PLASMA UREA NITROGEN AS AN INDICATOR OF AMINO ACID IMBALANCE IN RABBIT DIETS

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Abstract: In recent decades, recommendations on dietary protein content have been considerably reduced, while fibre content has been increased. Under these conditions, an adequate dietary amino acid balance could be crucial to optimise feed efficiency. Plasma urea nitrogen (PUN) level could be a good indicator of an amino acid imbalance and its potential has already been studied in other species, but not yet in rabbits. The main objective of the present work was to detect the possible interest of PUN in pinpointing amino acid deficiencies in rabbits. Two experimental diets were formulated from the same basal mixture, following all the recommendations for growing rabbits, except lysine, whose content was variable, following current guidelines in diet P8.1 or lower from those in P4.4 (with 8.1 and 4.4 g/kg dry matter of lysine and with 757 and 411 mg of lysine per MJ of digestible energy). Three different trials were designed: one where the animals were fed *ad libitum* (AL) and two others in which fasting periods of 10 h were included; one where feeding was restored at 08:00 h (Fast8h) and the other at 18:00 h (Fast18h). A total of 72 three-way crossbred growing rabbits (24 animals for each trial in a split-plot trial) up to a total of 12 recordings were used. Blood samples were taken every 4 h in AL trial and every hour after refeeding up to a total of six controls, in trials Fast8h and Fast18h. The differences between balanced and unbalanced diets in lysine were highest (*P*<0.001) between 04:00 h and 12:00 h in animals fed *ad libitum*, and at 3 h after refeeding (21:00 h) in Fast18h. These results suggest that PUN could be an adequate indicator to detect deficiencies in amino acids in growing rabbit diets.

Key Words: rabbit, amino acid, plasma urea nitrogen, feed intake.

INTRODUCTION

In rabbit production, genetic improvement of feed conversion ratio (FCR) has been performed indirectly by selection for average daily gain (ADG), as there is a negative genetic correlation between these two parameters (Blasco, 1989). In consequence, paternal lines consisting of animals with high ADG are now available (Baselga, 2004), and it is possible that their nutritional requirements may have been modified. In order to ensure the full expression of their genetic growth potential, diets must cover these demands for growth.

To cut feeding costs, the intention is to reduce FCR. In fact, FCR is still the factor that has the greatest economic weight in rabbit production (Cartuche *et al.,* 2014; Gidenne *et al.,* 2017). To optimise this index, and to avoid deficits associated with the lack of some limiting amino acid, a level of protein slightly above requirements has traditionally been provided in commercial growing diets. However, recommendations on the dietary protein content have been considerably reduced in recent decades (Carabaño *et al.,* 2009), while fibre content has been increased (Trocino *et al.,* 2013), with the aim of optimising diets, mainly to reduce the risk of digestive disorders associated with an increased ileal nitrogen flow (Maertens, 2009; Carabaño *et al.,* 2009 and 2010), but also minimising the excretion of nitrogen into the environment (Maertens *et al.,* 2005; Romero *et al.,* 2009).

As a consequence of this dietary protein limitation, selection by ADG could have been penalised (Marín-García *et al.,* 2019), affecting the phenotypic expression of high ADG animals and consequently the definition of the rankings. In fact, although FCR has improved considerably, differences in FCR between maternal and paternal lines described several years ago are quite similar to those currently observed (Marín-García et al, 2019; Feki *et al.,* 1996). This possible protein deficit was suggested in a previous study, where animals with high ADG (>55 g/d) fed according to current protein recommendations had lower body protein retention than expected as a function of their ADG (Marín-García *et al.,* 2016).

The knowledge of protein nutrition in rabbits is scarce in comparison to other species (de Blas *et al.,* 1998; Carabaño *et al.,* 2009; Villamide *et al.,* 2013), which is why the development of a suitable method to achieve this aim or to detect dietary amino acid imbalances would be very helpful. Formerly, amino acid requirements were determined by dose-response trials (de Blas *et al.,* 1998; de Blas and González-Mateos, 2010), where the optimal amino acid level recommendation will be the one resulting in the best performance (i.e., ADG).

PUN level, which corresponds to the amount of nitrogen in the form of urea circulating in the bloodstream, could be a good indicator of an amino acid imbalance. In theory, a diet with a deficiency in any essential amino acid would lead animals to catabolise the remaining amino acids, increasing the urea production in the liver that would be released into the bloodstream to further excretion, which would lead to an increase in PUN compared to a well-balanced diet (basal PUN from protein turnover). In fact, the potential interest of this parameter has already been studied in other species, such as pigs (Brown and Cline, 1974) or broilers (Donsbough *et al.,* 2010).

PUN level depends not only on the dietary amino acid imbalance, but also on the protein intake and the balance between hepatic production and renal excretion. However, there are no studies on the circadian evolution of this metabolite in rabbits, or on its possible use as an indicator of potential amino acid imbalances in this species.

Therefore, the main objectives of the present work were to study the circadian evolution of PUN, to evaluate its possible interest for the detection of amino acid deficiencies, and establish the appropriate methodology to optimise its use for this purpose in growing rabbits.

MATERIAL AND METHODS

The experimental protocols were approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (Boletín Oficial del Estado, 2013).

Experimental diets

Two experimental diets were formulated and pelleted from the same basal mixture. The first (P8.1) was a diet formulated following the recommendations for growing rabbits (de Blas *et al.,* 2010) including 8.1 g of lysine per kg of dry matter (DM) by adding L-lysine HCl (4.7 g per kg). The second diet (P4.4) had no L-lysine HCl added, so its lysine content was far from the current recommendations (4.4 g of lysine per kg of DM), ensuring a dietary amino acid imbalance associated with a lysine deficit and with 757 mg and 411 mg of Lysine per MJ of digestible energy. The ingredients of the basal mixture and the chemical composition of the experimental diets are summarised in Table 1.

Animals and experimental procedure

Three different trials were designed, as summarised in Figure 1. The first of these trials was performed with animals fed AL. This trial would allow us to determine the circadian evolution of the PUN in animals consuming diets hypothetically balanced or unbalanced, perhaps linked to feeding behaviour of the animal. Unlike other species, where animals eat only in 1 or 2 daily meals, growing rabbits are characterised by having many small daily events (Gidenne *et al.,* 2010; Bellier *et al.,* 1995; Prud'hon *et al.,* 1975). Consequently, in order to reduce individual variability in feeding behaviour, two other trials were designed in which fasting periods of 10 h were imposed, one where feeding was restored at 08:00h (Fast8h) and the other at 18:00 h (Fast18h).

Table 1: Ingredients and chemical composition of the experimental diets (P8.1 and P4.4)

Ingredients (g/kg)	Basal mixture	Chemical composition (g/kg dry matter)	P8.1[2]	P4.4[2]
Wheat grain	288	Dry matter	912	
Sunflower meal	165	Ash	88	
Soybean oil	40	Crude protein	158	
Cereal straw	83	Ether extract	61	
Alfalfa hay	360	Starch	183	
Defatted grape seed	45	Neutral detergent fibre	366	
L-Lysine HCl	0	Acid detergent fibre	216	
DL-Methionine	1.55	Acid detergent lignin	57	
L-Threonine	2.2	Digestible energy[3]	10.7	
L-Arginine	1.45	Digestible protein[3]	109	
Dicalcium phosphate	3.6	Amino acid composition:		
Sodium chloride	5.2	Aspartic acid	13.4	
Vitamin/mineral mixture[1]	5	Serine	60.1	
		Glutamic acid	20.9	
		Glycine	70.8	
		Histidine	2.51	
		Arginine	8.10	
		Threonine	6.88	
		Alanine	5.13	
		Proline	8.57	
		Cystine	1.56	
		Tyrosine	2.67	
		Valine	6.73	
		Methionine	3.23	
		Isoleucine	5.13	
		Lysine	8.10	4.40
		Leucine	8.80	
		Phenylalanine	6.47	

[1] Contains per kg of feed: vitamin A: 8375 IU; vitamin D3: 750 IU; vitamin E: 20 mg; Vitamin K3: 1 mg; vitamin B1: 1 mg; vitamin B2: 2 mg; vitamin B6: 1 mg; nicotinic acid: 20 mg; choline chloride: 250 mg; magnesium: 290 mg; manganese: 20 mg; zinc: 60 mg; iodine: 1.25 mg; iron: 26 mg; copper: 10 mg; cobalt: 0.7 mg; butyl hydroxylanysole and ethoxiquin mixture: 4 mg.
[2] P8.1: Basal mixture added with 4.7 g of L-Lysine HCl; P4.4: Basal mixture
[3] Calculated values from FEDNA (2010).

A total of 72 three-way crossbred growing rabbits (H×LP does inseminated with pooled semen from R bucks; lines H, LP and R from Universitat Politècnica de València, Spain, weaned at 28 d) were used, 24 animals for each trial. At 49 d of age (2225±22.6 g), animals were housed in individual cages (26×50×31 cm), kept at 10°C to 22°C throughout the experimental period (February) and under a photoperiod of 12 hours of light (06:00 to 18:00 h) and 12 h of darkness. Animals were fed using a commercial diet and no antibiotics in feed or water were used during the experiment. After one week of adaptation to the cage, one day before the first control (56 d of age), the animals were randomly divided into two groups and each group received one of the two experimental diets. The following day (57 d of age), feed intake was monitored and blood samples were taken from the central ear artery (1 mL in EDTA vials) every 4 h in the AL trial (08:00, 12:00, 16:00, 20:00, 00:00 and 04:00 h), and every hour after refeeding, up to a total of six controls, in the Fast8h (08:00, 09:00, 10:00, 11:00, 12:00 and 13:00 h) and Fast18h (18:00, 19:00, 20:00, 21:00, 22:00 and 23:00 h) trials. At 08:00 h on the following day (58 d of age), the animals were switched to the other experimental diet and, after a day of adaptation to the new diet, the protocol described for day 57 for feed intake monitoring and blood sampling was repeated on day 59. Experimental diets were introduced just one day
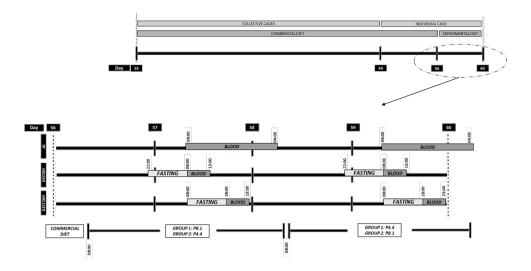
Figure 1: Representation of experimental design of the three trials. Blood sampling and control of feed intake were performed every 4 hours in AL trial and every hour in Rest8h and Rest18h trials. P8.1 and P4.4 are diets with 8.1 and 4.4 g of lysine per kg of dry matter, respectively.

before controls to avoid adaptation mechanisms. Blood samples were immediately centrifuged for five min at 700 *g*, and the supernatant plasma was frozen at –20°C until further analysis.

Chemical analysis

Determination of PUN was performed using a commercial kit (Urea/BUN-Color, BioSystems S.A., Barcelona, Spain). The samples were defrosted and tempered, after which 1 µL was pipetted into test tubes (a standard and a blank were included in each batch). Later, 1 mL of reagent A (sodium salicylate 62 mmol/L, sodium nitroprusside 3.4 mmol/L, phosphate buffer 20 mmol/L and urease 500 U/mL) was added to each sample, mixed thoroughly and incubated for 5 min at 37°C. Subsequently, 1 mL of reactant B (sodium hypochlorite 7 mmol/L and sodium hydroxide 150 mmol/L) was added, mixed thoroughly and incubated for a further 5 min at 37°C. Finally, the absorbance of each sample was read at 600 nm against the blank.

Chemical analyses of diets were performed following the Association of Official Agricultural Chemists' methods (AOAC, 2000): 934.01 for DM, 942.05 for ash, 976.06 for crude protein, and 920.39 with previous acid hydrolysis of samples for ether extract. Starch content was determined according to Batey (1982), by a two-step enzymatic procedure with solubilisation and hydrolysis to maltodextrins with thermostable α-amylase, followed by complete hydrolysis with amyloglucosidase (both enzymes from Sigma–Aldrich, Steinheim, Germany), and the resulting glucose was measured by the hexokinase/glucose-6 phosphate dehydrogenase/NADP system (R-Biopharm, Darmstadt, Germany). Neutral detergent fibre, acid detergent fibre and acid detergent lignin were analysed sequentially according to (Mertens, 2002), method 973.18 (AOAC, 2000) and Van Soest *et al.* (1991), respectively, with a thermostable α-amylase pre-treatment and expressed exclusive of residual ash, using a nylon filter bag system (Ankom, Macedon, NY, USA).

The amino acid content in diets was determined after acid hydrolysis with HCl 6N at 110°C for 23 h as previously described by Bosch *et al.* (2006), using a Waters high performance liquid chromatography system (Milford, MA, USA) consisting of two pumps (Mod. 515, Waters), an autosampler (Mod. 717, Waters), a fluorescence detector (Mod. 474, Waters) and a temperature control module. Aminobutyric acid was added as an internal standard after hydrolysis. Amino acids were derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and separated with

Table 2: *Ad libitum* trial (average of the two experimental diets): Feed intake during the previous four hours and plasma urea nitrogen (PUN) according to the time of day (least square means±standard errors).

	Time of the day						
	08:00 h	12:00 h	16:00 h	20:00 h	00:00 h	04:00 h	*P-value*
Feed intake (g/h)	4.84±0.33[a]	6.02±0.33[b]	8.03±0.33[c]	10.86±0.33[d]	8.91±0.33[c]	6.48±0.33[b]	<0.001
PUN (mg/dL)	13.44±0.46[a]	13.95±0.45[a]	14.26±0.46[ab]	15.85±0.46[c]	15.39±0.45[bc]	15.31±0.45[bc]	0.001

[a,b,c,d] Least square means in the same row with no common superscripts differ significantly at *P*<0.05.

a C-18 reverse-phase column Waters Acc. Tag (150×3.9 mm). Methionine and cystine were determined separately as methionine sulphone and cysteic acid, respectively, after performic acid oxidation followed by acid hydrolysis.

Statistical analysis

The PUN and previous feed intake data were statistically analysed with the GLM procedure of SAS, (2002). For each of the three changeover trials (balanced Latin square), the model included as fixed effects the animal (each of the 24 animals becomes a block), the control day (57 and 59 d of age), the experimental diet (P8.1 and P4.4) and time of day (6 levels), as well as the interaction of the experimental diet with the time of day. Least square means were obtained, with their standard errors, and compared using t-test, defining significance level at *P*<0.05. Orthogonal contrasts between experimental diets were also performed (t-test).

RESULTS

Ad libitum trial

Table 2 shows the daily evolution of feed intake and PUN every 4 h in the AL trial. Feed intake was the highest from 16:00 to 20:00 h (being 24% of daily intake), decreasing gradually before and after this period until 04:00 to 08:00 h, when it was the lowest (11% of the daily intake). PUN level showed a similar trend to feed intake, reaching the highest value at 20:00 h and the lowest at 08:00 h (a reduction of 15%).

Table 3 shows the average feed intake, PUN and PUN/intake ratio according to the experimental diet in the AL trial. Those animals fed with unbalanced P4.4 diet had lower intake (−11%), higher PUN (+16%), and higher PUN/intake ratio (+0.61±0.09 mg h/dL g) than those consuming the diet formulated according to the recommendations (P8.1).

Figure 2 shows the circadian evolution of feed intake according to the experimental diet. No significant differences between diets were observed from 00:00 h to 12:00 h. Differences appeared from 12:00 to 00:00 h, when feed intake of animals increased, being 19.7% higher with P8.1 than with P4.1 (*P*<0.001).

Figure 3 shows the circadian evolution of PUN (Figure 3a) and PUN/intake ratio (Figure 3b) for the two experimental diets. Differences between diets were significant (*P*<0.05) only at 04:00, 08:00 and 12:00 h for PUN and at 00:00, 08:00 and 12:00 h for PUN/intake ratio. Maximum differences between diets were observed at 08:00 h for both traits.

Table 3: *Ad libitum* trial (average of all sampling times): Feed intake during the previous 4 h) and plasma urea nitrogen (PUN) according to experimental diet, P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively (least square means±standard errors)

	P8.1	P4.4	*P*-value
Feed intake (g/h)*	7.96±0.19	7.09±0.19	0.002
PUN (mg/dL)	13.61±0.26	15.75±0.26	<0.001
PUN/intake ratio (mg h/dL g)	1.99±0.09	2.60±0.09	<0.001

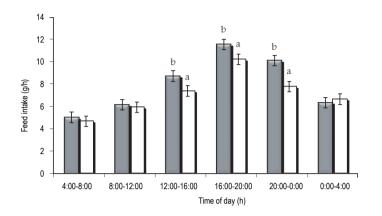* Significant interaction experimental diet and time of day (*P*<0.05).

Figure 2: *Ad libitum* trial: Feed intake (average during the previous 4 h) according to time of day and experimental diet (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) (least square means±standard errors). Bars for the same time of day with different letters differ significantly (*P*<0.05). ▨ P8.1, ▢ P4.4.

Fasting trials

Figure 4 represents the evolution of PUN when refeeding the animals after 10 h of fasting before 08:00 h (Fast8h) or 18:00 h (Fast18h) with the experimental diets. The PUN values increased after refeeding in both fasting trials and maximum values were recorded 3 or 4 h later. In Fast8h, PUN was only significantly higher for animals fed with P4.4 compared to those with P8.1 in the sampling performed 2 h after refeeding. In contrast, PUN was always significantly (*P*<0.05) higher in animals fed with P4.4 in trial Fast18h, this difference being maximum 3 h after refeeding (+11%).

DISCUSSION

Ad libitum trial

Total feed intake during the 24-h period (57 d of age) averaged 181 g, 13% higher than that previously reported for the 9th week of age (Bellier *et al.,* 1995; Prud'hon *et al.,* 1975). Moreover, the circadian distribution pattern of feed
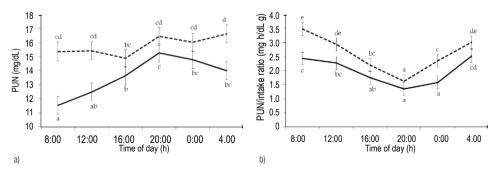

Figure 3: *Ad libitum* trial: Circadian evolution of (a) plasma urea nitrogen (PUN) and (b) PUN/intake ratio (dividing by feed intake during four hours before blood sampling) with the experimental diets (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) (least square means±standard errors). Values having different letters differ significantly (*P*<0.05). —— P8.1, ---- P4.4.
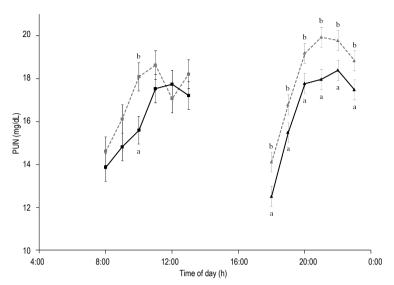
Figure 4: Fasting trials: Evolution of plasma urea nitrogen (PUN) of animals fed with the experimental diets (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) after 10 h of fasting before 8:00 h (Fast8h trial, left lines) or 18:00 h (Fast18h trial, right lines) (least square means±standard errors). Values for the same time of day and trial with different letters differ significantly (*P*<0.05). --■-- P4.4 Fast8h, –■– P8.1 Fast8h, --▲-- P4.4 Fast18h, –▲– P8.1 Fast18h.

intake was quite similar to that observed in earlier studies (Bellier *et al.,* 1995; Prud'hon *et al.,* 1975). These results suggest that the experimental management did not importantly affect feed intake.

In the case of the balanced diet, the circadian evolution of PUN followed a pattern very similar to that of feed intake (Figures 2 and 3a). It is consistent with PUN values depending on dietary protein quantity, as the higher the protein intake, the higher the PUN (Eggum, 1970), due to the catabolism of a greater amount of leftover amino acids.

On the other hand, PUN is also inversely related with dietary protein quality (Eggum, 1970). It is well known that a dietary deficiency in some essential amino acid results in a diversion of the excess of amino acids not used in protein synthesis towards catabolic pathways and, consequently, an increase in PUN. Accordingly, this parameter has been used to estimate the essential amino acid requirements, mainly in swine (Taylor *et al.,* 1982, 1985; Coma *et al.,* 1995, 1996). In the AL trial, the average PUN in growing rabbits receiving the P4.4 diet, with a lysine level 46% below the current recommendation, was 16% higher than in those receiving the P8.1 diet. Moreover, PUN values induced by diet P4.4 were continuously high throughout the day, probably because these values (averaging 15.75±0.26 mg/dL) represent a threshold to increase renal urea excretion for homeostasis when feed intake is dispersed over a day. Consequently, the differences in PUN between both diets were only significant from 04:00 to 12:00 h (averaging +2.35±0.52 mg/dL, *P*<0.01). This period could be appropriate for evaluating amino acid deficiencies in diets for growing rabbits under ad libitum feeding.

An essential amino acid deficiency also results in a reduction in feed intake (Forbes, 2007). Feed intake decrease with an unbalanced diet was also observed in the current trial from 12:00 to 00:00 h, when feed intake was higher (Figure 2). As feed intake could affect PUN and vice versa, some authors express PUN values per unit of feed intake (Brown and Cline, 1974). However, in the current trial, PUN/feed intake ratio showed higher residual variability than PUN (coefficient of variation of 47 and 21%, respectively) and the inclusion of previous feed intake as a covariate had no significant effect on PUN. Therefore, this correction did not improve the ability to differentiate between diets.
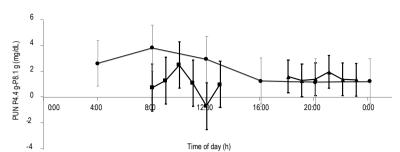
Figure 5: Evolution of the difference in plasma urea nitrogen (PUN) between the two experimental diets (P4.4 – P8.1; with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) during the different trials: Ad libitum trial and after a fasting of 10 h before 08:00 h (Fast8h) and at 18:00 h (Fast18h) (least square means and confidence interval). Values which confidence interval do not trespass the X-axis were significantly different from zero. —■— Fast8h, —▲— Fast18h, —●— Ad libitum.

Fasting trials

Mean values obtained for PUN were higher after fasting (averaging 17.7±0.13 mg/dL) than those observed *ad libitum* (averaging 14.7±0.18 mg/dL), probably because of very high feed intake during the first hour after refeeding (averaging 19.2±0.63 g), 77% higher than the maximum recorded in the AL trial (from 16:00 to 20:00 h). Moreover, PUN after fasting was higher in the Fast18h trial than in the Fast8h trial (18.2±0.15 mg/dL *vs.* 17.1±0.21 mg/dL respectively), as well as feed intake during the first hour after refeeding (22.8±0.94 g *vs.* 15.7±0.85 g, respectively), as expected, as the alimentary behaviour of growing rabbits is not uniform throughout the day, showing higher feed intake as evening approaches and lower in the morning, when they are practising caecotrophy (Prud'hon *et al.,* 1975; Bellier *et al.,* 1995; Hirakawa, 2001). These findings support the influence of feed intake on PUN mentioned above and suggest that overeating could induce an important transitory increase of PUN.

On the other hand, the PUN pattern after refeeding was similar to that described for pigs, where PUN increases for the first 3-4 h after feeding and thereafter reaches a plateau (Eggum, 1970). In fact, blood sampling for PUN determination in swine is recommended 3 h after feeding (Roth-Maier *et al.,* 2003; Scheinder *et al.,* 2006).

Figure 5 shows the evolution of PUN difference between the two experimental diets (P4.4 – P8.1; with 8.1 and 4.4 g of lysine per kg of DM, respectively) during the different trials. When animals fasted during the night (Fast8h trial), PUN was higher with diet P4.4 only 2 h after refeeding (+2.50±0.92 mg/dL; *P*=0.007). Differences between diets were unclear, probably because the effect of lysine deficiency could have been counteracted to a great extent by the higher feed intake recorded during the first hour after refeeding with diet P8.1 compared to diet P4.4 (18.5±1.21 g *vs.* 12.8±1.21 g, +47%; *P*<0.001), as well as by the practice of caecotrophy during the morning hours, as microbial protein from soft faeces are rich in this essential amino acid. Moreover, the possible asynchrony of this practice among individuals could also explain the high residual variability in PUN (SE for each diet in each time equal to 0.66 mg/dL). As a result, this protocol to determine PUN failed to improve the accuracy in assessing the amino acid imbalance.

In contrast, when animals fasted during the day (Fast18h trial), PUN was always higher with diet P4.4 (+1.5±0.27 mg/dL; *P*<0.01), and the difference was maximum 3 h after refeeding (+1.96±0.66 mg/dL; *P*=0.003). In addition, residual variability was lower than in the Fast8h trial (SE for each diet in each time equal to 0.46 mg/dL). Differences between diets were clear, probably because the effect of deficiency in lysine was not disturbed by differences between diets in feed intake during the first hour after refeeding (22.3±1.33 g *vs.* 23.3±1.33 g; *P*>0.05, for P8.1 diet and P4.4 diet, respectively) and by the interference of caecotrophy (Nicodemus *et al.,* 1999). Thus, fasting during the day might allow the maximising, homogenising and synchronising of feed intake in growing rabbits just after refeeding, while reducing residual variability in PUN.

CONCLUSIONS

In view of these results, it can be concluded that PUN could be an adequate indicator to detect deficiencies in amino acids in growing rabbit diets. The differences between balanced and unbalanced diets in lysine were highest between 04:00 and 12:00 h in animals fed *ad libitum*, and at 3 h after refeeding (21:00 h) when a fasting period of 10 h was applied.

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