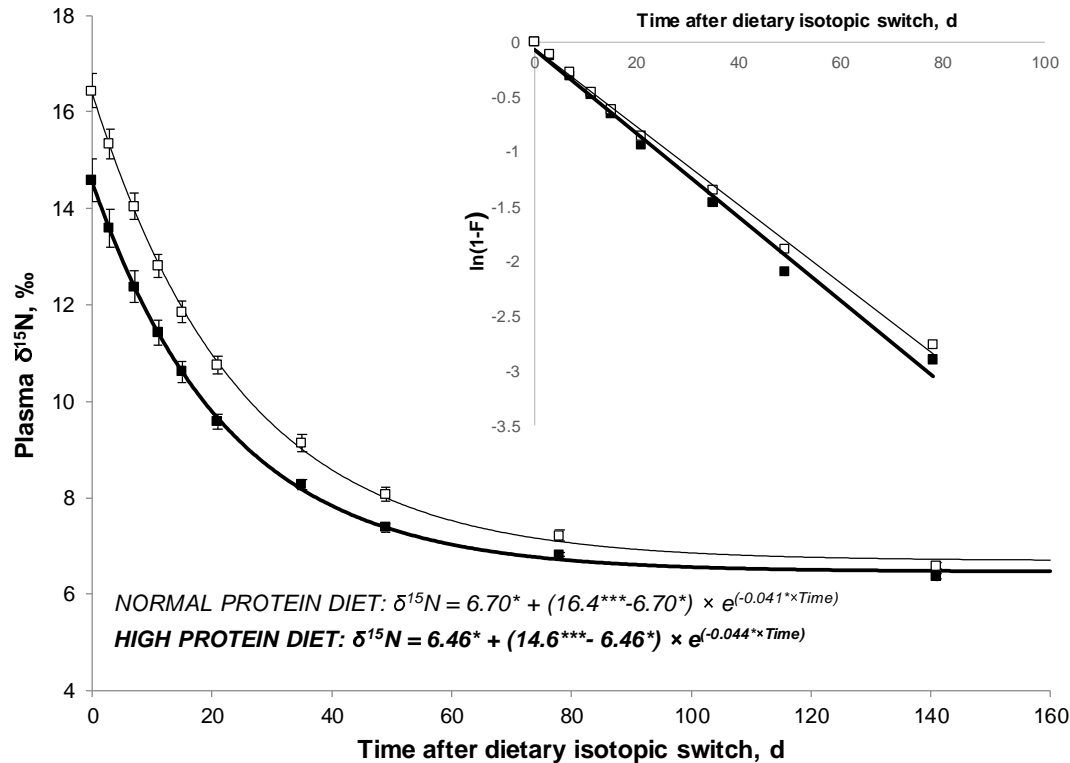


Figure 3. Kinetics of ^{15}N depletion in plasma proteins after a ^{15}N diet switch (see Material and Methods) in Charolais fattening bulls fed either Normal or High protein diets. Compared to animals fed Normal protein diets ($n = 18$), animals fed High protein diets ($n = 18$) showed lower plasma protein $\delta^{15}\text{N}$ values at time 0 (14.6 vs. 16.4‰; $P < 0.001$) and higher fractional rate of plasma protein ^{15}N -depletion (4.42 vs. 4.08 %/d; $P = 0.02$). Inset represents the reaction-progress variable approach [$\ln(1-F)$; see Material and Methods] diagnosing that a mono-exponential model was sufficient to adequately fit the ^{15}N -depletion kinetics in plasma proteins (Martinez del Rio and Carleton, 2012). Significant differences in model parameters across treatments are depicted by stars ($***P < 0.001$; $**P < 0.01$; $*P < 0.05$).

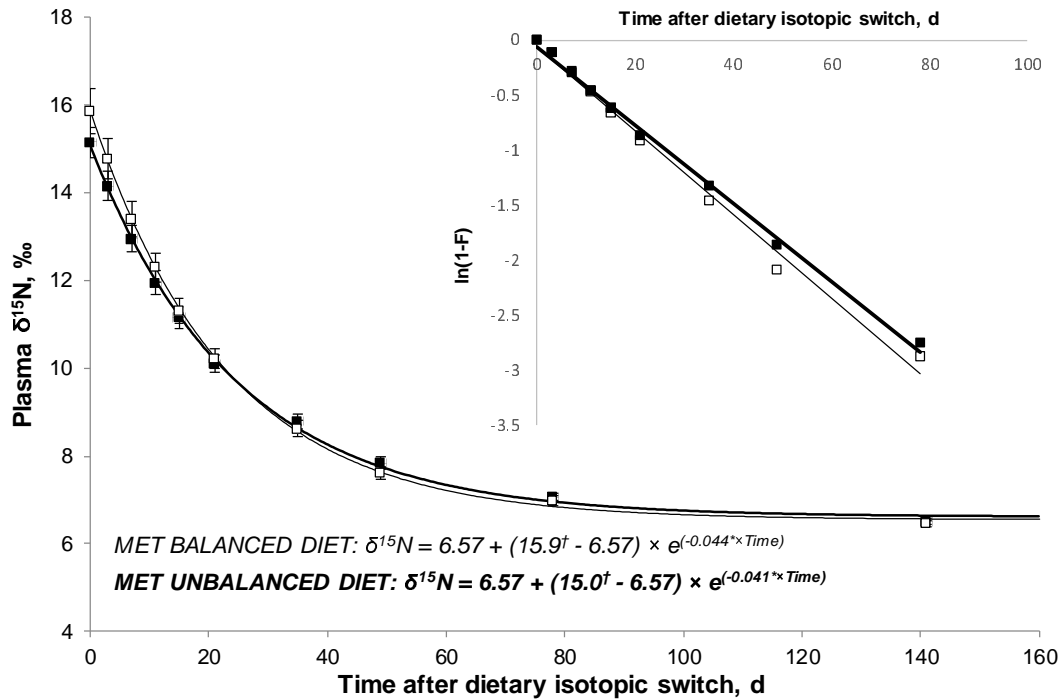


Figure 4. Kinetics of ^{15}N depletion in plasma proteins after a ^{15}N diet switch (see Material and Methods) in Charolais fattening bulls fed either balanced or unbalanced diets in terms of methionine content. Compared to animals fed diets unbalanced in methionine ($n = 18$), animals fed diets balanced in methionine ($n = 18$) showed higher plasma protein $\delta^{15}\text{N}$ values at time 0 (15.9 vs. 15.0‰; $P = 0.05$) and higher fractional rate of plasma protein ^{15}N -depletion (4.38 vs. 4.10%/d; $P = 0.05$). Inset represents the reaction-progress variable approach [$\ln(1-F)$; see Material and Methods] diagnosing that a mono-exponential model was sufficient to adequately fit the ^{15}N depletion kinetics in plasma proteins (Martinez del Rio and Carleton, 2012). Significant differences in model parameters across treatments are depicted by symbols ($^{\dagger}P < 0.10$; $^*P < 0.05$).

Supporting information

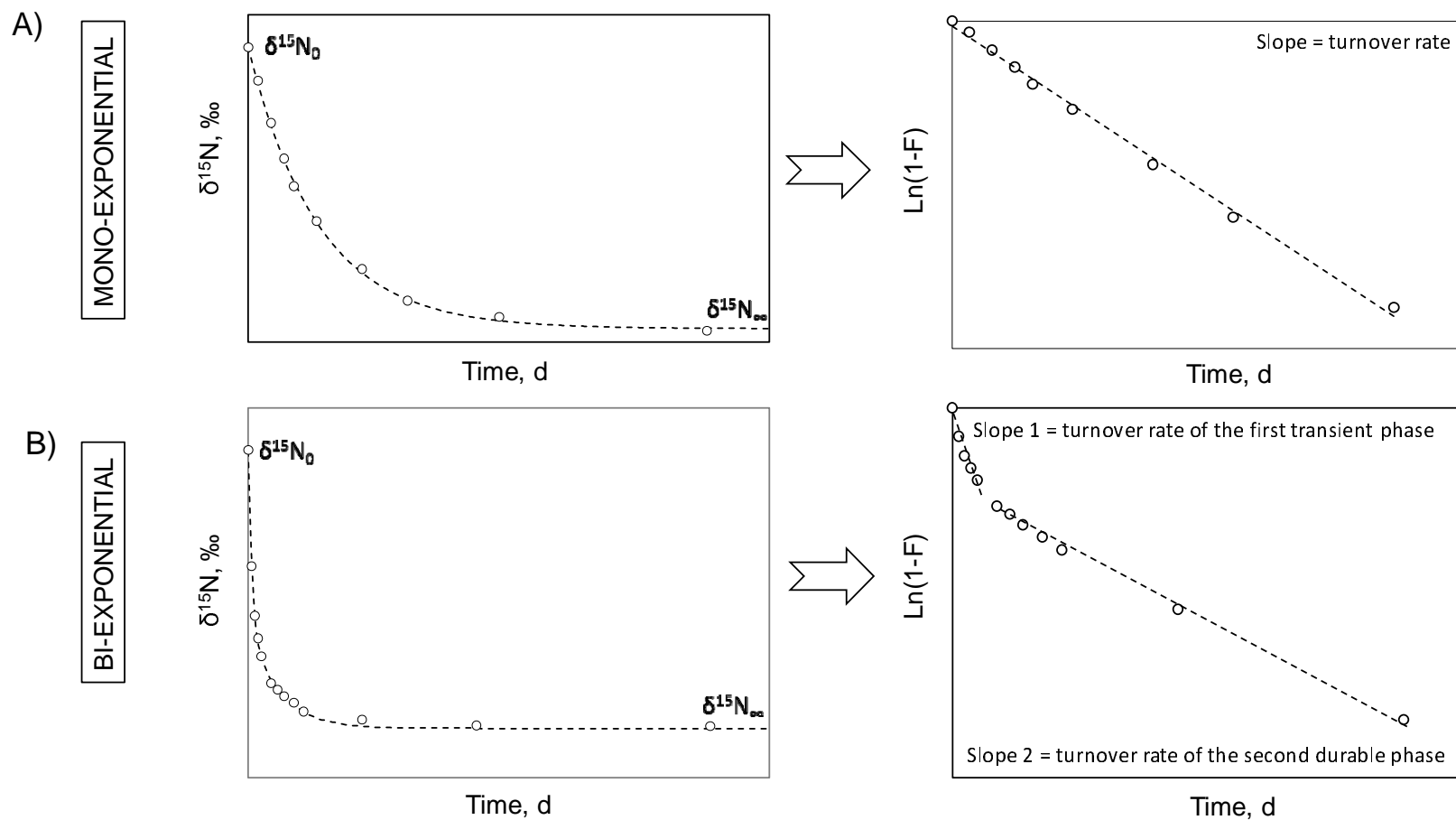


Figure S1. Modelling the kinetics of ^{15}N after a ^{15}N diet-switch in ruminants depending on whether a mono-exponential model is sufficient or a bi-exponential model is required to adequately fit the data. A) Mono-exponential model (like in plasma proteins) where $\delta^{15}\text{N}$ kinetics are adequately fitted according to $\delta^{15}\text{N}(t) = \delta^{15}\text{N}_\infty + (\delta^{15}\text{N}_0 - \delta^{15}\text{N}_\infty) \times e^{-k \times t}$ and a single slope is noted when the logarithm of the reaction

progress variable $(1-F = (\delta^{15}\text{N}(t) - \delta^{15}\text{N}_\infty)/(\delta^{15}\text{N}_0 - \delta^{15}\text{N}_\infty))$ is regressed on time. B) Bi-exponential model (like in urines) where $\delta^{15}\text{N}$ kinetics are adequately fitted according to $\delta^{15}\text{N}(t) = \delta^{15}\text{N}_\infty + (\delta^{15}\text{N}_0 - \delta^{15}\text{N}_\infty) \times [p \times e^{-k_1 \times t} + (1-p) \times e^{-k_2 \times t}]$ and two different slopes are noted when the logarithm of the reaction progress variables is regressed on time. The two different slopes represent the fractional turnover rates during a first transient (k_1) and a second durable (k_2) phases, respectively.