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The isotopic N turnover rate as a proxy to evaluate in the long-term the protein turnover in growing ruminants

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ABSTRACT:

Protein turnover is an energy-consuming process essential for ensuring the maintenance of living organisms. Gold standard methods for protein turnover measurement are based on intravenous infusions of stable isotopes. Although accurate they have inherent drawbacks precluding their generalization for large farm animals and during long time periods. We proposed here a non-invasive proxy of the whole-body fractional protein degradation (**WBFPDR**; protein turnover for a growing animal) in the long term and in a large number of beef cattle. The proxy is based on the rate at which urine-N and plasma proteins are progressively depleted in ^{15}N after a slight decrease in the isotopic N composition of diet (i.e. diet-switch). We aimed to test the ability of this proxy to adequately discriminate the WBFPDR of 36 growing-fattening young bulls assigned to different dietary treatments known to impact the protein turnover rate: the protein content and amino acid profile. To achieve this objective, the experimental diets were enriched with ^{15}N labeled-urea during 35 days while the animals were adapted to diets. After stopping the ^{15}N labeled-urea administration the animals were thereafter sampled for spot urines (n = 13) and blood (n = 10) over 5 months and analyzed for their ^{15}N enrichments in total N and plasma proteins, respectively. Adequately fitting the ^{15}N kinetics in plasma proteins and urines required mono- and bi-exponential models, respectively, and the model parameters were compared across dietary conditions using a non-linear mixed effect model. The single ^{15}N depletion rate found in plasma proteins represented their fractional synthesis rate, whereas the slowest depletion rate found in urines was interpreted as a proxy of the WBFPDR. The proxy here tested in urines suggested different WBFPDR values between Normal vs High protein diets but not between balanced vs unbalanced methionine diets. In contrast, the proxy tested in plasma indicated that both dietary conditions affected the fractional synthesis rate of plasma proteins. We consider that the rate at which urines are progressively

40 ^{15}N-depleted following an isotopic diet-switch could be proposed as a non-invasive proxy of
41 the long-term whole-body fractional protein degradation rate for large farm animals.
42
43 Keywords: protein turnover, isotope, ^{15}N, ruminant, beef cattle
44

45 **INTRODUCTION**

46 Protein turnover refers to the renewal of proteins in a cell, tissue or organism and
47 involves the continuous degradation and synthesis of proteins not leading to net changes in
48 protein mass. The protein accretion occurring in the growing animal is the result of a greater
49 intensity of protein synthesis over protein degradation. In the growing state, the protein turnover
50 rate (amount of replaced proteins per unit of time) equals to total protein degradation or
51 alternatively to the amount of freshly synthesized protein necessary to replace the degraded
52 protein. The protein turnover in growing ruminants is huge compared to the net protein
53 deposition with as high as 94% of the whole body protein synthesis only serving to
54 counterbalance the protein degradation occurring in fattening young bulls (Lobley, 2003).
55 Besides, this huge turnover is accompanied by a high-energy cost for the ruminant, with only
56 protein synthesis accounting for 23% of total energy expenditure in ruminants (Caton et al.,
57 2000). Nevertheless, this dynamic state represents an essential mechanism for life by enabling
58 maintenance services such as metabolic regulation, cellular repair and rapid adaptation against
59 environmental changes, among other functions. This dual facet of protein turnover ("service"
60 function for maintenance vs energy-consuming mechanism) explain why this animal trait has
61 been of interest for animal nutritionist and physiologist in the last decades.

62 Standard methods for whole-body protein turnover assessment are based on either
63 precursor or end-product methods (Waterlow, 1984) consisting in continuous or single dose
64 intravenous infusions of stable isotope labelled amino acids (**AA**) and the quantification of the

65 labelling in either the free AA precursor pool (plasma) or catabolic end-products (urine),
66 respectively. The main drawbacks associated to the evaluation of protein turnover rate through
67 these standard methods are i) short-term evaluation (from few hours [precursor method] to few
68 days [end-product method]), ii) invasive procedure and usually non-physiological conditions,
69 iii) the requirement of a controlled environment with confined animals and usually steady-state
70 conditions [precursor method] iv) expensive methods which precludes applying it to a big
71 number of large farm animals such as beef cattle. Alternative, less expensive and less invasive
72 methods have been explored, such as total urinary 3-methyl-histidine excretion, to evaluate the
73 whole-body protein degradation. However, 3-methyl-histidine urinary excretion is also based
74 on relatively short term evaluation (i.e. usually between 4 and 6 days), restricted to myofibrillar
75 muscle protein (myosin-actin) turnover, not applicable for sheep or pigs (cited in Nishizawa et
76 al., 1979) and subjected to an accurate estimation of the muscle mass in the body (Castro-Bulle
77 et al., 2007).

78 A new isotopic approach was originally developed in animal ecophysiology (Fry and
79 Arnold, 1982; Tieszen et al., 1983) to model the rate at which animal's tissues incorporate the
80 isotopic signature of a new diet in view of accurate food web assessment. It is currently being
81 applied mainly in marine animal species (Abimorad et al., 2014; Mohan et al., 2016) to evaluate
82 the metabolic turnover rate of animal's tissues. This isotopic method consists in following the
83 dynamic of isotopic change (i.e. enrichment or depletion) of animal's tissues immediately after
84 a change in the natural isotopic composition of the diet (i.e. diet-switch). The rate at which
85 animal's tissues will incorporate the isotopic signature of the new diet seems to be mostly driven
86 by their protein turnover rate (Carleton and Martinez del Rio, 2005; MacAvoy et al., 2005;
87 Braun et al., 2013) and some authors suggest that protein turnover rate might be predicted from
88 isotopic incorporation studies (Carleton et al., 2008). This approach has already been explored
89 in ruminant's tissues (Harrison et al., 2011; Bahar et al., 2014) but, as its original purpose, just

for assessing the time span needed for muscles to reflect the isotopic signature of the diet in view of meat authentication (i.e isotopic dietary reconstruction). However, the isotopic approach used in those studies in ruminant and marine animal species did not involve repeated measures from the same animal, but only one tissue sample per animal and individuals slaughtered at different times. Thus, these studies only informed about the turnover of specific sampled tissues and not of the whole animal body. Because most of the nitrogen from animal proteins is excreted through urines, once degraded and their AA partially catabolized, our hypothesis is that the urinary isotopic N turnover rate assessed from multi-point sampling from the same animal is a non-invasive proxy of the long term whole-body fractional protein degradation (**WBFPDR**; protein turnover in a growing animal).

Thus, the aim of this study was to adapt the tissue isotopic turnover approach (Braun et al., 2013; Abimorad et al., 2014) to the ruminant's urine and test it as a non-invasive, less disturbing proxy of the long term WBFPDR in a relatively large number of growing-fattening beef cattle. In addition, we also measured the isotopic turnover of plasma proteins as an indicator of the fractional protein synthesis rate (**FSR**) of plasma proteins. Because no gold standard method is currently available for measuring the WBFPDR in the long term (several months) we evaluated this proxy by assessing its ability to detect differences across two dietary factors known to impact the whole-body protein turnover in a different extent: i) the protein content and intake as a strong and well-known effect (Waterlow, 2006) and ii) the dietary amino acid profile as a less important modulator of protein turnover rate in cattle (Wessels et al., 1997). For this purpose, we measured the kinetics of nitrogen isotopic ($\delta^{15}N$) depletion in urines and plasma proteins over a 5-month period following a slight decrease in the $\delta^{15}N$ of the diet in 36 fattening young bulls fed diets formulated at two different protein and methionine contents. Preliminary results has been published as an abstract (Cantalapiedra-Hijar et al., 2018).

MATERIALS AND METHODS

The experiment was conducted at Herbipôle (Inra, UE 1414, Theix, France) in compliance with the National Legislation on Animal Care (Certificate of Authorization to Experiment on Living Animals, APAFIS#7180-2016101016361277 v4, Ministry of Agriculture, France).

Animals, diets and performance test

Thirty-six growing-fattening Charolais bulls (320±33kg and 266±22d) were assigned to one of the four experimental diets (n=9/treatment) resulting from a factorial 2 × 2 design: two dietary metabolizable protein levels (100% [Normal] vs 120% [High] of requirements; INRA, 2018) crossed with two dietary methionine content (unbalanced diet [2.0 g Met/100 g metabolizable protein] vs balanced diet [2.6 g Met/100 g metabolizable protein]). All diets were iso-NE per kg DM and consisted of 60% grass silage and 40% concentrate with amounts adjusted daily to ensure at least 10% of refusals.

Dietary isotopic N switch

The method here applied to assess the WBFPDR was based on the rate at which tissues and animal N pools incorporate a new dietary ^{15}N isotopic signature following a dietary isotopic switch (Carleton and Martinez del Rio, 2005; MacAvoy et al., 2005). However, because the range of natural ^{15}N abundance values (**$\delta^{15}N$**; $^{15}N/^{14}N$ ratio deviation from the international standard [atmospheric N_2]) in ruminant's feed remains quite narrow (between 0 and 5‰ on average according to our experience; Cantalapiedra-Hijar et al., 2015) we decided to create an artificial dietary isotopic switch by incorporating in the diet extrinsic ^{15}N labelled urea as previously reported (Bahar et al., 2014). The ^{15}N-urea once degraded into ^{15}N-ammonia and taken up by the rumen microorganisms will enrich the animal's tissues through the absorbed microbial ^{15}N-AA. Results from a preliminary trial aiming to define the conditions for implementing this method in ruminants showed that for a given animal the fraction of ^{15}N-urea bypassing directly to the urines, without being first incorporated into microbial proteins, was highly variable across time and individuals and led to problems for fitting the progressive

urinary ^{15}N-enrichment post-diet switch (data not shown). Thus, we decided to evaluate the animal ^{15}N turnover rates during a depletion (Abimorad et al., 2014) rather than enrichment (Bahar et al., 2014) phase. For this, animals were progressively enriched in ^{15}N over 35 days while adapting to their respective diet; each animal received daily in the morning (0830) a capsule (10 x 3mm) containing 20 mg of ^{15}N labelled urea (98% APE; Sigma–Aldrich, St. Louis, USA). This amount of ^{15}N labelled urea and time was chosen for enhancing the urinary ^{15}N between 10 and 15‰ above that of the basal diet according to results obtained from a preliminary trial. The capsule was mixed with around 200 grams of concentrate (the one assigned to the animal) in a small bucket within the feeder just before the only meal distribution (0900). Twenty minutes after administration it was systematically checked that the capsule had been swallowed by the animal. Less than 5% of capsules were administered by hand directly in the mouth when animals did not swallow it within the first twenty minutes. The rest of the diet was then distributed to the feeder. On day 36, animals no longer received the ^{15}N labelled urea, and blood and urine were sampled from that day (d0) onwards.

Sampling and ^{15}N analysis

Blood was sampled at 0900 by venipuncture from the caudal vein of all animals on d0, d3, d7, d11, d15, d21, d35, d49, d78 and d141 (n=10 per animal) after stopping the ^{15}N labelled urea administration (d0 refers to 24h after the last ^{15}N-urea administration). Blood was collected into 9 mL evacuated tubes (BD vacutainer, Plymouth, UK) containing lithium heparin as an anticoagulant, centrifuged within the first hour at 2500 g for 15 min at 4°C and stored at -20°C for determination of δ^{15}N values in total plasma proteins as previously described (Cantalapiedra-Hijar et al., 2015). Urinary spot samples were obtained from all animals between 0900 and 1000 on d0, d1, d2, d3, d4, d7, d9, d11, d14, d17, d35, d70, d142 (n = 13). For urinary sampling, animals were head blocked during 1h while eating in the morning and a bucket (30 x 30 x 15 cm) placed around the penis through two elastic ropes knotted on the back

of the animal. Most of animals urinated during the first hour and only few samplings were conducted beyond the first hour and always within the first three hours. Twenty mL of urine were then transferred into one tube containing 1 mL of 30%H_2SO_4 and filtered later in the lab through a 30 µm standard filter paper to remove fine particles. Filtered and acidified spot urine samples were stored at -20°C before determination of $\delta^{15}N$ values. Freeze-dried plasma proteins and liquid urinary samples pipetted onto nitrogen-free absorbent (chromosorb) were weighted in tin capsules and analyzed for N stable isotope composition ($\delta^{15}N$) by using an isotope-ratio mass spectrometer (Isoprime, VG Instruments, Manchester, UK) coupled to an elemental analyzer (EA Vario Micro Cube, Elementar, Germany). Tyrosine was used as internal standard and included in every run to correct for possible variations in the raw values determined by the mass spectrometer. Results were expressed using the delta notation.

Isotopic turnover rate modelling and statistical analysis

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

Mono-exponential model: $\delta^{15}N(t) = \delta^{15}N_\infty + (\delta^{15}N_0 - \delta^{15}N_\infty) \times \exp^{-k \times t}$ [1]

Bi-exponential model: $\delta^{15}N(t) = \delta^{15}N_\infty + (\delta^{15}N_0 - \delta^{15}N_\infty) \times [p \times \exp^{-k_1 \times t} + (1-p) \times \exp^{-k_2 \times t}]$ [2]

where t (d) is the time since the ^{15}N diet-switch, $\delta^{15}N(t)$ (‰) is the pool $\delta^{15}N$ value at time t, $\delta^{15}N_0$ (‰) is the pool initial $\delta^{15}N$ value, and $\delta^{15}N_\infty$ (‰) is the asymptotic value of the pool after the animal has reached isotopic steady state with its basal diet (without ^{15}N-urea administration). In the mono-exponential model, k (d^{-1}) is the fractional isotopic turnover rate of the pool, while in the bi-exponential model, k_1 and k_2 (d^{-1}) are two distinct fractional isotopic turnover rates and p and (1-p) t their respective contributions to the whole isotopic turnover.

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

$$\ln (1\text{-}F) = \ln [(\delta^{15}N(t) - \delta^{15}N_{\infty}) / (\delta^{15}N_0 - \delta^{15}N_{\infty})] = - k \times t$$

where (1-F) measures the remaining $\delta^{15}N$ distance to the new equilibrium as a proportion of the total isotopic distance between the initial and the asymptotic $\delta^{15}N$ reached at equilibrium. As illustrated in Appendix (Figure A1), we decided what model was required on the basis of a visual inspection of a plot of ln(1-F) against time, depending on whether ln(1-F) was a decreasing linear function of time with slope equal to -k (mono-exponential model) or a sequence of 2 lines of increasingly shallow slopes equal to $-k_1$ and $-k_2$ (bi-exponential model). This graphical analysis was further supported by the Akaike Information criterion, where lower values indicates a superiority of one model over another.

The model parameters (i.e., $\delta^{15}N_{\infty}$, $\delta^{15}N_0$ and k for the mono-exponential model, and $\delta^{15}N_{\infty}$, $\delta^{15}N_0$, k_1, k_2 and p for the bi-exponential model) were estimated using a non-linear procedure and statistically analyzed through a non-linear mixed-effect model (nlme package; Pinheiro and Bates, 2000) fitted by maximum likelihood in the R software (R Development Core Team, 2015) with experimental dietary factors (protein content, methionine content and their interaction) considered as fixed effects and the animal as a random effect. To avoid over-parametrization in the most complex model (bi-exponential) and avoid a failure for the model to converge, the random effect of animal was only considered on those parameters where significant animal variability was noted through graphical and statistical analysis (Pinheiro and Bates, 2000). Significant effects were declared when the $P \leq 0.05$ and a trend was considered when $0.05 > P < 0.10$.

RESULTS

No significant interaction (P>0.05) between protein level and methionine concentration was observed for any of the variables here analyzed. Thus, for the shake of clarity, results are presented independently for each of these two experimental factors. During the length of the applied approach (around 5 months) bulls fed High protein diets had on average higher (P = 0.005) daily gains (1.82 kg/d) compared to those fed Normal protein diets (1.63 kg/d on average). On the other hand, methionine balanced diets tended (P = 0.09) on average to promote higher daily gains (1.78 kg/d) than bulls fed unbalanced diets (1.67 kg/d).

Urinary isotopic turnover rate

For $\delta^{15}N$ kinetics in urines, the reaction progress approach clearly identified two independent slopes with a cut-off between d4 and d7 (Insets in Figures 1 and 2). This demonstrated the existence of two distinct rates, a rapid and a slow, of urinary $\delta^{15}N$ depletion after the diet-switch, and justified the need of a bi-exponential model to adequately fit these data. The individual $\delta^{15}N$ kinetics were indeed correctly fitted for almost all animals ($r2 \geq 0.96$; n=34) using a bi-exponential model, except for 2 animals that were badly fitted with no apparent explanation and that were therefore excluded from the analyses. When all data were pooled and analyzed through a mixed-effect bi-exponential model, only a significant effect of the dietary protein content was observed on the fractional rates of ^{15}N-depletion in urines, where animals fed High protein diets showed higher values 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 of $\delta^{15}N$ turnover compared to animals fed Normal protein diets (Figure 1). No effect of methionine content was observed on any model parameter ($P > 0.10$; Figure 2).

Plasma isotopic turnover rate

For $\delta^{15}N$ kinetics in plasma proteins, the reaction progress approach showed a single slope (Inserts in Figures 3 and 4). This demonstrated the existence of a single, homogenous rate of $\delta^{15}N$ depletion in plasma proteins after the diet-switch, and justified that a mono-exponential

model was sufficient to adequately fit these data. The individual $\delta^{15}N$ kinetics in plasma proteins were indeed correctly fitted ($r2 \geq 0.98$; n=36) through a mono-exponential asymptotic model. When all data were pooled and analyzed through a mixed-effect asymptotic model, lower plasma protein $\delta^{15}N$ values were observed in animals fed High compared to Normal protein diets (Figure 3) on first day (d0; $P = 0.04$) and at equilibrium (d142; $P = 0.09$). In contrast, the effect of balancing diets in terms of methionine content tended ($P = 0.09$) to have a higher $\delta^{15}N$ value in plasma proteins on d0 (Figure 4) but not at equilibrium ($P = 0.47$). The fractional ^{15}N-depletion rate in plasma proteins was higher in animals fed High compared to Normal protein diets (4.42 vs 4.08%/d; $P = 0.02$) and in those fed diets balanced compared to unbalanced in methionine (4.38 vs 4.10%/d; $P = 0.05$).

DISCUSSION

Following a diet change, animal's tissues progressively assimilate the isotopic signature of the new diet (Carleton and Martinez del Rio, 2005). The rate at which this occur is known as the isotopic turnover rate and depends largely on how fast the metabolic tissue replacement occurs (Tieszen et al., 1983; Arneson et al., 2006). We adapted the tissue isotopic turnover approach, extensively explored by ecologists (Martinez del Rio and Carleton, 2012), to the case of ruminant's urines and plasma proteins to respectively evaluate the WBFPDR (protein turnover in a growing animal) and plasma protein FSR of 36 growing-fattening cattle in the long term (5 months). This isotopic approach is simple to set up, relatively cheap (estimated to be less than 300 dollars per bull), allowing to obtain measurements over long periods (several months) and without perturbing animals (unlike classic tracer methods with invasive procedures under non-physiological conditions). Although the mechanistic interpretation of isotopic turnover rates is always a complex issue (Martinez del Rio and Anderson-Sprecher, 2008), it is proposed that the rates at which urines and plasma proteins are progressively ^{15}N-depleted following an isotopic diet-switch represent non-invasive (or less invasive) proxies of the long-

263 term WBFPDR and plasma protein FSR, respectively. Such proxies could be very useful for
264 future studies on feed efficiency (protein turnover as an energy-consuming process), animal
265 robustness (protein turnover as a maintenance service) and meat quality (in vivo protein
266 turnover associated to the rate of post mortem proteolysis and thus to meat tenderness) carried
267 out on large number of animals.
268
269 *Biological meaning of isotopic turnover rates*
270 The simplicity of the method here described may contrast with the need to address some
271 methodological considerations for interpreting our results. The rate at which animal's tissues
272 will incorporate the isotopic signature of the new diet seems to be mostly driven by their protein
273 turnover rate according to many previous reports (Carleton and Martinez del Rio, 2005;
274 MacAvoy et al., 2005; Braun et al., 2013). Moreover, this has been confirmed by mechanistic
275 models (Poupin et al., 2013; Martinez del Rio and Carleton, 2012), demonstrating that the main
276 driver determining the rate of assimilation of the new dietary $\delta^{15}N$ value into a tissue after a
277 diet-switch is the protein FSR of that tissue. Thus, the isotopic turnover rate we found in the
278 plasma protein pool (i.e., the k model parameter; Figure 3 and 4) represented its own FSR (i.e.,
279 the sum of its fractional degradation rate and fractional growth rate) and may be proposed as a
280 way to indirectly evaluate the liver FSR of plasma proteins. In contrast, to the best of our
281 knowledge the isotopic turnover rate of urine has never been analyzed mechanistically and, as
282 further discussed, its biological meaning is mainly ascribed to the WBPFDR. Some
283 considerations and assumptions need, however, to be discussed to support our biological
284 interpretation.
285 First, the urinary $\delta^{15}N$ kinetics post diet-switch did not only reflect the N metabolism of
286 the animal but also that of its symbiotic rumen microbiota. Urines would theoretically also drain
287 the previously ^{15}N enriched microbiota as they renovate their own structural proteins and N

pools (amino acids but also purine derivatives). However this would have quantitatively a short-term impact on urinary $\delta^{15}N$ kinetics due to the high protein turnover rate of rumen microbiota (127 to 686%/d in rumen bacteria; Wallace and McPherson, 1987). Second, because of the complex and extensive urea recycling in ruminants we should not exclude the fact that the clearance rate of the previously administered ^{15}N labelled urea from the whole body after stopping its administration might contribute in an undetermined extent to the urinary ^{15}N depletion rate during the first hours. In this sense, it has been demonstrated in beef cattle that most infused ^{15}N-labelled urea is excreted through urines within the first 48h (Wessels et al., 1997), and so its impact on urinary ^{15}N depletion rate would be very short. Third, differences in the N absorption, and thus in the urinary N excretion, expected across diets (Normal vs High protein diets) might contribute to the observed differences in the urinary ^{15}N depletion rate. Indeed, the dietary N influx into the plasma pool, while being much smaller than the endogenous AA influx by protein degradation (Lobley, 2003), had the largest $\delta^{15}N$ difference with body AA at the beginning of the diet-switch (dietary AA were largely ^{15}N depleted compared to the body AA previously ^{15}N enriched during the administration of ^{15}N labelled urea). In consequence, the urinary $\delta^{15}N$ kinetics were probably mainly driven by the dietary AA influx during the very first days (k_1 phase), before being mostly driven by the endogenous AA influx released from the whole-body protein degradation thereafter (k_2 phase). All these three factors (rumen microbiota metabolism, isotopic labelled urea clearance rate and differences in N absorption across treatments) would have thus a limited short impact on the urinary ^{15}N turnover rate only during the first transient phase (k1). We think that the ^{15}N depletion rate in urines during the second durable phase (k2) is mostly driven by the release rate of endogenous ^{15}N labeled AA from degradation of previously labeled body proteins. This progressive release of previously labeled AA will translate first into the free plasma AA pool and then into the urea-N pool resulting from their catabolism. Therefore, we assume that the urinary ^{15}N depletion rate

313 during the second durable phase is mostly the consequence of the WBFPDR, whereas the rate
314 during the first transient phase is likely a mix from the previously evoked mechanisms together
315 with (in some undetermined extent) the protein degradation rate of some very fast animal
316 turning-over N pools.

317 A limitation to our interpretation could be that some N-containing compounds in urine
318 are metabolically unrelated to protein degradation and AA oxidation. This could represent a
319 potential issue particularly in ruminants where urinary urea-N excretion, the main urinary
320 nitrogenous component, may origin in a high extent from rumen ammonia production (INRA,
321 2018) and so from nitrogenous compounds unrelated to protein degradation and AA oxidation.
322 It can be acknowledge that our modelled k2 likely integrates the outflow rate of those minor
323 urinary nitrogenous compounds non related to AA oxidation and with an endogenous (animal)
324 origin (e.g. creatine, creatinine, endogenous purine derivatives and 3-methylhistidine and other
325 free AA). Such an endogenous nitrogenous compounds were likely previously labelled during
326 ^{15}N urea administration likewise body proteins and progressively released from the animal body
327 through the urines. However it should be stressed that i) its contribution to total urinary N
328 remains relatively modest in productive animals (0.05 g N/kg BW/d; INRA, 2018) and ii) for
329 some of these nitrogenous compounds their release rate is closely related to protein turnover
330 rate (e.g. 3-methyl-histidine as an index of muscle protein degradation and endogenous purine
331 derivatives from nucleic acid turnover rate).

332 For those urinary nitrogenous compounds non related to AA oxidation and with a
333 ruminal origin (mostly purine derivatives [allantoin and uric acid], hippuric acid and rumen
334 ammonia mostly transformed into urea) it can be argued, as previously mentioned, that it would
335 have probably an impact on the first transient phase of the urinary ^{15}N depletion rate (k1) but
336 as long as the plasma absorption of such a compounds remains relatively constant across the
337 study they will not have an effect on the second transient phase of this depletion (k2). Taking

all this together, we can consider that even if k2 does not strictly correspond only to the WBFPDR, it may reflect quantitatively this flux and can be proposed as a proxy of it.

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

Isotopic turnover rates across dietary factors impacting protein metabolism

Dietary protein content determines the dietary AA flux to the organism and is the major dietary determinant of the whole-body protein turnover rates in humans (Waterlow, 2006) and farm animals (Lobley, 2003) through the action of catabolic and anabolic hormones. In the present study, the urinary ^{15}N-depletion rates during both the first transient (k_1) and second durable (k_2) phases increased on average around 28% when increasing the metabolizable protein content by 20%. This finding is in line with the expected increases in dietary AA influx and WBFPDR that have been well recorded in k_1 and k_2, respectively. When a similar diet-switch approach was applied on adult rats fed diets at or much above protein requirements (10 vs 30 % CP), the urinary turnover rate of ^{15}N increased more than two folds, in agreement with an expected higher protein turnover rate (Braun et al., 2013). Our values for the WBFPDR (average k2 = 9.1%/d) are, however, higher than those reported in the few studies using standard isotopic methods in growing ruminants, which themselves encompasses a wide range (Lobley et al., 1980 [3.88-8.00%/d whole-body protein FSR in growing heifers]; Liu et al., 1995 [6.15 -7.63%/d WBFPD rate in growing lambs]; Wessels et al., 1997 [calculated WBFPD rate of around 1.8%/d] in growing steers) but still lower than other estimates (Davis et al., 1980 [19.7%/d WBFPD rate in growing lambs]). Although the methods are not comparable (few days vs several months) and our higher values compared to other cattle studies (Wessels et al., 1997; Lobley et al., 1980) could be just the consequence of using late maturing breeds with high

363 growth rates (1.70 kg/d on average for the studied period) and retaining between 35-45% of
364 their energy as protein (Geay and Robelin, 1979), the obtained values will need further
365 confirmation and interpretation.

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

377 On the other hand, no changes in the rate at which urines are progressively depleted in
378 ^{15}N following an isotopic diet-switch were found between unbalanced vs balanced diets in terms
379 of methionine content. In contrast, plasma protein ^{15}N-depletion rate was slightly increased,
380 indicating that protein FSR was enhanced for plasma proteins and likely also at other different
381 body levels. Our findings may indicate that the observed trend for AA balanced diets to improve
382 beef cattle performances observed in this experiment (Cantalapiedra-Hijar et al., 2018) and
383 others (Wessels et al., 1997) could stem from an increased whole-body protein synthesis rather
384 than a reduction in the whole-body protein degradation.

385 In conclusion, we believe that the rate at which animal's urines are depleted in ^{15}N after
386 stopping the administration of ^{15}N labelled urea in the diet (i.e. isotopic turnover rate after diet
387 switch) can be proposed as a non-invasive and simple proxy to evaluate in the long term the

388 whole-body protein turnover rate in a high number of animals. Further studies are warranted to
389 explore the potential and limits of this new promising isotopic approach for evaluating protein
390 turnover.

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396 LITERATURE CITED

397 Abimorad, E. G., C. Ducatti, D. Castellani, R. K. Jomori, M. C. Portella, and D. J. Carneiro.
398 2014. The use of stable isotopes to investigate the effects of supplemental lysine and
399 methionine on protein turnover and amino acid utilization in pacu, Piaractus mesopotamicus,
400 juveniles. Aquaculture, 433: 119-124.

401 Arneson, L. S., S. MacAvoy, and E. Basset. 2006. Metabolic protein replacement drives tissue
402 turnover in adult mice. Can. J. Zool. 84: 992-1002

403 Bahar, B., S. M. Harrison, A. P. Moloney, F. J. Monahan, and O. Schmidt. 2014. Isotopic
404 turnover of carbon and nitrogen in bovine blood fractions and inner organs. Rapid Commun.
405 Mass Spectrom. 28:1011-1018.

406 Braun, A., K. Auerswald, A. Vikari, and H. Schnyder. 2013. Dietary protein content affects
407 isotopic carbon and nitrogen turnover. Rapid Commun. Mass Spectrom. 27: 2676-2684.

408 Cantalapiedra-Hijar, G., I. Ortigues-Marty, B. Sepchat, J. Agabriel, J. F. Huneau, and H.
409 Fouillet. 2015. Diet–animal fractionation of nitrogen stable isotopes reflects the efficiency
410 of nitrogen assimilation in ruminants. Br. J. Nutr. 113: 1158-1169.

411 Cantalapiedra-Hijar, G., L. Bahloul, C. Chantelauze, V. Largeau, N. Khodorova, H. Fouillet,
412 and I. Ortigues-Marty. 2018. Improved cattle performance by methionine-balanced diets
413 does not results from decreased protein degradation. Proc. Eur. Assoc. Anim. Prod. (in press)
414 Carleton, S. A., and C. Martinez del Rio. 2005. The effect of cold-induced increased metabolic
415 rate on the rate of 13C and 15N incorporation in house sparrows (Passer
416 domesticus). Oecologia, 144: 226-232.
417 Carleton, S. A., Kelly, L., Anderson-Sprecher, R., and C. Martinez del Rio. 2008. Should we
418 use one-, or multi-compartment models to describe 13C incorporation into animal
419 tissues?. Rapid Commun. Mass Spectrom. 22: 3008-3014.
420 Castro Bulle, F. C. P., P. V. Paulino, A. C. Sanches, and R. D. Sainz. 2007. Growth, carcass
421 quality, and protein and energy metabolism in beef cattle with different growth potentials
422 and residual feed intakes. J. Anim. Sci. 85: 928-936.
423 Caton, J. S., M. L. Bauer, and H. Hidari. 2000. Metabolic components of energy expenditure in
424 growing beef cattle-review. Asian-Australas. J. Anim. Sci. 13: 702-710.
425 Cerling, T. E., L. K. Ayliffe, M. D. Dearing, J. R. Ehleringer, B. H. Passey, D. W. Podlesak,
426 A.M. Torregrossa, and A. G. West. 2007. Determining biological tissue turnover using stable
427 isotopes: the reaction progress variable. Oecologia, 151: 175-189.
428 Connell, A., A. G. Calder, S. E. Anderson, and G. E. Lobley. 1997. Hepatic protein synthesis
429 in the sheep: effect of intake as by use of stable-isotope-labelled glycine, leucine and
430 phenylalanine. Br. J. Nutr. 77: 255-271.
431 Davis, S. R., T. N. Barry, and G. A. Hughson. 1981. Protein synthesis in tissues of growing
432 lambs. Br. J. Nutr. 46: 409-419.
433 Fry, B., and C. Arnold. 1982. Rapid 13 C/12 C turnover during growth of brown shrimp
434 (Penaeus aztecus). Oecologia, 54: 200-204.

435 Geay, Y., and J. Robelin. 1979. Variation of meat production capacity in cattle due to genotype
436 and level of feeding: genotype-nutrition interaction. Liv. Prod. Sci. 6: 263-276.
437 Harrison, S. M., O. Schmidt, A. P. Moloney, S. D. Kelly, A. Rossmann, A. Schellenberg, F.
438 Camin, M. Perini, J. Hoogewerff, and F. J. Monahan. 2011. Tissue turnover in ovine muscles
439 and lipids as recorded by multiple (H, C, O, S) stable isotope ratios. Food Chem. 124: 291-
440 297.
441 INRA, 2018. INRA feeding system for ruminants. Wageningen Academic Publishers,
442 Wageningen, the Netherlands, 640 pp.
443 Liu, S. M., G. E. Lobley, N. A. MacLeod, D. J. Kyle, X. B. Chen, and E. R. Ørskov. 1995.
444 Effects of long-term protein excess or deficiency on whole-body protein turnover in sheep
445 nourished by intragastric infusion of nutrients. Br. J. Nutr. 73: 829-839.
446 Lobley, G. E., V. Milne, J. M. Lovie, P. J. Reeds, and K. Pennie. 1980. Whole body and tissue
447 protein synthesis in cattle. Br. J. Nutr. 43: 491-502.
448 Lobley, G. E. 2003. Protein turnover—what does it mean for animal production?. Can. J. Anim.
449 Sci. 83: 327-340.
450 MacAvoy, S. E., S. A. Macko, and L. S. Arneson. 2005. Growth versus metabolic tissue
451 replacement in mouse tissues determined by stable carbon and nitrogen isotope
452 analysis. Can. J. Zool. 83: 631-641.
453 Martinez del Rio, C. M. D., and S. A. Carleton. 2012. How fast and how faithful: the dynamics
454 of isotopic incorporation into animal tissues. J. Mammal. 93: 353-359.
455 Martinez del Rio, C. M. D., and Anderson-Sprecher, R. 2008. Beyond the reaction progress
456 variable: the meaning and significance of isotopic incorporation data. Oecologia, 156: 765-
457 772.
458 Mohan, J. A., S. D. Smith, T. L. Connelly, E. T. Attwood, J. W. McClelland, S. Z. Herzka, and
459 B. D. Walther. 2016. Tissue-specific isotope turnover and discrimination factors are affected

460 by diet quality and lipid content in an omnivorous consumer. J. Exp. Mar. Biol. Ecol. 479:
461 35-45.
462 Nishizawa, N., Y. Toyoda, T. Noguchi, S. Hareyama, H. Itabashi, and R. Funabiki. 1979. N τ-
463 Methylhistidine content of organs and tissues of cattle and an attempt to estimate fractional
464 catabolic and synthetic rates of myofibrillar proteins of skeletal muscle during growth by
465 measuring urinary output of N τ-methylhistidine. Br. J. Nutr. 42: 247-252.
466 Pinheiro, J. C., and D. M. Bates. 2000. Linear mixed-effects models. In Mixed-effects models
467 in S and S-Plus (ed. J. Chambers, W. Eddy, Hardle, S. Sheather, L. Tierney), pp. 3-56.
468 Springer, New York, USA.
469 Poupin, N., J .F. Huneau, F. Mariotti, D. Tomé, C. Bos, and H. Fouillet, H. 2012. Isotopic and
470 modeling investigation of long-term protein turnover in rat tissues. Amer. J. Physiol. Regul.
471 Integr. Comp. Physiol. 304: R218-R231.
472 Raggio, G., G .E. Lobley, R. Berthiaume, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre.
473 2007. Effect of protein supply on hepatic synthesis of plasma and constitutive proteins in
474 lactating dairy cows. J. Dairy Sci. 90: 352-359.
475 Steinbock, H. L., and H. Tarver. 1954. Plasma protein. 5. The effect of the protein content of
476 the diet on turnover. J. Biol. Chem. 209: 127-132.
477 Tieszen, L. L., T. W. Boutton, K. G., Tesdahl, and N. A. Slade. 1983. Fractionation and turnover
478 of stable carbon isotopes in animal tissues: implications for δ13C analysis of
479 diet. Oecologia 57: 32-37.
480 Tsahar, E., N. Wolf, I. Izhaki, Z. Arad, and C. Martinez del Rio. 2008. Dietary protein
481 influences the rate of 15N incorporation in blood cells and plasma of Yellow-vented bulbuls
482 (Pycnonotus xanthopygos). J. Exp. Biol. 211: 459-465.
483 Wallace, R. J., and C. A. McPherson. 1987. Factors affecting the rate of breakdown of bacterial
484 protein in rumen fluid. Br. J. Nutr. 58: 313-323.

485 Wessels, R. H., E. C. Titgemeyer, and G. St Jean. 1997. Effect of amino acid supplementation
486 on whole-body protein turnover in Holstein steers. J. Anim. Sci. 75: 3066-3073.
487 Waterlow, J. C. 1984. Protein turnover with special reference to man. Exp. Physiol. 69: 409-
488 438.
489 Waterlow, J. C. 2006. Protein turnover. CAB Interantional, Nosworthy Wag Wallingford,
490 Oxfordshire OX10 8DE, UK.

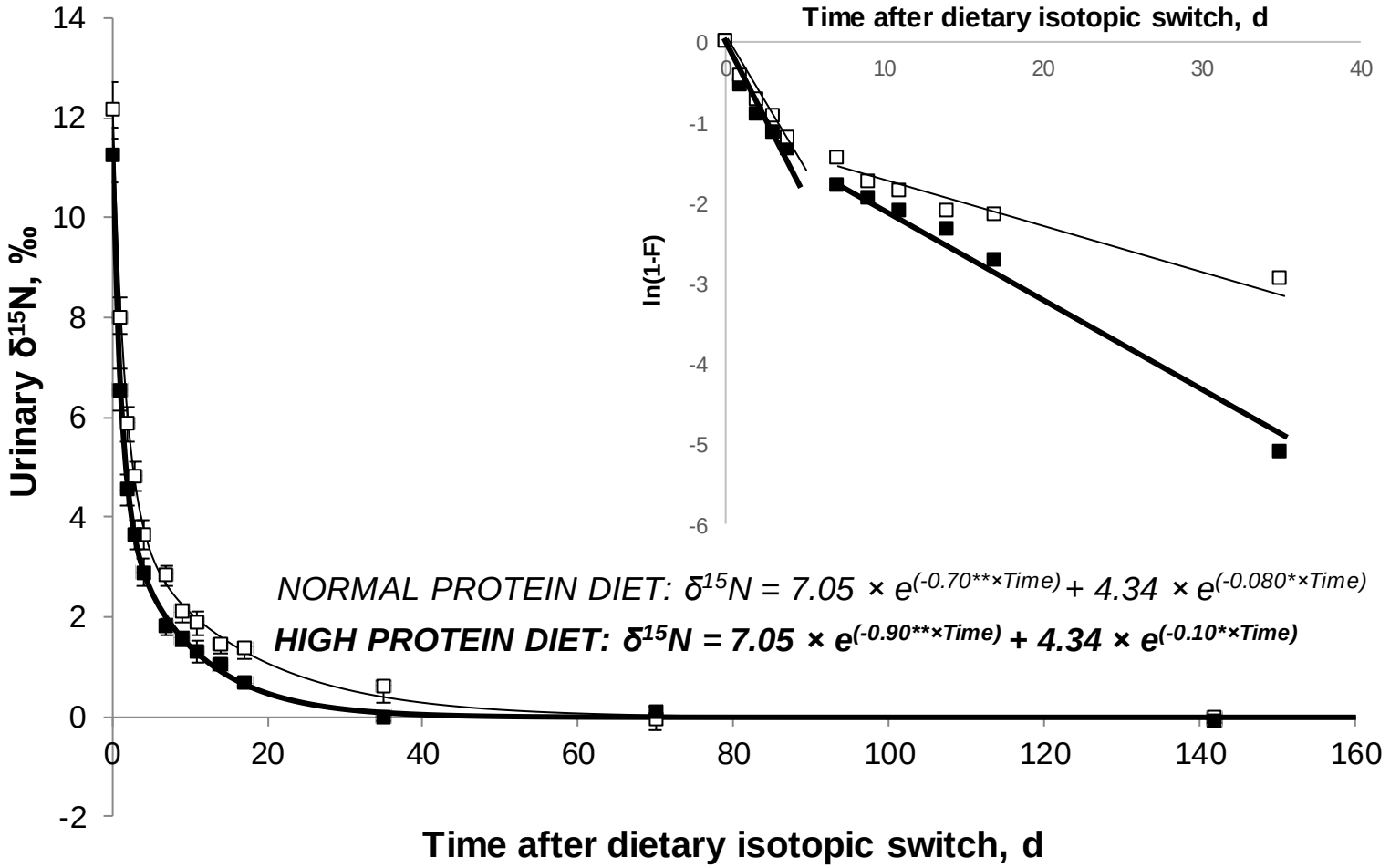

Figure 1. Kinetics of ^{15}N-depletion in urines after a ^{15}N diet-switch (see Material and Methods) in Charolais fattening bulls fed either Normal (thin line) or High (thick line) protein diets. Compared to animals fed Normal protein diet (n = 17), animals fed High protein diets (n = 17) showed higher 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 adequatly 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 (**$P < 0.01$; *$P < 0.05$).

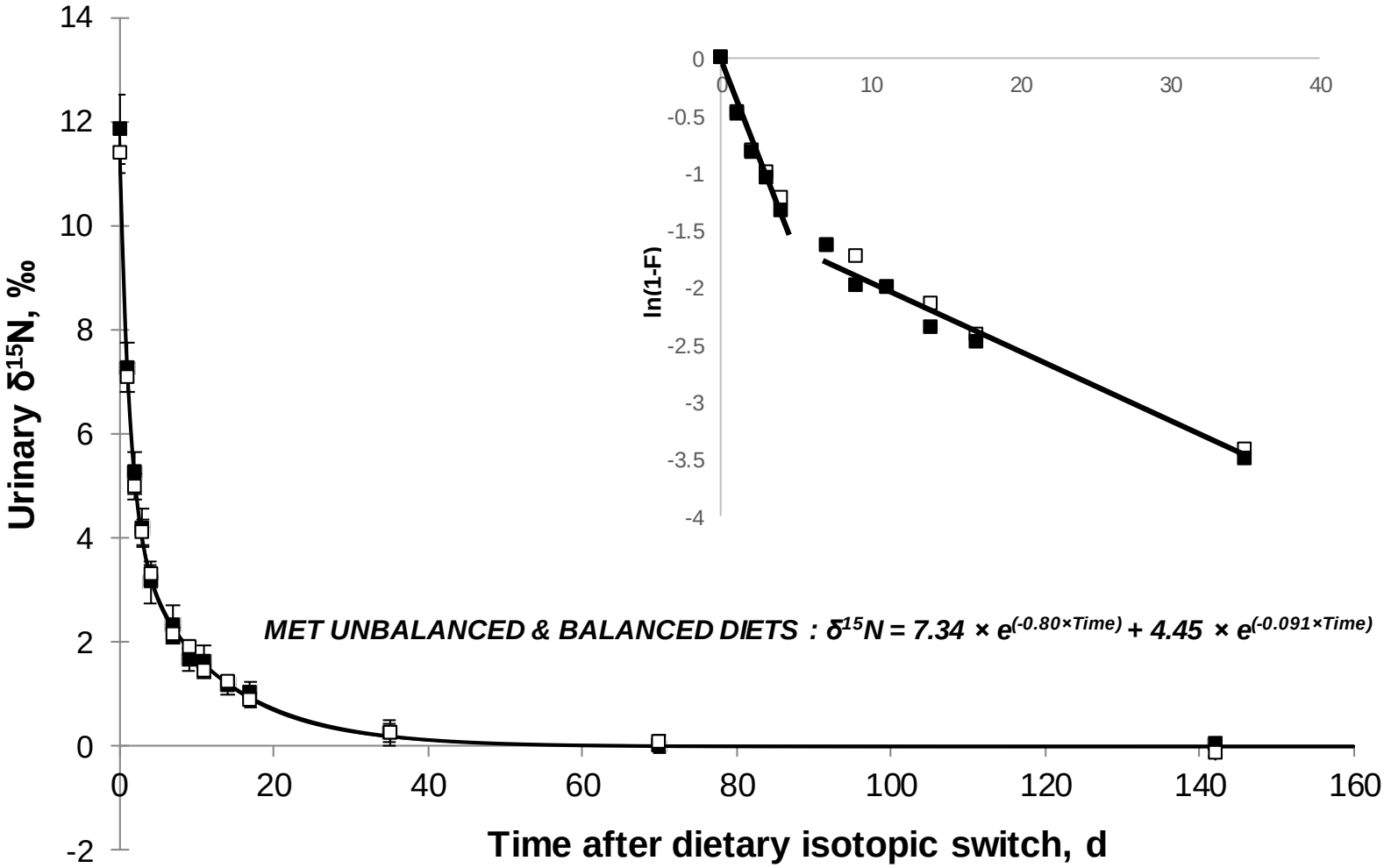

Figure 2. Kinetics of ^{15}N-depletion in urines after a ^{15}N diet-switch (see Material and Methods) in Charolais fattening bulls fed either balanced (thin line) or unbalanced (thick line) diets in terms of methionine content. Compared to animals fed diets unbalanced in methionine (n = 17), animals 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 adequatly 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).

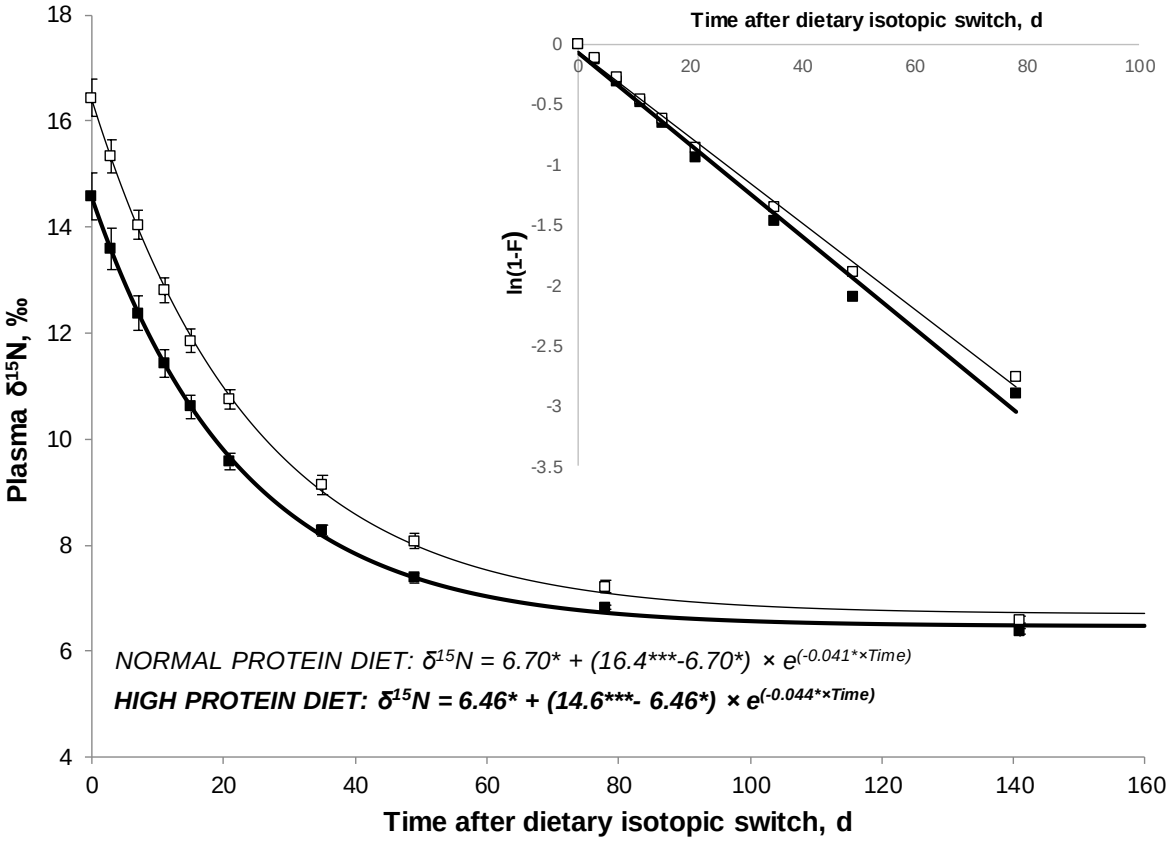

Figure 3. Kinetics of ^{15}N depletion in plasma proteins after a ^{15}N diet switch (see Material and Methods) in Charolais fattening bulls fed either Normal or High protein diets. Compared to animals fed Normal protein diets (n = 18), animals fed High protein diets (n = 18) showed lower plasma protein $\delta^{15}N$ 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 adequatly 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 (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

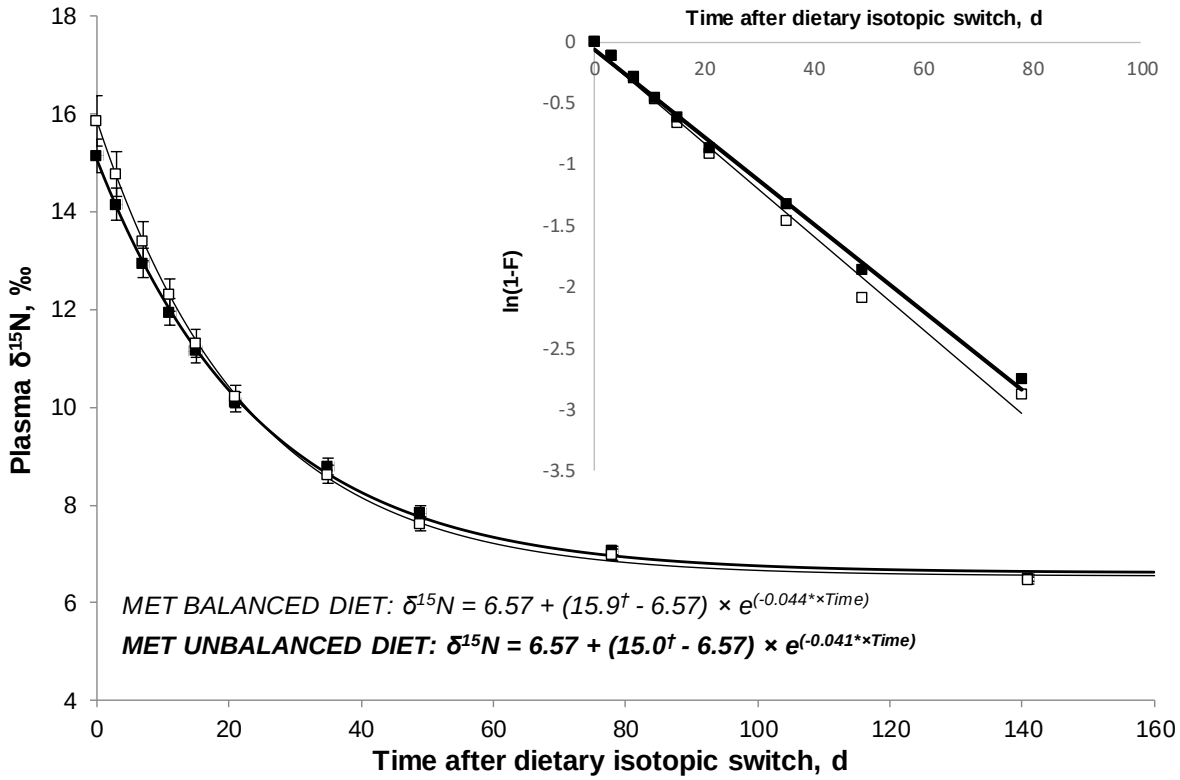

Figure 4. Kinetics of ^{15}N depletion in plasma proteins after a ^{15}N diet switch (see Material and Methods) in Charolais fattening bulls fed either balanced or unbalanced diets in terms of methionine content. Compared to animals fed diets unbalanced in methionine (n = 18), animals fed diets balanced in methionine (n = 18) showed higher plasma protein δ^{15}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 adequatly 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 ($^{\dagger}P < 0.10$; $^{*}P < 0.05$).

APPENDIX (Figure A1): Modelling the kinetics of ^{15}N depletion 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 adequatly fit the data A) Mono-exponential model (like in plasma proteins) where $\delta^{15}N$ kinetics are adequately fitted according to $\delta^{15}N(t) = \delta^{15}N_\infty + (\delta^{15}N_0 - \delta^{15}N_\infty) \times e^{-k \times t}$ and a single slope is noted when the logarithm of the reaction progress variable ($1\text{-}F = (\delta^{15}N(t) - \delta^{15}N_\infty)/(\delta^{15}N_0 - \delta^{15}N_\infty)$) is regressed on time. B) Bi-exponential model (like in urines) where $\delta^{15}N$ kinetics are adequately fitted according to $\delta^{15}N(t) = \delta^{15}N_\infty + (\delta^{15}N_0 - \delta^{15}N_\infty) \times [p \times e^{-k_1 \times t} + (1\text{-}p) \times e^{-k_2 \times t}]$ 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.