LYSINE, SULPHUR AMINO ACIDS AND THREONINE REQUIREMENTS OF GROWING RABBITS FROM A LINE SELECTED BY GROWTH RATE

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I started it tomorrow but I will finish yesterday

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GP por apostar por mí sobre todas las cosas. Haré que estés orgulloso.
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GP existir en un futuro.
GP por hacerme sentir que fui el mejor regalo que te hicieron los papás.Continúa con ese corazón.Eres única!
GP haber sido.
GP alegrarnos la existencia. Intentaré devolverte toda la felicidad que nos das.
GP tener esa alma festivalera, aportándome siempre la frescura que necesito. Eres eterno verano.
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GP esas fiestas imposiblemente locas.
GP ser mi tía/hermana. Lo que parece amor/odio, es solo amor.
GP no llamar al 112 cuando relativizo sobre el espacio-tiempo. Espero que esto siga.
GP coger aire fresco a >3000m.
GP saber entender que tenía que devorar los libros y quererme igual, o más. Catalán!
GP demostrarme que un océano no es absolutamente nada.
GP enseñarme que la amistad tiene un nombre: Sara.
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GP tu apoyo incondicional en todo el trabajo que llevamos.
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GP seguir existiendo.
GP volverme más loco. Por las noches temáticas. Por soltar cuando aún estábamos a tiempo.

Me he dejado un GP, no podía resumirte en una sola línea. Escribiera lo que escribiera siempre me sonaba pobre (será quizás la presión de la tesis). Quizás cualquier madre se hubiera desesperado cuando su hijo (con 6 meses) mandaba a su padre al hospital por alguna "trastada" (como lo llamabas tú), o quizás, se hubiera molestado cuando tiraba maderas a todos en las actuaciones de navidad, o cuando nadie se quería quedar con su hijo porque tenían que estar atentos… o se hubiera desanimado cuando suspendía hasta el patio y te decían aquello: "estudiar no es lo suyo…". Quizás se hubiesen cansado de contratar al profesor particular número 10^9 (el cual no conseguía que estudiara)… Quizás, otras se hubiesen enfadado (más) cuando sus hijos se llenaban de piercings…y tatuajes… Quizás hubieran puesto pegas para que se fueran de erasmus, quizás otras no hubieran aguantado que su hijo sea un viajero de corazón. Quizás otras madres hubieran forzado a sus hijos a ver la vida con sus mismos ojos. Quizás nadie podría haber custodiado mejor mi locura. Quizás yo soy yo, gracias a ti. Quizás porque, aunque todos lo crean, la mejor madre del mundo la tengo yo. (Ahora puedes quitar los quizás).

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ABSTRACT

Studies on protein nutrition in rabbits are scarce compared with other species, the estimation of their requirements is relatively old and no data are reported at ileal level. Moreover, their peculiar feeding behaviour, including caecotrophy, complicates more this study. In addition, in recent years, a relationship has been established between dietary crude protein and Epizootic Rabbit Enteropathy (one of the most important disease that affects this species). As a consequence, the protein content of diets has been reduced. On the other hand, there has been a loss of effectiveness of the selection for growth rate in the paternal lines, which could be related with a possible nutritional deficit in high growth rate rabbits when using the current commercial diets. Plasma urea nitrogen (PUN) could be a good indicator of an amino acid imbalance, but its use has not yet been tested in rabbits. Thus, the main hypothesis of this thesis concerns that growing rabbits selected by growth rate could have amino acid requirements different from current recommendations, and that another amino acid combination would maximize productive traits. To evaluate this hypothesis, a total of 1464 growing rabbits were used (mainly from the R line, a paternal line selected by growth rate, and, to a much lesser extent, from some other maternal lines with lower growth rates), 32 diets were formulated differing in the content of the three typically limiting amino acids (lysine, sulfur amino acids and threonine). Faecal and ileal digestibility trials were performed with 69 and 71 animals, respectively. Nutrient retention during their growing period was evaluated in 126 animals. Finally, PUN was analyzed in more than 2700 samples. From the first trial,

we obtained indication about the possible existence of some limiting amino acid when current moderate protein diets are used in growing rabbits with high growth rates, as retained protein to retained energy ratio was clearly lower for animals of the paternal lines (this suggested a deviation of a certain proportion of protein as a source of energy, which is inefficient). Before considering what combination of amino acids would improve their utilization, it was necessary to evaluate the possible interest of PUN to detect deficiencies in amino acids and establish the appropriate methodology to optimize its use for this purpose in growing rabbits. In the second study we verified the applicability of PUN in rabbits and checked the highest differences between diets: from 04:00h to12:00h in animals fed *ad libitum* and at three hours after refeeding (21:00h) when a fasting period of 10 h was applied. At this point, knowing that amino acid deficiency could exist and that PUN could be an indicator of it, we formulated a matrix of 27 diets (in a 3x3x3 factorial design, with three levels of the three amino acids mentioned above, using the current recommendations and increasing or decreasing them by 15%), searching for the combination minimizing PUN values and, hypothetically, improving the protein assimilation. From this trial, the diet MHL (8.1, 6.6 and 5.7 g/kg dry matter of lysine, sulphur amino acids and threonine, respectively) emerged as a candidate to replace the current recommendations (diet MMM; 8.1, 5.8 and 6.9 g/kg dry mater of lysine, sulphur amino acids and threonine, respectively). However a question arises: could this new combination improve productive traits? Finally, in the last study we verified the usefulness of this combination since it significantly improved both the growth rate and the feed conversion ratio. After digestibility

assessment, we propose levels of 5.3, 4.3 and 2.9 g/kg dry matter of apparent ileal digestible lysine, sulphur amino acids and threonine, respectively. These data are intended to improve current knowledge on protein nutrition and move towards precision protein nutrition.

RESUMEN

Los estudios sobre nutrición proteica en conejos son escasos en comparación con otras especies, el cálculo de las necesidades es relativamente antiguo, no están ofrecidos a nivel ileal y sus peculiares hábitos alimenticios complican su conocimiento. Además, en los últimos años, se ha establecido relación directa entre este nutriente con una de las principales enfermedades que afectan esta especie (la Enteropatía Epizoótica del Conejo), y como consecuencia, el contenido proteico de las dietas se ha reducido. Por otro lado, se ha comprobado una pérdida en la efectividad de la selección por velocidad de crecimiento en las líneas paternales la cual podría estar relacionada con una mala estimación de los requerimientos proteicos (especialmente en los animales con elevadas velocidades de crecimiento). Finalmente, el nitrógeno ureico plasmático (PUN) podría ser una buena herramienta para estimar el grado de aprovechamiento proteico del pienso, pero su uso no ha sido comprobado aún en esta especie. Con todo ello, la principal hipótesis de esta tesis es que existe una mala estimación de las necesidades de aminoácidos en conejos con elevadas velocidades de crecimiento y que otra combinación de aminoácidos podría maximizar su rendimiento. Para evaluar esta hipótesis se utilizaron un total de 1464 conejos de engorde (provenientes principalmente de la línea R, línea paternal seleccionada por velocidad de crecimiento, y, en mucha menor medida, de líneas maternales con menores velocidades de crecimiento), se formularon un total de 32 dietas (donde principalmente diferían el contenido de los tres aminoácidos típicamente limitantes; lisina, aminoácidos azufrados y treonina), se

realizaron 69 y 71 ensayos de digestibilidad fecal e ileal, respectivamente, se valoró la retención de nutrientes de 126 animales durante su crecimiento y por último se analizaron los niveles de PUN de más de 2700 muestras. Con el primero de los trabajos obtuvimos la confirmación del primer indicio: las dietas actuales parecían presentar deficiencias en aminoácidos para los conejos con altas velocidades de crecimiento, comprobando que aquellos animales que crecían más depositaban proporcionalmente más energía y menos proteína (esto sugería una desviación de cierta proporción de proteína como fuente de energía, siendo poco eficiente). Antes de plantearnos qué combinación de aminoácidos mejoraría este problema, tendríamos que poner a punto la técnica para detectar dichas deficiencias. En el segundo trabajo no solo comprobamos que el PUN podría ser utilizado, en esta especie, sino que se estableció la mejor metodología: A las 08:00h (bajo una alimentación *ad libitum*) o a las 21:00h (bajo un ayuno de 10h y un restablecimiento del alimento a las 18:00h). Ahora ya, sabiendo que podría existir dicha deficiencia y que además podemos utilizar el PUN, seleccionamos de una matriz de 27 piensos (donde habían tres niveles de lisina, azufrados y treonina), aquel que optimizaba los valores de PUN y que previsiblemente mejoraría la asimilación proteica. De este experimento surge la Dieta MAB (8.1, 6.6 y 5.7 g/kg materia seca de lisina, aminoácidos azufrados y treonina, respectivamente) como candidato a sustituir a las actuales recomendaciones, Dieta MMM (8.1, 5.8 y 6.9 g/kg materia seca de lisina, aminoácidos azufrados y treonina, respectivamente). Pero hasta ahora solo podíamos decir que el MAB reduce los nivele de urea: ¿Está esto relacionado con unos mejores parámetros productivos? Finalmente en el último trabajo se comprobó la superioridad productiva de esta combinación, ya que mejoró significativamente tanto la velocidad de crecimiento como el índice de conversión. Tras la valoración digestiva, podemos aconsejar unos niveles de 5.3, 4.3 y 2.9 g/kg de materia seca para la lisina, aminoácidos azufrados y treonina a nivel ileal aparente, arrojando más información sobre la nutrición proteica de precisión y pretendiendo atajar la problemática que nos planteábamos al inicio de la tesis.

RESUM:

Els estudis sobre nutrició proteica en conills són escassos en comparació amb altres espècies, el càlcul de les necessitats és relativament antic, no estan oferts a nivell ileal i els seus peculiars hàbits alimentaris compliquen el seu coneixement. A més, en els últims anys, s'ha establert relació directa entre aquest nutrient amb una de les principals malalties que afecten aquesta espècie (la enteropatia epizoótica del Conill), i com a conseqüència, el contingut proteic de les dietes s'ha reduït. D'altra banda, s'ha comprovat una pèrdua en l'efectivitat de la selecció per velocitat de creixement en les línies paternals la qual podria estar relacionada amb una mala estimació dels requeriments proteics (especialment en els animals amb elevades velocitats de creixement). Finalment, el nitrogen ureic plasmàtic (PUN) podria ser una bona eina per estimar el grau d'aprofitament proteic del pinso, però el seu ús no ha estat comprovat encara en aquesta espècie. Amb tot això, la principal hipòtesi d'aquesta tesi és que hi ha una mala estimació de les necessitats d'aminoàcids en conills amb elevades velocitats de creixement i que una altra combinació d'aminoàcids podria maximitzar el seu rendiment. Per avaluar aquesta hipòtesi es van utilitzar un total de 1464 conills d'engreix (provinents principalment de la línia R, línia paternal seleccionada per velocitat de creixement, i, en molta menor mesura, de línies maternals amb menors velocitats de creixement), es van formular un total de 32 dietes (on principalment diferien el contingut dels tres aminoàcids típicament limitants; lisina, aminoàcids ensofrats i treonina), es van realitzar 69 i 71 assajos de digestibilitat fecal i ileal, respectivament, es va valorar la retenció de

nutrients de 126 animals durant el seu creixement i finalment es van analitzar els nivells de PUN de més de 2700 mostres. Amb el primer dels treballs vam obtenir la confirmació del primer indici: les dietes actuals semblaven presentar deficiències en aminoàcids per als conills amb altes velocitats de creixement, comprovant que aquells animals que creixien més dipositaven proporcionalment més energia i menys proteïna (això suggeria una desviació de certa proporció de proteïna com a font d'energia, sent poc eficient). Abans de plantejar-nos què combinació d'aminoàcids milloraria aquest problema, hauríem de posar a punt la tècnica per detectar aquestes deficiències. En el segon treball no només vam comprovar que el PUN podria ser utilitzat, en aquesta espècie, sinó que es va establir la millor metodologia: A les 08: 00h (sota una alimentació ad libitum) oa les 21: 00h (sota un dejuni de 10h i a un restabliment de l'aliment a les 18: 00h). Ara ja, sabent que podria existir aquesta deficiència i que a més podem utilitzar el PUN, seleccionem d'una matriu de 27 pinsos (on havien tres nivells de lisina, ensofrats i treonina), aquell que optimitzava els valors de PUN i que previsiblement milloraria l'assimilació proteica. D'aquest experiment sorgeix la Dieta MAB (8.1, 6.6 i 5.7 g / kg matèria seca de lisina, aminoàcids ensofrats i treonina, respectivament) com a candidat a substituir les actuals recomanacions, Dieta MMM (8.1, 5.8 i 6.9 g / kg matèria seca de lisina, aminoàcids ensofrats i treonina, respectivament). Però fins ara només podíem dir que el MAB redueix els anivelli d'urea: Està això relacionat amb uns millors paràmetres productius? Finalment en l'últim treball es va comprovar la superioritat productiva d'aquesta combinació, ja que va millorar significativament tant la velocitat de creixement com l'índex de conversió. Després de la valoració digestiva, podem aconsellar uns nivells de 5.3, 4.3 i 2.9 g / kg de matèria seca per la lisina, aminoàcids ensofrats i treonina a nivell ileal aparent, llançant més informació sobre la nutrició proteica de precisió i pretenent tallar la problemàtica que ens plantejàvem a l'inici de la tesi.

INTRODUCTION

Genetic background

Rabbit meat production is based on a three-way crossing scheme (Baselga, 2004). Crossbred females of commercial farms are usually inseminated using semen of rabbit males coming from paternal lines as a terminal sire. The result of this crossbreed is the commercial growing rabbit. As litter size and feed conversion ratio (FCR) have been presented as the most important economical traits in rabbit farming, genetic progress on these traits are intended to be enhanced in maternal and paternal lines, respectively (Estany et al., 1992; Cartuche et al., 2014). The first of the two crosses is usually performed with two different maternal lines in order to obtain crossbred females with high heterosis and low inbreeding rates for the maternal traits (Estany et al., 1992; Cifre et al., 1998; Sánchez et al., 2008). Currently, maternal lines have about nine young born alive (Ragab, 2012), and there are evidences that rabbit selection programmes by litter size seem to work (Quevedo et al., 2006a). The second cross is performed with the crossbred females mentioned above and males coming from paternal lines. Paternal lines are usually selected for average daily gain (ADG) because a genetic negative correlation exists between ADG and FCR (Blasco, 1989). An adequate selection of rabbit males from paternal lines has a great impact on feed efficiency and competitiveness of the commercial farms (50 % of the genetic of commercial growing rabbits derives from these lines).

R line is the paternal line improved from the Universitat Politècnica de València. R line comes from the cross of two paternal lines, one founded in 1976 with California rabbits reared by Valencian farmers and other founded in 1981 with rabbits belonging to specialized paternal lines (Estany et al., 1992). The selection criteria used for this line from this moment was individual ADG from 28 to 63 d of age. As a consequence, ADG of this line is on average 55 g/d during the growing period. Although paternal lines have better growth traits than maternal lines [e.g. García-Quirós et al. (2014) observed better ADG (on av. +10.6 g/d) and FCR (on av. -0.25 g dry matter (DM)/g)], some studies have showed that the genetic progress in the paternal lines seems to be lower to that expected from the heritability of this trait being the phenotypical improvement observed one third of the expected in the last years (Costa et al., 2004; Quevedo et al., 2006; Project AGL2017-85162-C2-1-R, Marco-Jiménez and Pascual, 2017). These data are suggesting the lack on effectiveness of selection in these paternal lines, and a question arises: What could be affecting this genetic advance?

At this respect, there are different aspects that could be causing a lower selection progress: i) reaching to a biological threshold at the genetic selection level, ii) problems associated to a limited reproductive capacities in these lines or iii) a possible nutritional deficits in high growth rate rabbits with the current commercial diets for growing rabbit.

The first hypothetical cause (i) seems unlikely as some studies have indicated that there is still enough genetic variability to be allow a genetic progress on in rabbits (Baselga, 2004) and, furthermore, nowadays exist animals with really high growth rate (>87 g/d).

With respect to the second probable cause (ii), reproductive features are objectively lower in paternal lines. There are some studies where R line showed lower litter size (Gómez et al., 1999), ovulation rate (Cifre et al., 1994; Vicente et al., 2012), and fertility (Piles and Tussel, 2012) than maternal lines. If the offspring of a generation is not numerous enough, the response to selection could be unsatisfactory. This explanation seems more plausible than the previous one and there is a research line addressing to evaluate this hypothesis (Project AGL2017-85162-C2-1-R, Marco-Jiménez and Pascual, 2017).

Independently of the second cause, the third one (iii) seems to have a relationship with a lack of genetic response effectiveness. It would be logical to think that an animal with high growth rate has different nutrient requirements than other with less growth rate, especially on protein requirements, because there is a relationship between growth and protein requirements.

This PhD thesis has as main aim to explore this last aspect: Is there any nutritional deficit in animals with high growth rate during the growing period that could be affecting the right elaboration of the genetic rankings, and consequently the genetic progress?

General nutrition on growing rabbits

Nowadays, recommendations for each dietary nutrient of growing rabbits have been established. The concentration of a certain nutrient could be expressed as the quantity (g per kg, %, etc.) in the "fresh" diet or corrected by the dry matter. With the aim to unify all results, main data of this thesis has been presented in DM basis.

The current recommendations are based on a longer number of studies, which are usually summarized in recommendation tables, in order to facilitate feed formulation. Among them, one of the most used tables is that published in the book "Nutrition of the Rabbit" (2010), included in the chapter "Feed Formulation" (de Blas and González-Mateos, 2010), which will be our starting point in this thesis. As it can be seen in Table 0.1, the current recommendation for the digestible energy (DE) in growing rabbits is on average 11.3 MJ/kg DM. Respect to the fibre, 378, 211 and 56 g/kg DM are on average the recommendations for neutral detergent fibre, acid detergent fibre and acid detergent lignin, respectively. Although the knowledge about protein nutrition in rabbits is scarce in comparison to other species (Carabaño et al., 2000), current protein recommendations are stablished as on av. 167 and 116 g/kg DM of crude protein (CP) and digestible protein (DP), respectively.

Rabbits regulate feed intake mainly based on the dietary DE content. Thus, an increase in the DE level of the feed determines a decrease in ingestion and, on the contrary, a decrease in the level of DE of the feed is accompanied by an increase in its consumption (Gidenne et al., 2010). Is for this reason that seems to be more interesting to know the DP/DE ratio, and it is proposed as 9.5-10 g/MJ (Carabaño et al., 2009), 9.8-10.8 g/MJ (de Blas and González-Mateos, 2010) and 10.5-11 g/MJ (Xiccato and Trocino, 2010).

Table 0.1: Nutrient requirements of intensively reared growing rabbits summarized by de Blas and González-Mateos (2010)

Digstible energy (MJ/kg DM)	11.3
Crude protein (g/kg DM)	167 (158-178)
Digestible protein (g/kg DM)	116 (111-122)
Crude fibre (g/kg DM)	172 (167-178)
Neutral detergent fibre (g/kg DM)	378 (367- 389)
Soluble neutral detergent fibre (g/kg DM)	128
Acid detergent fibre (g/kg DM)	211 (200-222)
Acid detergent lignin (g/kg DM)	56
Starch (g/kg DM)	167 (156-178)
Ether extract (g/kg DM)	Free

However, in the last years there have been changes. Although some studies did not verify the favorable effect of dietary protein reduction on the health status of growing rabbits (Xiccato et al., 2004; García-Palomares et al., 2006; Trocino et al., 2013), there are many studies that related the excess of this nutrient with higher incidence of mucoid enteropathy (Chamorro et al., 2007; Gidenne et al., 2013; Martínez-Vallespín et al., 2011; Xiccato et al., 2011). For this reason, in last years dietary content of protein in rabbit feeds have been reduced while fibre content was increased (Trocino et al., 2013). In the case of DE content, the quantity has been more or less maintained, and therefore, the ratio expressed above could be modified.

In this context, where rabbits with high growth rates are fed using a commercial feeds (formulated to ensure an adequate growth and health of crossbred animals, which could be penalizing animals with high growth rates; Carabaño et al., 2009), a more detailed protein requirements should be studied. From this moment, we will proceed to talk about amino acid requirements and not about protein requirements, but it is necessary to understand a few the protein metabolism.

Protein metabolism

Proteins are macro-biomolecules consisting of one or more amino acids chains (Creighton, 1992). Proteins are synthesized from amino acids, which become available either from the end products of digestion (McDonald et al., 2010). There exist two types of amino acids (Neurath and Hill, 1982); those that the animal can synthesize (non-essential) or those that it cannot synthesize by itself (essential). A priori, only essential aminoacids are susceptible to become limiting when the diet does not provide enough quantity (D'Mello, 1995). The most prevalent limiting amino acids are lysine, sulphur amino acids and threonine for poultry, pigs and rabbits (Bonato et al., 2015; Van Milgen and Dourmad, 2015; de Blas and González-Mateos, 2010).

As it can observed (Figure 0.1), from the total crude protein (CP) ingested only a part, that is digested by enzymatic action and converted to amino acids and peptides (McDonald et al., 2010), is absorbed (into stomach and mainly in small intestine; D'Mello, 1994). This proportion corresponds with ileal digestible CP. The rest (ileal indigestible CP) goes to large intestine where could be used or modified by the microbiota action, that could be used via caecotrophy (faecal digestible CP), or eliminated in faeces (faecal indigestible CP). Digestible amino acid requirements could be calculated by either considering (real) or not (apparent) the endogenous flow.

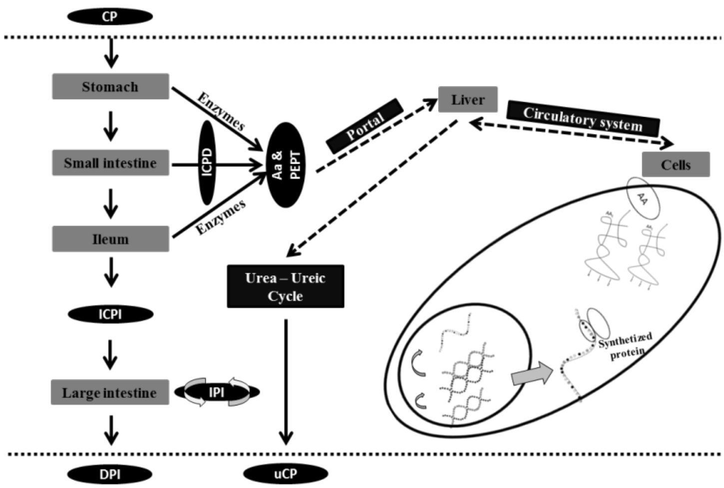

Figure 0.1. The protein evolution in monogastric. CP=Crude protein; ICPD= ileal digestible CP; ICPI= ileal indigestible CP; IPI= faecal digestible CP; DPI= faecal indigestible CP; uCP= CP excreted by urine; Aa= Amino acid; PEPT=Peptides

It is therefore logical to think that ileal digestibility allows us to know how much quantity of amino acids are available for the animal (Lemme et al., 2004) and, therefore, provides more information on both the requirements of animals and the balance of diet. Precision nutrition will imply knowledge of this digestible fraction of the diet. However, there are different peculiarities (i.e. caecotrophy) regarding rabbit digestive physiology that make the amino acid study more complicate.

Lagomorphs practice caecotrophy, as ingestion of faeces for nutritional purposes. Rabbits produce two kinds of faeces, soft and hard faeces. Soft faeces (coming from the caecum) are excreted and systematically ingested according to a circadian rhythm, which is the opposite to that of feed intake and hard faeces excretion (Lebas and Laplace, 1974, 1975; Bellier and Gidenne, 1996). Furthermore, there exists a circadian pattern of feed intake of growing rabbits, eating more quantity during the evening and night. Therefore, both feed intake and caecotrophy are not uniform along day (Bellier et al., 1995). Caecotrophy plays an important role in rabbit nutrition, providing proteins and B vitamins from bacteria. Soft faeces contain greater proportions of protein, minerals and vitamins than hard faeces, while hard faeces are higher in fibrous components (Carabaño et al., 2010). For this reason, caecotrophy suppose and additional source of water, protein and vitamins for the rabbit. The contribution of soft faeces to the total CP intake is around 17% (Fraga, 1998; Carabaño et al., 2000). Furthermore, protein of soft faeces is rich in essential amino acids such as lysine, sulphur amino acids and threonine (Proto, 1976; Spreadbury, 1978; Nicodemus et al., 1999; García et al., 2004). That is why the protein study in this species is substantially complicated. Despite the difficulty of its measurement, it is interesting to study that digestible protein at the ileal level, since they correspond to the amino acids that are available for protein synthesis.

Once the amino acids reaches to the cytoplasm of the different cells, they are available for the transferor RNA (tRNA). Inside nucleus, and dependent of animal requirements (Labadam et al., 2001), replication and transcription of DNA are produced, and when messenger RNA (mRNA) is formed, it is sent to cytoplasm, and protein synthesis starts (Eagle, 1959). For this reason, the protein synthesis depends of two aspects: animal requirements (defined for the DNA transcribed to cover the needs of the cell) and free amino acid available in the cell (result of digestion). At this moment two things can happen: synthesis could occur correctly or not, i.e., the synthesis could meet all the requirements of the cell or not.

Once protein synthesis has been performed, which is the subsequent destiny of this protein in growing animals? Protein produced could be used for maintenance or growth. Digestible protein required for maintenance in growing rabbits is estimated to be 2.9 g day^{-1} kg^{-1} live weight $(LW)^{0.75}$ (Partridge et al., 1989; Fernández and Fraga, 1996; Motta Ferreira et al., 1996; Fraga, 1998). On the other hand, it could be used to cover growth requirements, which vary according to the animals' growth rate. The efficiency of utilization of ileal digestible CP for growth is estimated to be 0.56 (Partridge et al., 1989; Fernández and Fraga, 1996; Motta Ferreira et al., 1996; Fraga, 1998). The protein destined for growth can be measured as the retained nitrogen. Retained nitrogen can be measured as the difference between the nitrogen input and the nitrogen output (Xicatto and Troccino, 2010). In order to know how much protein has been retained by one animal, it is necessary to know the initial and final quantity of protein in the body, and usually it is evaluated using the comparative slaughter technique. Retained protein could be different in function of the diet composition, the digestibility, and effectiveness in the body protein synthesis. However, retained protein provides the highest degree of knowledge about what happened with that protein that was digested by the animal.

Previously we discussed about protein synthesis and how the animal manages its own protein production. However, does it always happen in a "perfect" way? Obviously not. Amino acid that are not utilized (e.g. excess of protein or presence of some limiting amino acid; Harper et al., 1970) and the ones coming fromcell renewal are catabolyzed and the resulting NH_4^+ submitted to the liver again (via alanine or glutamine in order to avoid its toxicity), where it is transformed into urea/uric acid (Graph 0.1). The urea cycle requires the utilization of three molecules of ATP per molecule of urea (Ottaway and Apps, 1984; Bohinski, 1979), and uricotelic animals lose about 15 per cent of the energy of the amino acids from which is derived (Leningher et al., 1983). Low dietary protein/energy ratiohas implications with growth traits (Hussein et al., 2010), health status (Carabaño et al., 2009) and reproductive traits (Frobish et al., 1966). Therefore, a deficient amino acid supply results in a reduction in animal's performance while an oversupply is costly and leads to an excessive nitrogen excretion with a potentially negative environmental impact (Langner et al., 1983; Smith et al., 2000; Van Milgen and Dourmad, 2015; Hou et al., 2016). Different nutrition strategies have the possibility to increase the biological value of feed and reduce nitrogen excretion (Yano et al., 1999). On the other hand, if urea nitrogen (or ureic N) has a relationship with the part of the non-utilized but digested protein, could urea nitrogen be used as a measurement of dietary amino acid imbalance?

After amino acid degradation, urea/uric acid travels by the circulatory system. For this reason, it is logical to think that is easy to know a possible imbalance in the protein utilization (or nitrogen excretion) by determining urea/ureic nitrogen content in the blood, concentrated in plasma (plama urea nitrogen; PUN) or serum (serum urea nitrogen; SUN), depending whether clotting factors are included or not (Barelli et al., 2007). However, looking in the bibliography, no studies have been found that use this metabolite in rabbits. Although it is presented as a great candidate, it is necessary to know its potential, at least in other species.

Table 0.2 summarizes all studies where urea nitrogen could be related with growth or reproductive traits. Around 14.000 animals have been used in 76 different trials (60 studies). About 67% and 33% of the experiments have been conducted in pigs or in poultry, respectively. From them, only 5% of the studies compared different protein source, 17% compared different levels of CP and the 78% remaining compared different amino acid levels (on this order: lysine, sulphur amino acids, valine, tryptophan and threonine). Of the total of these studies, 86% showed a PUN reduction as a positive response. As a summary, PUN has been showed as a good indicator of amino acids imbalance in pigs and poultry. However, its effectiveness has not demonstrated in rabbits, which peculiarities must be taken into account . Effectiveness should be demonstrated first to be able to use it as a tool.

Table 0.2: Literature studies where urea nitrogen was measured and it can related with growth or reproductive traits

PIG		POULTRY					
GROWING		SOW		BROILER		HEN	
Dean et al.	(2007)	Atinmo et al.	(1974)	Lin et al.	(2014)	Azzam et al.	(2015)
Guzik et al.	(2002)	Coma et al.	(1996)	Liu et al.	(2014)	Dai et al.	(2012)
Kerr et al.	(2003)	Cuaron et al.	(1984)	Nworgu et al.	(2007)	Guo et al.	(2019)
Lenehan et al.	(2003)	Dong et al.	(2014)	Si et al.	(2016)	He et al.	(2015)
Nieto et al.	(2015)	Figueroa et al.	(2002)	Tian et al.	(2014)	Qi et al.	(2017)
Toledo et al.	(2014)	Gao et al.	(2004)	Yang et al.	(2009)	Wang et al.	(2017)
Waguespack et al.	(2012)	Jiang et al.	(2011)	Yang et al.	(2016)	Yu et al.	(2010)
Yin et al.	(2001)	Lewis and Speer	(1973)	Yuan et al.	(2012)		
Zerves et al.	(2002a)	Li et al.	(2013)	Yuan et al.	(2015)		
Zerves et al.	(2002b)	Park et al.	(2010)				
		Peters and Maham	(2001)				
		Qi et al.	(2017)				
		Rempel et al.	(2018)				
		Roth-Maier et al.	(2003)				
		Schneider et al.	(2006)				
		Sohail et al.	(1978)				
		Soltwedel et al.	(2006)				
		Velayudha et al.	(2018)				
		Woerman and Speer	(1976)				
		Yang et al.	(2000)				
		Zhang et al.	(2001)				

Current amino acid recommendations

As we have previously indicated, it would be necessary to know in depth the amino acid requirements. Usually, amino acid requirements have been determined using dose-response methods, which show the response of the performance to ascending contents of the amino acid studied. Most of these studies did not take into account the interaction of the amino acid studied with the others, and this may cause the method to be somewhat more imprecise.

Independently, the majority of studies were performed more than 25 years ago, so a review of these requirements could be necessary. In addition, these studies were performed at total dietary level or at apparent faecal digestible level. Nevertheless, for all described above, amino acid formulation of feeds should be done at ileal digestible level. In order to achieve this goal in practice, it is necessary to evaluate the amino acid content on main raw materials used in rabbit feeds (Villamide and Fraga, 1998, Villamide et al., 2009, 2010) and the amino acid requirements at ileal digestible level. Growing rabbit requirements have been provided by different authors, initially by Lebas (1989), and they were updated and completed in the chapter of "Feed formulation" (de Blas and González-Mateos, 2010) of the book "Nutrition of the Rabbit" (2010). However, although we have information about the amount of amino acid apparent and true digestible at ileal level in the main raw materials (Villamide et al., 2010), the recommendations for the growing rabbit in those sources are still in total dietary content and apparent faecal digestible content (Table 0.3). In addition, the amino acid requirements could be different for animals with different growth rates, due to their different tissue accretion, intestinal growth and maintenance of mucosa functionality both in other species (Armero, 1988) and in rabbits (Carabaño et al., 2009), and predictably they do not have the same relation between maintenance and growth requirements.

Table 0.3: Essential amino acid requirements (g/kg dry matter) of intensively reared growing rabbits, summarized by de Blas and González-Mateos (2010)

	Total dietary content	Apparent faecal digestible content
Lysine	8.1	6.3
Sulphur amino acids	5.8	4.4
Threonine	6.9	4.8

Finally, the current framework has the following main points: (i) genetic selection in paternal lines seems to be slow; (ii) there are indications of nutritional requirements are dependent on the growth rate of the animals, that are fed using common diets; (iii) it seems that the PUN could be a good indicator of the use of dietary amino acids, but its use in rabbits has not been shown yet; (iv) and amino acid requirements were studied many years ago and they are expressed at total dietary or at apparent faecal digestible level, and possible interactions between amino acid did not taken into account. In this global context, this PhD thesis appears. The hypothesis of this thesis is that current growing rabbits characterized by a high growth rate would have different amino acid requirements from current recommendations, and that there would be another amino acid combination that would maximize growth traits.

The main objective of this thesis is to provide the amino acid recommendations, using PUN as a tool, for growing rabbits selected by growth rate, aiming to improve rabbit amino acid utilization, and consequently, growth rate and feed conversion ratio, resulting in a reduction of nitrogen excretion and contributing to effectiveness of genetic selection by growth rate. In order to achieve this aim, specific objectives are:

O.1. To assess the effect of common diet on protein digestibility and retention in growing rabbits selected by growth rate, and to obtain indices revealing the presence of limiting amino acids.

O.2. To investigate the viability of PUN as an indicator of amino acid imbalance in rabbit feeds, and to establish the best methodology to use it.

O.3. To determine the best combination of the main limiting amino acids (lysine, sulphur amino acids and threonine), able to reduce PUN.

O.4. To compare thatcombination obtained in O.3 with the current recommendations, regarding their effects on growth parameters. Finally, to establish the new amino acid recommendations at ileal level, if aplicable.

PAPER 1

A moderate protein diet does not cover the requirements of growing rabbits with high growth rate

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Abstract

Genetic selection has increased the growth rate and requirements of growing rabbits, while the protein content of commercial feeds has been adjusted to avoid digestive disorders. The aim of this work was to evaluate how a diet with moderate levels of protein [146 g crude protein (CP)/kg] could be affecting protein and amino acids acquisition in terms of growth rate of the animals. From 189 weaned rabbits (28 days old), only 41 animals were selected at 42 days, in order to ensure the greatest variability for growth rate. To achieve this goal, animals came from three genetic lines: H and LP (maternal lines selected by litter size) and R (paternal line selected for growth rate), characterised by normal, moderate and high growth rate from weaning to slaughtering, respectively. Apparent faecal digestibility of dry matter (DM), CP and gross energy (GE) of the diet from 49 to 53 days of age, as well as the ileal apparent digestibility of DM, CP and amino acids at 63 days of age, was determined in all the selected animals. Protein, energy and amino acids retained in the empty body during the growing period were also determined by slaughtering 15 weaning rabbits at 28 days of age, and the 41 selected animals at 63 days of age. Animals from the R line showed higher feed intake than those from maternal lines, as well as lower feed conversion ratio, even below that expected from their growth rate. Apparent faecal digestibility of GE and apparent ileal digestibility of DM, CP and cystine of the diet were higher in LP than in H rabbits ($P<0.05$), showing intermediate values in R rabbits. However, apparent ileal digestibility of glutamic acid and glycine was significantly higher in R than in H rabbits ($P<0.05$), showing intermediate values in LP rabbits. As expected, both daily protein and energy retained in the empty body increased as growth increased. However, R growing rabbits seem to have lower protein retained and higher energy retained in the empty body than that expected from their growth. In fact, retained protein to retained energy ratio was clearly lower for R growing rabbits. These results seem to show the possible existence of some limiting amino acid when current moderate protein diets are used in growing rabbits with high growth rates, recommending a review of the amino acid requirements for the growing rabbits from paternal lines.

Key words: Rabbit, growth rate, protein, amino acids, retention and digestibility.

Introduction

There is a need to reduce feeding costs in rabbit farms by improving feed conversion ratio (FCR), and paternal lines, designed to improve feed efficiency, play a relevant part in achieving this goal (Cartuche et al., 2014). Usually, selection for feed efficiency in current rabbit genetic programmes is done indirectly, selecting by average daily gain (ADG), as there is a negative genetic correlation between these two traits (Blasco, 1989; Baselga, 2004). Currently, the animals from these parental lines show ADG values above 55 g/d during the growing period (Marín-García et al., 2016).

Commercial feeds for growing rabbits are designed to ensure an adequate ADG for three-way crossbred animals [usually with crude protein (CP) levels over 150 g/kg]. In any case, Carabaño et al.

(2009) reported that protein levels around 140 g/kg did not impair growth performance in growing rabbits with ADG rates of up to 55 g per day. However, since the onset of epizootic rabbit enteropathy, the protein content of commercial feeds has been decreased (to moderate levels of 140-150 g/kg) to reduce the risk of digestive troubles (Trocino et al., 2013). In this context, animals with a high ADG could have difficulties proving their growth potential with these diets.

Genetic progress in the paternal rabbit lines for feed efficiency may be linked to different improvements in utilisation of the feed nutrients, e.g. an improvement in the digestive efficiency at the ileal (or even faecal) level, or higher efficiencies in use of the digested nutrients for maintenance or growth. In growing rabbits, part of the digestible protein ingested (DPI) is used to cover the maintenance requirements [2.9 g DP day^{-1} kg^{-1} live weight $(LW)^{0.75}$] and a second part is retained in the body (DPr; with an estimates DPr/DPI efficiency of 0.56; Partridge et al., 1989). However, as these processes are not completely efficient, a fraction of the DPI, together with the renewed amino acids of the maintenance, is used by oxidative degradation as energy source and nitrogen is excreted in the form of urea. The lower this latter fraction, the greater the efficiency of the rabbits in using the DPI. In any case, independently of a possible increase in efficiency, animals with a higher growth rate would also have higher requirements for maintenance (due to their higher live weight) and for growth (due to their higher ADG), and there will possibly be a greater amount of DPI that will be used as an energy source. For all these reasons, high growth rate animals should have a greater protein input than animals with lower growth, as otherwise protein retention and growth could be penalised.

Pascual et al. (2008), comparing growing rabbits from a paternal line that differed in 11 generations of selection for ADG, and given a feed with 161 g CP/kg, observed that there were no significant differences in relative growth of any of the body components studied, with both genetic types showing similar growth patterns and carcass composition. However, Pascual et al. (2007), when comparing growing rabbits that differed in 16 generations of selection for ADG, but given a feed with 145 g CP/kg, observed that the most selected animals had greater dissectible fat percentage and lower meat to bone ratio of the hind leg. These results lead us to hypothesise that when diets with a moderate protein content are used, growing rabbits with a high growth rate may not be able to correctly cover their protein requirements, which may be affecting the correct elaboration of the rankings and genetic progress in the paternal lines.

Therefore, the aim of this work was to evaluate how a current commercial growing rabbit diet, with moderate levels of digestible protein, could be affecting protein and amino acids acquisition (considering both digestibility and retention) depending on the growth rate of the animals (using genetic lines differing for this trait).

Material and methods

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005). The experimental protocols followed the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [Boletín Oficial del Estado, 2013].

Experimental diet

Table 1.1 shows the ingredients and chemical composition of the experimental diet used in this work. The feed was formulated to have a moderate CP content, but ensuring the minimum recommendations for DP [111 g per kg dry matter (DM)], and the main essential amino acids (8.1, 5.8 and 6.9 g of lysine, sulphur amino acids and threonine per kg DM, respectively), as proposed by de Blas and Mateos (2010), and these objectives were essentially achieved. Although the DP to digestible energy (DE) ratio was intended to be 10.5 g/MJ, a value of 9.5 g/MJ was obtained (because of DE content higher than expected), which is at the limit of recommendation from Carabaño et al. (2009) and below those from de Blas and González-Mateos (2010) and, particularly, from Xiccato and Trocino (2010), which are 9.5-10, 9.8-10.8 and 10.5-11 g/MJ, respectively. A version of the diet including 5 g/kg DM of alfalfa hay marked with ytterbium was also manufactured.

Animals

One-hundred and eighty-nine weaned rabbits (28 days old), from three genetic lines (H, LP and R), developed at the Institute of Animal Science and Technology of the Universitat Politècnica de València, were used, in order to ensure a wide range for the growth rate of the rabbits during the growing period (63 animals from each genetic line). These lines differ greatly in their genetic background and growth rate during the growing period. Line H, which was founded following hyper-prolificacy criteria at birth (Cifre et al., 1998) and selected by litter size at weaning over 17 generations, is characterised by a large litter size at weaning but a normal growth rate during the growing period. Line LP, founded by hyper-longevity criteria (Sánchez et al., 2008) and selected by litter size at weaning over 7 generations, is characterised by a greater robustness than other lines and good growth rate during the growing period. Finally, Line R, which was obtained after two generations of randomly mating from a pool of animals of two commercial sire lines (Estany et al., 1992), and then selected by ADG in the growing period over 38 generations, is characterised by a high growth rate during the growing period.

Experimental procedure

Throughout the experimental period (February to March), animals were kept at 15ºC to 22ºC, with a photoperiod of 12 hours of light and 12 hours of darkness. At 28 days of age, 15 weaned rabbits (5 per genetic line) were slaughtered by intracardiac puncture with sodium thiopental (75 mg/kg of LW) to determine the empty body characteristics at this age. The rest of the animals were housed in individual cages and fed with the experimental diet until 63 days of age. Mortality and morbidity (presence of diarrhoea) were controlled daily and feed intake and LW were recorded weekly. Any animal presenting any digestive anomaly, weight loss or low ingestion was ruled out of the trial. At 42 days of age, from the total of growing rabbits, 50 were initially selected for the trial (15, 18 and 17 of the H, LP and R lines, respectively), although only 41 of these animals -without any anomaly- gave us complete information at the end of the trial (11, 17 and 13 of the H, LP and R lines, respectively). The initial selection criteria within genetic type consisted of choosing those animals that could provide the greatest variability in growth rate during the growing period, based on the ADG shown in the first two weeks (28 to 42 days of age).

At 42 days of age, selected animals were housed in individual metabolic cages of 52x44x32 cm and, after a week of acclimatisation, a faecal digestibility trial was conducted according Perez et al. (1995). From 49 to 53 days of age, feed consumption was controlled and faeces were collected. Faeces were stored in identified plastic bags and frozen at -20ºC until analysis. From 53 days of age, all the animals began to receive the feed marked with ytterbium until slaughtering at 63 days of age. At this age, animals were weighed and slaughtered by intracardiac injection of sodium thiopental (75 mg/kg of LW) between 19:00 to 23:00 h, to minimise the influence of caecotrophy on the composition of digestive contents (Merino and Carabaño, 2003). Samples of ileal content were taken from the distal part of the small intestine (around 20-30 cm before the ileo-caeco-colic valve) for each animal, frozen at -20ºC, freeze-dried and ground. The whole digestive tract was emptied and reintroduced into the body of the slaughtered animal. Empty bodies, obtained at 28 and 63 days of age, were weighed and placed in plastic bags, identified and frozen at -40ºC. Frozen empty bodies were crushed and homogenised in a cutting machine (Tecator, Abusson, France), and one sample per animal was freeze-dried and stored at -40ºC until analysis.

Chemical analysis

Feed was analysed for DM, ash, CP, neutral detergent fibre (aNDFom), acid detergent fibre (ADFom), lignin (sa), starch, gross energy (GE) and amino acid content. Faeces were analysed for DM, CP and GE, and ileal samples for DM, CP, and amino acid content. Finally, empty bodies samples were analysed for DM, CP, GE and amino acid content. Samples were analysed according to the methods of AOAC (2000): 934.01 for DM, 942.05 for ash and 976.06 for CP. Starch content was determined according to Batey (1982). The aNDFom (assayed with a thermo-stable amylase and expressed exclusive of residual ash,), ADFom (expressed exclusive of residual ash) and lignin (determined by solubilisation of cellulose with sulphuric acid) were analysed sequentially (Van Soest et al., 1991). GE was determined by adiabatic bomb calorimetry (Gallenkamp Autobomb, Loughborough, UK).

The amino acid content was determined after acid hydrolysis with HCL 6N at 110◦C for 23h as previously described by Bosch et al. (2006), using a Waters (Milford, Massachusetts, USA) HPLC system 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 internal standard after hydrolysation. The amino acids were derivatised with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and separated with a C-18 reverse-phase column Waters AcQ Tag (150 mm × 3.9 mm). Methionine and cystine were determined separately as methionine sulphone and cysteic acid, respectively, after performic acid oxidation followed by acid hydrolysis (Alagón et al., 2016).

Calculations and statistical analysis

From the information obtained from those weaned rabbits slaughtered at 28 days of age (empty body weight and CP, GE and amino acid contents in the empty body), there were fitted linear regression equations using LW at 28 days of age as independent variable for each of them. Values for all these variables at 28 days of age for the animals slaughtered at the end of retention trial (63 days of age) were estimated for each animal by means of these equations to properly determine nutrient retention during the growing period.

Table 1.1: Ingredients and chemical composition of the experimental diet with moderate protein content

Ingredients (g/kg)		Chemical composition (g/kg DM)	
Barley grain	155	Dry matter (DM; g/kg)	921
Wheat grain	50	Ashes	67
Soybean meal 30%CP	170	Neutral detergent fibre	304
Wheat bran	170	Acid detergent fibre	159
Alfalfa hay	160	Acid detergent lignin	23
Beet pulp	190	Starch	157
Soybean hulls	30	Crude protein	158
Defatted grape seed	20	Digestible protein[3]	111
Molasses	10	Digestible energy[3] (MJ/kg DM)	11.65
Lard	30	Amino Acid content:	
L-lysine HCl	1.7	Aspartic acid	15,65
DL-methionine	0.3	Serine	8,57
L-threonine	1	Glutamic acid	36,98
Calcium carbonate	1	Glycine	9,78
Salt	2	Histidine	3,56
Antimicrobials[1]	4	Arginine	7,37
Oligoelement and vitamin premix[2]	5	Threonine	6,93
		Alanine	7,19
		Proline	10,38
		Cystine	2,50
		Tyrosine	3,57
		Valine	8,35
		Methionine	3,21
		Isoleucine	6,01
		Lysine	8,27
		Leucine	11,41
		Phenylalanine	7,60

[1] 40 ppm of tiamulin fumarate (Caliermutin 2%, Laboratorios Calier S.A., Barcelona, Spain), 120 ppm of neomycin sulphate (Hipramix neomicina14%, Hipra S.A., Girona, Spain), 29 ppm of lincomycin 7 hydrochloride and 29 ppm of spectinomycine sulphate (Linco-Spectin 880, Zoetis Spain, S.L., Madrid, Spain)[2] Supplied 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.[3] Experimentally determined according to Perez et al. (1995). Using 41 helthy growing rabbits of 49 days of age weaned at 28 days. Faeces were collected during 4 days after a period of 7 days of adaptation to diets

Data on performance [ADG, daily feed intake (DFI) and FCR], apparent digestibility coefficients (both faecal and ileal) and nutrients retained in the empty body of the animals (energy, protein and amino acids) were analysed using a GLM procedure from SAS (SAS Institute, 2009), including the genetic type as the only fixed effect. In the case of apparent digestibility coefficients and nutrients retained in the empty body, linear effect of ADG or daily empty body weight gain (DEBWG) were also respectively determined.

The evolution of DFI, FCR, retained protein and energy, and retained protein to retained energy ratio according to the DEBWG of animals were evaluated. As the moderate protein diet used in this work should theoretically meet the requirements of the animals coming from the maternal lines (H and LP), linear regressions for these variables with DEBWG were obtained by fitting only the data from the maternal lines. Linear regressions were obtained using a REG procedure of SAS, extrapolating to high DEBWG values. Finally, other relationships of interest with the DEBWG, such as the proportion of digestible protein intake addressed to different life functions or the total protein ingested, retained, used in maintenance or not used, were determined with a REG procedure of SAS using data from all animals.

Results

Table 1.2: Performance of the experimental population according to genetic type (n=41)

		Genetic type				
	Range	H	LP	R	SEM	P-value
Body weight at 28 d (g)	265 – 910	506.8	607.4	618.8	38.3	0.1126
Body weight at 63 d (g)	1500 – 3425	1967[a]	2189[b]	2930[c]	71	0.0001
Daily feed intake (g/d)	89.6 – 186.5	110.8[a]	118.2[a]	154.2[b]	3.9	0.0001
Average daily gain (g/d)	34.14 – 69.86	41.50[a]	44.53[a]	64.73[b]	1.22	0.0001
Daily empty body weight gain (g/d)	26.39 – 65.48	34.76[a]	37.86[a]	55.14[b]	1.25	0.0001
Feed conversion ratio	2.10 – 3.11	2.672[a]	2.657[a]	2.381[b]	0.046	0.0001

SEM: standard error of the means. Means within a row with different letter were significantly different (P<0.05).

Table 1.2 shows the data on the main groth traits controlled in this work. Through the use of various genetic types and the choice of animals from a larger population at 42 days of age, the data set used in this study showed a wide range for all traits. In fact, high coefficients of variation for ADG (20 to 25%), DFI (18%) and FCR (8%) were obtained. Regarding the effect of the genetic type, LW at 63 days of age of the R line rabbits was higher than for those of the LP line (+741 g; P<0.05) and that of the LP higher than those of the H line (+222 g; P<0.05). In addition, DFI, ADG, DEBWG and FCR of growing rabbits of R line was significantly better than those obtained for LP and H lines (on av. +40 g/d, +22 g/d, +19 g/d and -0.28, respectively; P<0.05). When ADG was included into the model as a covariate, the effect of genetic type on DFI (+35%; P<0.001) disappeared while differences in FCR (-11%; P<0.001) remained, as could be also deduced from Figure 1.1, which shows the effect of DEBWG on DFI and FCR. The extrapolated regression lines obtained with DFI and FCR data from maternal lines show how R growing rabbits seem to have a slightly lower DFI but a clearly lower FCR than that expected from their DEBWG.

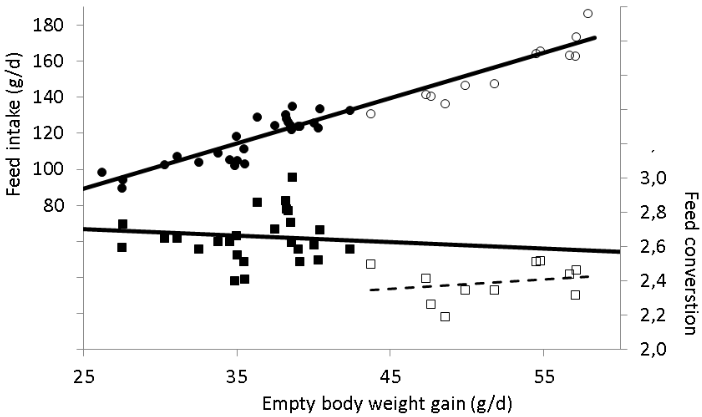

Figure 1.1: Relationship between daily empty body weight gain with daily feed intake represented as circles) and feed conversion ratio (represented as squares). Data from both maternal lines H and LP are represented in black, defining the regresion line that was extrapolated for the entire daily empty body weight gain range. Data from the paternal line R are presented in white.

The effect of genetic type and ADG on the main coefficients of apparent faecal and ileal digestibility is presented in Table 1.3. Apparent faecal digestibility of GE and apparent ileal digestibility of DM, CP and cystine were significantly higher in LP than in H rabbits (+0.019, +0.125, +0.076 and +0.140, respectively; $P<0.05$), showing intermediate values in R rabbits. However, apparent ileal digestibility of glutamic acid and glycine were significantly higher in R than in H rabbits (+0.051 and +0.152, respectively; $P<0.05$), showing intermediate values in LP rabbits. The ADG did not affect significantly the apparent faecal or ileal digestibility.

Table 1.3: Coefficients of apparent faecal and ileal digestibility according to the genetic line (H, LP and R) and average daily gain (ADG; g/d) (n=41)

	H	LP	R	P-value	ADG
Faecal coefficients: (49 to 53 days old)					
Dry matter	0.615 ± 0.006	0.631 ± 0.006	0.629 ± 0.006	0.1775	0.002 ± 0.002
Crude protein	0.691 ± 0.008	0.705 ± 0.007	0.704 ± 0.008	0.3762	0.002 ± 0.003
Gross energy	0.620 ± 0.006[a]	0.639 ± 0.006[b]	0.636 ± 0.006[ab]	0.0651	0.002 ± 0.002
Ileal coefficients: (63 days old)					
Dry matter	0.306 ± 0.023[a]	0.431 ± 0.018[b]	0.368 ± 0.024[ab]	0.0006	0.000 ± 0.001
Crude protein	0.617 ± 0.015[a]	0.693 ± 0.012[b]	0.652 ± 0.016[ab]	0.0017	0.000 ± 0.001
Alanine	0.587 ± 0.037	0.640 ± 0.029	0.684 ± 0.039	0.2045	0.002 ± 0.002
Arginine	0.721 ± 0.024	0.749 ± 0.019	0.773 ± 0.026	0.3464	0.001 ± 0.001
Aspartic acid	0.578 ± 0.038	0.639 ± 0.030	0.683 ± 0.040	0.1736	0.002 ± 0.002
Cystine	0.485 ± 0.046[a]	0.625 ± 0.042[b]	0.525 ± 0.049[ab]	0.0843	-0.002 ± 0.019
Glutamic acid	0.808 ± 0.016[a]	0.837 ± 0.013[ab]	0.859 ± 0.017[b]	0.1147	0.001 ± 0.001
Glycine	0.419 ± 0.041[a]	0.521 ± 0.033[ab]	0.571 ± 0.044[b]	0.0451	0.003 ± 0.002
Histidine	0.621 ± 0.029	0.669 ± 0.023	0.683 ± 0.031	0.3000	0.001 ± 0.002
Isoleucine	0.678 ± 0.028	0.719 ± 0.022	0.749 ± 0.029	0.2340	0.001 ± 0.002
Leucine	0.723 ± 0.022	0.761 ± 0.017	0.783 ± 0.023	0.1663	0.004 ± 0.004
Lysine	0.688 ± 0.029	0.743 ± 0.023	0.767 ± 0.030	0.1620	0.001 ± 0.002
Methionine	0.743 ± 0.042	0.775 ± 0.035	0.759 ± 0.045	0.8454	0.000 ± 0.002
Phenylalanine	0.751 ± 0.023	0.779 ± 0.018	0.761 ± 0.022	0.6356	-0.001 ± 0.001
Proline	0.686 ± 0.026	0.728 ± 0.020	0.753 ± 0.027	0.1968	0.001 ± 0.001
Serine	0.636 ± 0.027	0.673 ± 0.022	0.710 ± 0.028	0.1915	0.001 ± 0.001
Threonine	0.516 ± 0.041	0.550 ± 0.032	0.621 ± 0.043	0.2077	0.003 ± 0.002
Tyrosine	0.681 ± 0.027	0.700 ± 0.021	0.714 ± 0.029	0.7070	0.000 ± 0.001
Valine	0.619 ± 0.029	0.669 ± 0.023	0.706 ± 0.031	0.1387	0.002 ± 0.002
Sulphur amino acids	0.632 ± 0.039	0.717 ± 0.036	0.631 ± 0.037	0.1119	0.000 ± 0.002

Means within a row with different letter were significantly different (P<0.05).

Table 1.4: Retained protein (g/d), protein content of the empty body weight gain (mg/g) and amino acid content of the retained protein (g/g) according to the genetic line (H, LP and R) and daily empty body weight gain (DEBWG; g/d) from 28 to 63 days old (n=41)

	H	LP	R	P-value	DEBWG
Retained protein	6.48 ± 0.28[a]	7.42 ± 0.22[b]	9.35 ± 0.27[c]	0.0001	0.145 ± 0.010*
Protein	196.3 ± 3.4[b]	201.7 ± 2.76[b]	179.6 ± 3.3[a]	0.0001	–0.949 ± 0.209*
Alanine	0.051 ± 0.001	0.052 ± 0.001	0.053 ± 0.001	0.6145	$7\cdot10^{-5} \pm 8\cdot10^{-5}$
Arginine	0.064 ± 0.002	0.062 ± 0.001	0.064 ± 0.002	0.7628	$1\cdot10^{-5} \pm 10\cdot10^{-5}$
Aspartic acid	0.068 ± 0.002	0.071 ± 0.001	0.068 ± 0.002	0.4595	$-3\cdot10^{-5} \pm 13\cdot10^{-5}$
Cystine	0.025 ± 0.002	0.025 ± 0.001	0.020 ± 0.002	0.2754	$-9\cdot10^{-5} \pm 13\cdot10^{-5}$
Glutamic acid	0.125 ± 0.003	0.126 ± 0.003	0.122 ± 0.003	0.7395	$-9\cdot10^{-5} \pm 19\cdot10^{-5}$
Glycine	0.075 ± 0.003	0.067 ± 0.003	0.074 ± 0.003	0.1141	$9\cdot10^{-5} \pm 20\cdot10^{-5}$
Histidine	0.021 ± 0.001	0.021 ± 0.001	0.021 ± 0.001	0.9653	$-2\cdot10^{-5} \pm 4\cdot10^{-5}$
Isoleucine	0.030 ± 0.001	0.030 ± 0.001	0.030 ± 0.001	0.9952	$-4\cdot10^{-5} \pm 7\cdot10^{-5}$
Leucine	0.066 ± 0.002	0.069 ± 0.002	0.072 ± 0.002	0.2773	$1\cdot10^{-5} \pm 1\cdot10^{-5}$
Lysine	0.058 ± 0.002	0.061 ± 0.002	0.059 ± 0.002	0.3637	$-3\cdot10^{-5} \pm 13\cdot10^{-5}$
Methionine	0.020 ± 0.001	0.019 ± 0.001	0.018 ± 0.001	0.5102	$-2\cdot10^{-5} \pm 7\cdot10^{-5}$
Phenylalanine	0.031 ± 0.002	0.030 ± 0.001	0.032 ± 0.001	0.6996	$1\cdot10^{-5} \pm 9\cdot10^{-5}$
Proline	0.049 ± 0.002	0.047 ± 0.002	0.050 ± 0.002	0.6118	$1\cdot10^{-5} \pm 12\cdot10^{-5}$
Serine	0.036 ± 0.002	0.038 ± 0.002	0.038 ± 0.002	0.8553	$3\cdot10^{-5} \pm 12\cdot10^{-5}$
Threonine	0.036 ± 0.001	0.036 ± 0.001	0.036 ± 0.001	0.9690	$-1\cdot10^{-5} \pm 7\cdot10^{-5}$
Tyrosine	0.022 ± 0.001	0.024 ± 0.001	0.024 ± 0.001	0.2900	$4\cdot10^{-5} \pm 9\cdot10^{-5}$
Valine	0.043 ± 0.001	0.043 ± 0.001	0.043 ± 0.001	0.9752	$-5\cdot10^{-5} \pm 8\cdot10^{-5}$
Sulphur amino acids	0.045 ± 0.003	0.043 ± 0.003	0.038 ± 0.003	0.3382	$-10\cdot10^{-5} \pm 19\cdot10^{-5}$

Means within a row with different letter were significantly different (P<0.05).

Retained protein, protein content of the empty body weight gain and amino acid content of the retained protein depending on the genetic type and on the DEBWG of growing rabbits are shown in Table 1.4. As expected, daily retained protein increased with the growth rate (+0.145 g/d of retained protein for each +1 g/d of DEBWG; P<0.05), with R rabbits showing greater daily protein retention than LP rabbits (+1.9 g/d; $P<0.05$), and these latter greater than H rabbits (+0.9 g/d; $P<0.05$). However, the amount of protein retained per unit of empty body weight gain was significantly lower in R than in LP and H rabbits (on av. -20 mg per g of empty body weight gain), a significant lineal decrease of protein being retained per unit of empty body weight gain as DEBWG increased (-0.95 mg per g of empty body weight gainfor each +1 g/d of DEBWG; $P<0.05$). On the other hand, no effects of the genetic type and DEBWG were observed in the amino acid profile of the retained protein.

Finally, Figure 1.2 shows the evolution of daily protein and energy retained in the empty body, as well as their ratio, according to the DEBWG. As expected, both daily retained protein and energy increased as the DEBWG increased (Figure 1.2a). However, when regression lines were obtained with the data from maternal lines and extrapolated, R growing rabbits seem to have lower retained protein and higher retained energy in the empty body than that expected from their DEBWG. In fact, retained protein to retained energy ratio was clearly lower for R growing rabbits (Figure 1.2b).

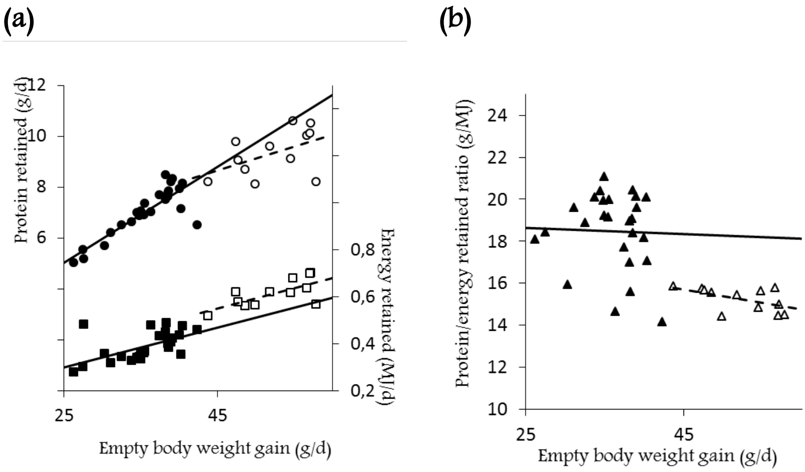

Figure 1.2: Relationship between daily empty body weight gain with (A) the retained protein (represented as circles) and the retained energy (represented as squares), and (B) the retained protein / retained energy ratio. Data from both maternal lines H and LP are represented in black, defining the solid regresion line that was extrapolated for the entire empty body weight gain range. Data from the paternal line R are presented in white and fitted to the broken line.

Discussion

The main aim of this work was to determine whether growing rabbits with a high growth rate are able to correctly cover their protein requirements when the current diets with a moderate protein content are used. This condition is very important for the correct elaboration of the genetic rankings in the paternal lines, which could significantly affect the genetic progress and the efficiency in rabbit farms. To assess this hypothesis, we have built a population of growing rabbits with a wide ADG using three different genetic types, which a priori should differ in this parameter. This premise has been achieved, providing a population distributed homogeneously from

34 to 70 g/d of ADG and from 26 to 65 g/d of DEBWG. In fact, the differences found in growth traits among the different genetic types, although affected by the initial selection of the animals, showed the expected behaviour for these genetic types. R growing rabbits showed greater DFI and ADG and lower FCR than H ones, with LP animals presenting intermediate values. These results agree with those obtained for these same lines by García-Quirós et al. (2014) and Mínguez et al. (2016).

As expected, feed intake was highly correlated with the growth rate of the animals (increasing 2.5 g/d of DFI per g/d of DEBWG) independently of the genetic type. However, although feed efficiency improved as the growth rate of animals increased, animals from line R seems to show a lower FCR than expected. The regression lines of Figures 1.1 and 1.2 were obtained only with the data of the animals from the maternal lines, with DEBWGbelow 43 g/d, as the diet offered should easily cover the requirements of these animals (Carabaño et al., 2009), and the growing rabbits from the R line had much lower FCR than expected from this projection (on av. –0.28). Although an increase in the growth rateleads indirectly to the improvement of FCR, there are other factors such as the criteria of foundation (Savietto et al., 2014) or the continued genetic selection for ADG of this line, which could explain the greater feed efficiency of these animals.

A possible explanation for the improvement in the FCR could be an improvement in the digestive efficiency of the animals that show higher growth rate. However, ADG of animals did not affect the apparent digestibility coefficients of all controlled nutrients. Hypothetically, slight increase in digestibility when increasing growth rate could have hidden because of the associated increasing feed intake, as an increase in feed intake normally leads to a faster digestive passage rate and shorter exposure period to digestive enzymes (Carabaño et al., 2010), but no significant correlations were observed in the current work between digestibility coeficients and feed intake. Growing rabbits of the line H showed a worse utilisation of the dietary DM, GE, CP and some amino acids than those from LP or R line. Savietto et al. (2012) found slight and ambient-dependent increase in faecal digestibility of neutral detergent fibrein rabbit does from the LP line compared to other maternal lines. In any case, differences in digestive efficiency observed in this study seem to be more related to the genetic type used than to growth rate.

As expected, a positive lineal relationship between DEBWG and daily retained protein was observed. Although there are not many studies where the effect of growth rate on protein retention has been studied, an increase in ADG is always associated with an increase in the total amount of protein retained in the body (Gidenne and Perez, 2000; Birolo et al., 2016). However, retained protein per unit of empty body weight gain decreased as DEBWG increased and the animals of the R line showed lower protein content on the empty body weight gain than the animals from the H and LP lines,. However, no changes were observed in the amino acid profile of the retained protein depending of the genetic type or DEBWG of the growing rabbits, unlike what was reported in pigs by Armero (1998), who indicated that the content of some amino acids in muscle could be different depending on genetic type. Although there is a global negative linear correlation between the growth rate and protein content of the empty body weight, this effect is only detected in animals of the R line, not in H and LP lines, where a diet with a moderate protein level would have no difficulties in covering the requirements correctly. These results could be showing that, when diets with a moderate level of protein are used, amino acid supply could be limiting the correct development of animals with high growth rate from paternal lines. An amino acid supply limiting the protein accretion could have two explanations that are not mutually exclusive. On the one hand, the dietary DP to DE ratio was only 9.5 g/MJ, the lower limit of the less demanding recommendation (9.5-10 g/MJ; Carabaño et al., 2009). On the other hand, an imbalance in the dietary content of amino acids could have occurred. In fact, in Figure 1.2 we can see this issue in a more graphic way. Rabbits from maternal lines linearly increase the daily amount of protein retained as their growth rate increases. However, animals having a growth rate above 50 g/d of ADG (approx. 43 g/d of DEBWG), all of the R line, show lower protein retention than expected from their growth rate. When some amino acid is limiting the protein retention, the remaining non-retained amino acids would be derived for using as energy source (Leningher et al., 1983), which would be deposited as body fat. In fact, animals from R lineanimals showed greater energy retention and, particularly, lower retained protein to retained energy ratio than expected from their growth rate. These results would be in agreement with those observed by Pascual et al. (2007 and 2008) when analysing the body composition of growing rabbits from R line that only differed in their degree of selection (16 and 11 generations of difference, respectively), and therefore in their growth rate. Pascual et al. (2008), using a feed with 161 g CP/kg that should theoretically cover the protein requirements of high growth rate animals, observed that there were no significant differences in relative growth of any of the body components studied, with both generations showing similar growth patterns and carcass composition. However, Pascual et al. (2007), using a feed with 145 g CP/kg close to that used in the present work and the current commercial feeds, observed that the most selected animals had greater dissectible fat percentage and lower meat to bone ratio. These results would confirm the hypothesis of the existence of some limiting amino acid in animals with high growth rate when the current fattening feeds with moderate levels of protein are used.

In spite of showing similar digestive efficiency than the maternal lines and lower protein retention than expected, animals from R line showed better FCR than that expected from their growth rate .. This paternal line has been selected for ADG for many generations and FCR has been improved indirectly because a genetic negative correlation exists between both traits (Baselga, 2004). However, as mentioned above, retention protein per unit of emty body weight gain is lower as the growth rate of these animals increases and the remaining amino acids are addressed to body fat deposition, which is much less efficient. Thus, although generally FCR improves as growth rate increases (since proportion of nutrients addresed to maintenance decreases and that addressed to growth increases), FCR did not improve or even impared as the growth rate increases within line R (dotted line in Figure 1.1). Some previous works (Costa et al., 2004; Quevedo et al., 2006) have already observed that the FCR response to the selection in line R was lower than expected. These results could corroborate the hypothesis of a possible existence of a limiting amino acid that could be affecting the expression of the genetic potential of high growth rate rabbits in the paternal lines.

Conclusions

From the results of this work, we can conclude that the amount of protein retained in the body increases with the growth rate of the animals, but is lower than that expected from 50 g/d of ADG when moderate protein diets are used, increasing the amount of the protein used for fat accretion,. The most plausible reason is the existence of some limiting amino acid when such diets are used in animals with high growth rates. For this reason, determining amino acid requirements during the growing period could be necessary to allow adequate expression of the genetic potential of animals in the paternal lines, and the proper development of the genetic rankings in order to improve the genetic progress in ADG and FCR.

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PAPER 2

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 parameter has already been studied in other species, but not yet in rabbits. The main objective of the present work was to study the possible interest in detecting 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 far from these in P4.4. Three different trials were designed: one where the animals were fed ad libitum (AL) and two others in which fasting periods of 10 hours were included; one where feeding was restored at 08:00h (Fast8h) and the other at 18:00h (Fast18h). A total of 72 three-way crossbred growing rabbits (24 animals for each trial in a split-plot trial) were used. Blood samples were taken every 4 hours 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 between 04:00h and 12:00h in animals fed ad libitum, and at three hours after refeeding (21:00h) in Fast18h. These results suggest that PUN could be an adequate indicator to detect deficiencies in amino acids in growing rabbit diets.

Keywords: 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 growth rate (GR), as there is a negative genetic correlation between these two parameters (Blasco, 1989). In consequence, paternal lines consisting of animals with high GR 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). 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-. 2010), but also minimising the excretion of nitrogen into the environment (Romero et al., 2009).

As a consequence of this dietary protein limitation, selection by GR could have been penalised (Marín-García et al., 2019), by affecting the phenotypic expression of high GR 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 has been suggested in a previous study, where animals with high GR (>55 g/d) fed according to current protein recommendations had lower body protein retention than expected as a function of their GR (Marín-García et al., 2016).

The knowledge about protein nutrition in rabbits is scarce in comparison to other species (Carabaño et al., 2009; de Blas et al., 1998), 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), where the amino acid level recommendation will be the one resulting in the best performance (i.e., GR).

Plasma urea nitrogen (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 at 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 does not only depend 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 to detect deficiencies in amino acids and establish the appropriate methodology to optimise its use for this purpose in growing rabbits.

Material and methods

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005). The experimental protocols followed 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. The ingredients of the basal mixture and the chemical composition of the experimental diets are summarised in Table 2.1.

Animals and experimental procedure

The experimental protocols followed the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [Boletín Oficial del Estado, 2013], being approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València and authorised by the General Directorate of Agriculture, Livestock and Fisheries of the Government of the Valencian Community.

Three different trials were designed, as summarised in Figure 2.1. 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 events, 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 hours were imposed, one where feeding was restored at 08:00h (Fast8h) and the other at 18:00h (Fast18h).

Table 2.1: Ingredients and chemical composition of the experimental diets (P8.1 and P4.4)

Ingredients (g/kg)	Basal mixture	Chemical composition (g/kg DM)	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 fiber	366	
L-Lysine HCl	0	Acid detergent fiber	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) were used, 24 animals for each trial. At 49 days of age (2225±22.6 g), animals were housed in individual cages (26×50×31 cm), kept at 10ºC to 22ºC throughout the experimental period (February) and under a photoperiod of 12 hours of light (06:00 to 18:00h) and 12 hours of darkness. After one week of adaptation to the cage, one day before the first control (56 days of age) the animals were randomly divided into two groups and each group received one of the two experimental diets. The following day (57 days of age), feed intake was monitored and blood samples were taken from the central ear artery (1 mL in EDTA vials) every 4 hours in AL trial (08:00, 12:00, 16:00, 20:00, 00:00 and 04:00h), and every hour after refeeding, up to a total of six controls, in trials Fast8h (08:00, 09:00, 10:00, 11:00, 12:00 and 13:00h) and Fast18h (18:00, 19:00, 20:00, 21:00, 22:00 and 23:00h). At 08:00h of the following day (58 days 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 before controls to avoid adaptation mechanisms.

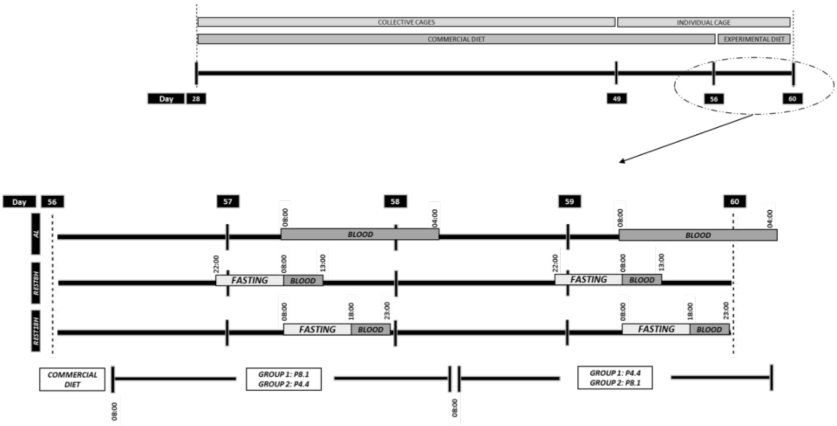

Figure 2.1: Representation of experimental design of the three trials. Blood sampling and control of feed intake were performed each 4 hours in AL trial and each hour in Rest8h and Rest18h trials. P8.1 and P4.4 are diets with 8.1 and 4.4 g of lysine per kg of dry matter, respectively

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 (in each batch a standard and a blank were included). 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 minutes at 37°C. Subsequently, 1 mL of reactant B (sodium hypochlorite seven mmol/L and sodium hydroxide 150 mmol/L) was added, mixed thoroughly and incubated for a further 5 minutes 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 pretreatment 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 ºC for 23 hours as previously described Bosch et al. (2006), using a Waters HPLC 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 a C-18 reverse-phase column Waters Acc. Tag (150 mm x 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 change-over 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 days of age), the experimental diet (P8.1 and P4.4) and time of the day (6 levels) as well as the interaction 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 2.2: *Ad libitum* trial: Feed intake (average during the previous four hours) and plasma urea nitrogen (PUN) in function of the time of the day (least square means ± standard errors)

	Time of the day						
	08:00h	12:00h	16:00h	20:00h	00:00h	04:00h	*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.

Table 2.2 shows the daily evolution of feed intake and PUN each four hours in the AL trial. Feed intake was the highest from 16:00 to 20:00h (being 24% of daily intake), decreasing gradually before and after this period until 04:00 to 08:00h, when it was the lowest (11% of the daily intake). PUN level showed a similar trend to feed intake, reaching the highest value at 20:00h and the lowest at 008:00h (a reduction of 15%).

Table 2.3. *Ad libitum* trial: Feed intake (average during the previous four hours) and plasma urea nitrogen (PUN) in function of 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).

Table 2.3 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).

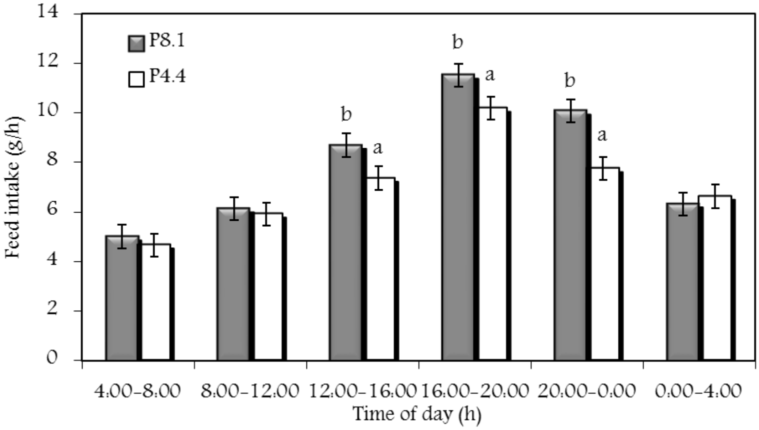

Figure 2.2: *Ad libitum* trial: Feed intake (average during the previous four hours) in function of time of day and experimental diet (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) (least square means ± standard errors). Bars for the same time of day with different letters differ significantly (*P*<0.05)

Figure 2.2 shows the circadian evolution of feed intake according to the experimental diet. No significant differences between diets were observed from 00:00h to 12:00 h. Differences appeared from 12:00 to 00:00h, when feed intake of animals increased, being 19.7% higher with P8.1 than with P4.1 (P<0.001).

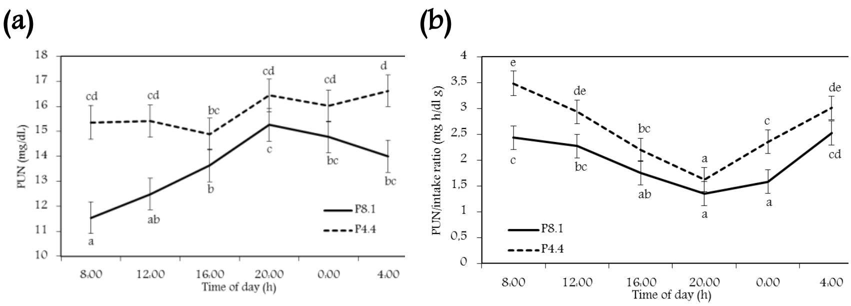

Figure 2.3: Ad libitum trial: Circadian evolution of (a) plasma urea nitrogen (PUN) and (b) PUN/intake ratio (dividing by feed intake during four hours before blood sampling) with the experimental diets (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) (least square means ± standard errors). Values having different letters differ significantly (P<0.05)

Figure 2.3 shows the circadian evolution of PUN (Figure 2.3a) and PUN/intake ratio (Figure 2.3b) for the two experimental diets. Differences between diets were significant only at 04:00, 08:00 and 12:00h for PUN and at 00:00, 08:00 and 12:00h for PUN/intake ratio. Maximum differences between diets were observed at 08:00h for both traits.

Fasting trials

Figure 2.4 represents the evolution of PUN when refeeding the animals after 10 hours of fasting before 08:00h (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 three or four hours 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 hours after refeeding. In contrast, PUN was always significantly higher in animals fed with P4.4 in trial Fast18h, this difference being maximum 3 hours after refeeding (+11%).

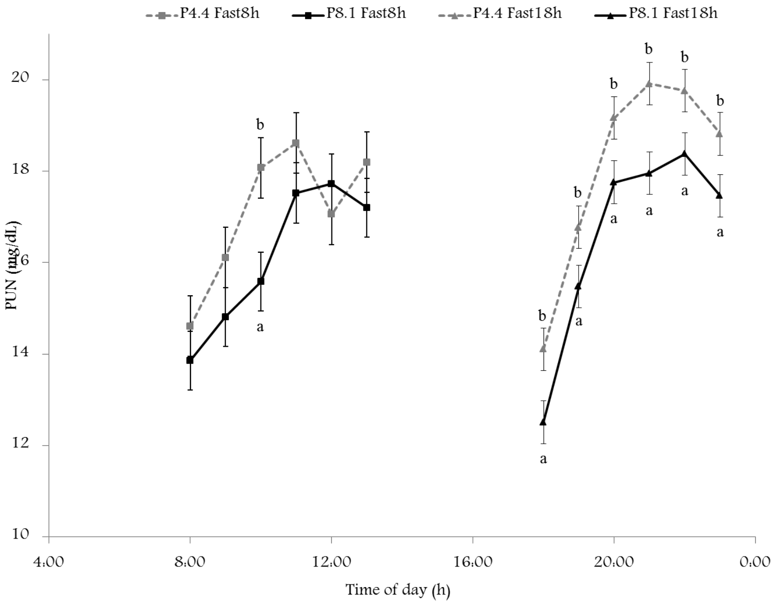

Figure 2.4: Fasting trials: Evolution of plasma urea nitrogen (PUN) of animals fed with the experimental diets (P8.1 and P4.4 with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) after 10 h of fasting before 8:00h (Fast8h trial, left lines) or 18:00h (Fast18h trial, right lines) (least square means ± standard errors). Values for the same time of day and trial with different letters differ significantly ($P<0.05$)

Discussion

Ad libitum trial

Total feed intake during the 24-hours period (57 days of age) averaged 181 g, 13% higher than that previously reported during the 9th week of age (Bellier et al., 1995; Prud'hon et al., 1975). Moreover, the circadian distribution pattern of feed intake was quite similar to that observed in the cited studies. 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 (Figure 2.2 and 2.3a). 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 (Coma et al., 1995&1996; Taylor et al., 1982&1985). 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 12:00h (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). This feed intake decrease with an unbalanced diet was also observed in the current trial from 12:00 to 00:00h, when feed intake was higher (Figure 2.2). 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.

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 20:00h). 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 [Bellier et al., 1995; Prud'hon et al., 1975; 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).

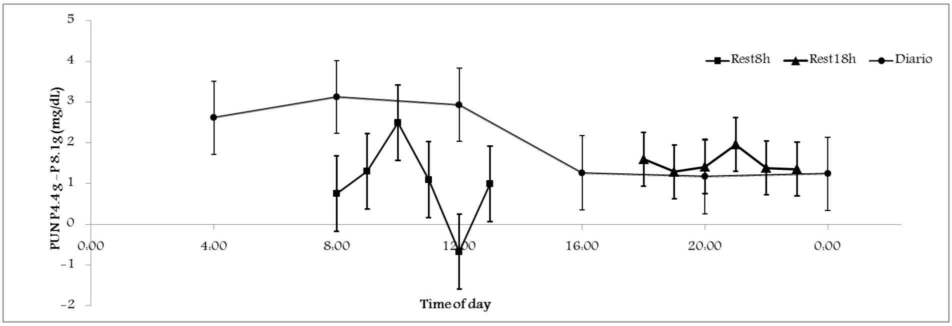

Figure 2.5: Evolution of mean difference in plasma urea nitrogen (PUN) between the two experimental diets (P4.4 – P8.1; with 8.1 and 4.4 g of lysine per kg of dry matter, respectively) during the different trials: *Ad libitum* trial and after a fasting of 10 h before 08:00h (Fast8h) and at 18:00h (Fast18h) (least square means and confidence interval). Values which confidence interval do not trespass the X-axis were significantly different from zero

When animals fasted during the night (Fast8h trial), PUN was higher with diet P4.4 only 2 hours 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 (30). Moreover, the possible asynchrony of their 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 unbalance.

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 hours 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. 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 04:00h and 12:00h in animals fed ad libitum, and at three hours after refeeding (21:00h) when a fasting period of 10 h was applied.

Acknowledgements

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PAPER 3

Plasmatic urea nitrogen in growing rabbits with different combinations of dietary levels of lysine, sulphur amino acids and threonine

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Abstract

The aim of this work was to evaluate the plasmatic urea nitrogen (PUN) induced by the different dietary combinations of the three main limiting amino acids in order to search for the combination minimizing PUN and, hypothetically, improving performance of rabbits with high growth rates. Twenty-seven experimental diets were formulated starting from the same basal mixture. The contents of lysine, sulphur amino acids and threonine were variable and three different levels were established for each of them. The first one, according with the current recommendations (Medium, M), and two other levels were on average 15% higher (High, H) or lower (Low, L). Diets were named with three letters, with first, second and third letters indicating lysine, sulphur amino acids and threonine levels respectively. Nine-hundred and eighteen weaned rabbits (28 days old) were used (34 per diet), distributed in 6 batches. Animals were from two different genetic lines in order to obtain a great variability in growth rate. At weaning, animals were fed ad libitum with a commercial feed for growing rabbits until 46 days old. At 08.00h of the day 47 each collective cage was randomly switched to one of the 27 experimental diets, provided ad libitum. At 08:00h of day 48, blood samples were taken from the central ear artery, then the animals were subjected to 10 hours of fasting and a second blood sample was extracted at 21.00h. From this moment, animals were switched again to commercial feed provided ad libitum until 63 days old. At 08:00h, PUN was higher with the L level of lysine (+1.08±0.165 mg/dL, +9.7%, $P<0.001$), unaffected by the level of sulphur amino acids and increased with the level of threonine. At

21:00h, minimum PUN (14.72±0.661 mg/dL) was observed with the MHL diet, although its difference with the 3 nearest values (LHM, MMH and LLL diets) was not significant (-1.29±0.759 mg/dL, P=0.090). These results suggest that, in relation to the usual recommendations, the content of sulphur amino acids should be raised (to 6.6 g/kg DM) and that of threonine should be reduced (to 5.7 g/kg DM), independently on growth rate. To confirm this hypothesis, studies on the effects on growth performance and protein retention are necessary.

Keywords: Growing rabbits, plasma urea nitrogen, amino acid, lysine, sulphur amino acid and threonine.

Introduction

Selection for growth rate (with the aim to improve feed to gain ratio) has been done in paternal rabbit lines. However, some study suggests a loss on the effectiveness in this selection (Quevedo et al., 2006), which could be related to protein nutrition. Thus, with a diet containing 179 g CP/kg DM, Pascual et al. (2008) did not observed differences in relative growth, while when a diet with 161 g CP/kg DM was used, animals more selected by growth rate had greater dissectible fat percentage and lower meat to bone ratio (Pascual et al., 2007). Marín-García et al. (2019a) showed that animals selected by growth rate had lower protein retention and higher energy retention than expected. On the other hand, as a consequence of the irruption of the epizootic rabbit enteropathy, dietary protein content has tended to be reduced nowadays (Carabaño et al., 2009). In this context, the presence of some limiting amino acid could be suggested to explain the above commented findings.

The most frequently limiting amino acids are lysine, sulphur amino acids (methionine and cystine) and threonine. The requirements in these amino acids for growing rabbits are considered well-known (de Blas and González-Mateos, 2010). However, these current recommendations have been established from dose-response studies and in these studies the interaction between amino acids is not taken into account (i.e., it has been demonstrated in broilers that the requirements of glycine increases if dietary levels of methionine or arginine are low; McDonald et al., 1988). Independently, most studies on amino acid requirements in growing rabbits were performed more than 25 years ago, so a review of these requirements seems necessary.

A large number of studies showed that low levels of plasmatic urea nitrogen (PUN), which corresponds to the amount of nitrogen in form of urea circulating in the bloodstream, could be related with performance in pigs (Roth-Maier et al., 2004; Nieto et al., 2015) or in broilers (Donsbough et al., 2010). Marín-García et al. (2019b) have demonstrated that PUN could be an indicator to detect amino acid deficiencies in diets for growing rabbits and have proposed an appropriate methodology to determine it.

The aim of this work was to evaluate PUN induced by the different dietary combinations of the three main limiting amino acids (using the current recommendations and increasing or decreasing them by

15%), in order to search for the combination minimizing PUN and, hypothetically, improving performance of rabbits with high growth rates.

Material and methods

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005). The experimental protocols followed the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes [Boletín Oficial del Estado, 2013].

Experimenal diets

Twenty-seven experimental diets were formulated starting from the same basal mixture (Table 3.1). This basal mixture was formulated following the recommendations of all nutrients for growing rabbits (de Blas and González-Mateos, 2010), with a moderate content of CP (155 g/kg DM). The contents of lysine, sulphur amino acids and threonine were variable and three different levels were established for each of them (Table 3.2). The first one, according with the current recommendations (Medium, M), and two other levels were on average 15% higher (High, H) or lower (Low, L). Diets were named with three letters, with first, second and third letters indicating lysine, sulphur amino acids and threonine levels respectively.

Table 3.1: Ingredients of basal mixture and average chemical composition of the 27 experimental diets

Ingredients	*g/kg*	*Chemical composition*		*g/kg DM*
Wheat bran	300	Dry matter (g/kg)		907
DDGS corn	50	Ash		104
Bakery by-product	30	Crude protein		155
Sunflower meal	36	Crude fat		29.7
Alfalfa meal	334	Neutral detergent fiber		455
Beet pulp	80	Acid detergent fiber		262
Straw	136	Acid detergent lignin		52.9
Beet molasses	13.85	Amino acid composition:		
L-Arginine	3.1		Aspartic acid	12.95
L-Histidine	1.5		Serine	5.74
Calcium carbonate	6.55		Glutamic acid	22.96
Sodium clorhide	4		Glycine	6.53
Vitamin/Trace			Histidine	3.57
element mixture[1]	5		Arginine	9.28
			Threonine	VAR
			Alanine	6.69
			Proline	8.04
			Cystine	2.37
			Tyrosine	2.90
			Valine	7.03
			Methionine	VAR
			Isoleucine	5.07
			Lysine	VAR
			Leucine	9.70
			Phenylalanine	5.58

[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.

VAR: Levels of these amino acids vary depending on experimental diets.

Animals and experimental procedures

Nine-hundred and eighteen weaned rabbits (28 days old) were used (34 per diet), distributed in 6 batches. Animals were from two different genetic lines in order to obtain a great variability in growth rate. These lines were line H [founded following a criterion of hyper-prolificacy at birth (Cifre et al., 1998) and then selected by litter size at weaning over 17 generations, characterised by a large litter size at weaning but a standard growth rate] and line R [obtained after two generations of randomly mating from a pool of animals of three commercial paternal lines (Estany et al., 1992) and then selected by average daily gain during the growing period over 38 generations, characterised by a high growth rate].

Table 3.2: Levels (g/kg DM) of the three studied amino acids

Level	Lysine	Sulphur amino acids (Cystine + Methionine)	Threonine
High (H)	9.4	6.6 (2.37 + 4.23)	7.8
Medium (M)	8.1	5.8 (2.37 + 3.43)	6.9
Low (L)	6.7	4.9 (2.37 + 2.53)	5.7

Throughout the experimental period (April to December), animals were kept at 15ºC to 22ºC, with a photoperiod of 16 hours of light and 8 hours of darkness. At weaning, animals were identified, weighed and allocated to a collective cage of 7 animals (by distributing animals from every mother and line among different cages). Then, animals were fed ad libitum with a commercial feed for growing rabbits until 46 days old. At 08.00h of the day 47 each collective cage was randomly switched to one of the 27 experimental diets, provided ad libitum. Following the methodology described by Marín-García et al. (2019b), at 08:00h of day 48 (after 24 hours receiving the experimental diet), blood samples were taken from the central ear artery (1 mL in EDTA vials). Subsequently, the animals were subjected to 10 hours of fasting and a second blood sample was extracted at 21.00h (3 hours after refeeding). Blood samples were immediately centrifuged during 5 minutes at 700 G, and the supernatant plasma was frozen at -20°C until further analysis. From this moment, animals were switched again to commercial feed provided ad libitum until 63 days old and then weighed to calculate the average daily gain during the growing period.

Chemical analysis

The 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 (in each batch a standard and a blank were included). 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 minutes 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 other 5 minutes at 37°C. Finally, the absorbance of each sample was read at 600 nm against the blank.

The amino acid content in diets was determined after acid hydrolysis with HCl 6N at 110 °C for 23 hours as previously described Bosch et al. (2006), using a Waters HPLC 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. The amino acids were derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm x 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

Data were analyzed using the GLM procedure of SAS (SAS, 2009), separately for each sampling time (8:00 and 21:00h), according to the next model:

$$Yijklm = m + \alpha * Li + \beta * Sj + \gamma * Tk + \Omega * Li * Sj * Tk + \varepsilon * Bl + \theta * ADGijkl + eijklm$$

where m is the mean, Li = lysine effect (3 levels), Sj = sulphur amino acid effect (3 levels), Tk = threonine effect (Thr level), Li*Sj*Tk = all possible interactions of these main effects, Bl = batch effect (6 levels), ADGijkl = average daily gain from 28 to 63 days old (covariate) and "eijklm" =error.

Results

Table 3.3 shows PUN values according to sampling time and the levels of the studied amino acids. Average PUN was highly dependent on the sampling conditions (mean ± standar error of mean of 11.47±0.088 and 18.29±0.152 mg/dL at 08:00 and 21:00h, respectively, P<0.001).

At 08:00h, PUN was higher with the L level of lysine (+1.08±0.165 mg/dL, +9.7%, P<0.001), unaffected by the level of sulphur amino acids and increased with the level of threonine. No significant effects of double or triple interactions and covariate (average daily gain) were detected (P>0.2).

Table 3.3: Plasmatic urea nitrogen (PUN) according to sampling time and the dietary level (H: High, M: Medium; L: Low) of the different amino acids. Least square means ± standard errors

	08:00h			
	H	M	L	P value
Lysine	10.93±0.136[a]	11.18±0.138[a]	12.13±0.136[b]	<0.001
Sulphur Amino Acids	11.44±0.137	11.47±0.135	11.33±0.136	0.759
Threonine	11.90±0.136[c]	11.36±0.137[b]	10.97±0.136[a]	<0.001
	21:00h			
	H	M	L	P value
Lysine	18.57±0.217[b]	17.40±0.217[a]	17.63±0.215[a]	<0.001
Sulphur Amino Acids	17.78±0.219	17.56±0.214	18.26±0.216	0.061
Threonine	18.12±0.217	17.94±0.217	17.54±0.214	0.150

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

At 21:00h, PUN was higher with the H level of lysine (+1.05±0.262 mg/dL, +6%, P<0.001) and no significant effects of the levels of sulphur amino acids and threonine were observed. Nevertheless, interactions sulphur amino acids*threonine and lysine*sulphur amino acids*threonine as well as covariate (average daily gain) were significant ($P<0.001$). Figure 3.1 shows PUN values obtained with the 27 experimental diets. Minimum PUN (14.72±0.661 mg/dL) was observed with the MHL diet, although its difference with the 3 nearest values (LHM, MMH and LLL diets) was not significant (-1.29±0.759 mg/dL, $P=0.090$).

Figure 3.2 shows the relationship between PUN at 21:00h with average daily gain for MHL diet compared with diets varying only in the level of one amino acid. PUN tended systematically to increase with average daily gain being regularly lower with MHL diet.

Discussion

In the current study, PUN varied depending on the sampling conditions, being widely higher when blood samples were taken at 21:00h, i.e. 3 hours after refeeding following a 10-hours fasting period, than at 8:00h under ad libitum feeding. These results closely with those obtained by Marín-García et al. (2019b) for these same times and feeding managements, as a result of low feed intake during morning (when rabbits are practicing caecotrophy) and the overeating at evening after a fasting period, since the higher is the protein intake the higher is the PUN (Eggum, 1970), because of the catabolism of greater amount of leftover amino acids. Additionally, the essential amino acid content in microbial protein recycled through soft faeces could improve the quality of dietary protein and reduce protein catabolism, although the amino acid supply from soft faeces does not seem to be enough to alter the amino acid pattern of conventional diets (Villamide et al., 2010).

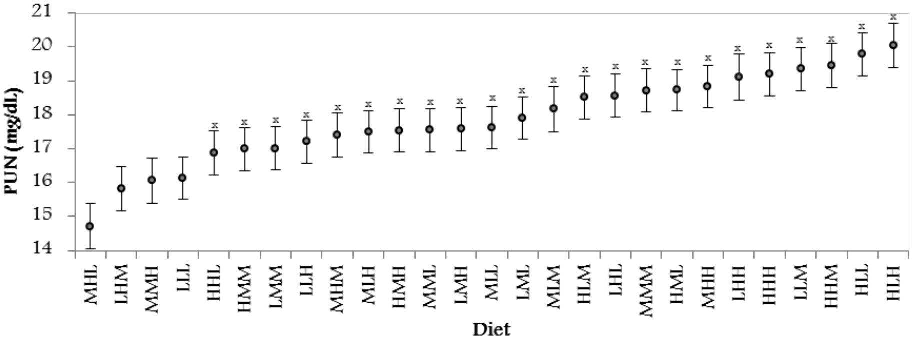

Figure 3.1: Plasmatic urea nitrogen (PUN) at 21:00h according to the experimental diet. First, second and third letters indicate lysine, sulphur amino acids and threonine levels respectively (H: high; M: medium; L: low). Least square means ± standard errors. [X] Value is significantly higher than the minimum, obtained with MHL diet (P<0.05)

From results at 21:00h, when there is not caecotrophy interference, MHL diet seemed the candidate to be the best balanced diet in order to minimize PUN. Although other three diets (LHM, MMH and LLL diets) induced PUN values not significantly higher than MHL diet, there are some indications in the results obtained at 8:00h that these diets would be suboptimal. Thus, L lysine diets (as LHM and LLL diets) caused higher PUN values at 8:00h probably because of a lysine deficiency not compensated with supply from soft faeces; according the literature (Nicodemus et al., 1999; García et al., 2004, 2005) lysine content in soft faeces averages 4.9% of CP, although with some diets this value is low than in L lysine diets (4.3% of CP). Moreover, increasing threonine level (as in LHM and MMH diet) provoked higher PUN values at 8:00h compared to L threonine diets, which could be interpreted as a result of an excess of threonine; in fact, soft faeces are particularly rich in threonine, the only essential amino acid to whose supply they contribute significantly more than to CP supply (Nicodemus et al., 1999), averaging 5.5% of CP according the above cited literature and usually being higher than in H threonine diets (5.3% of CP).

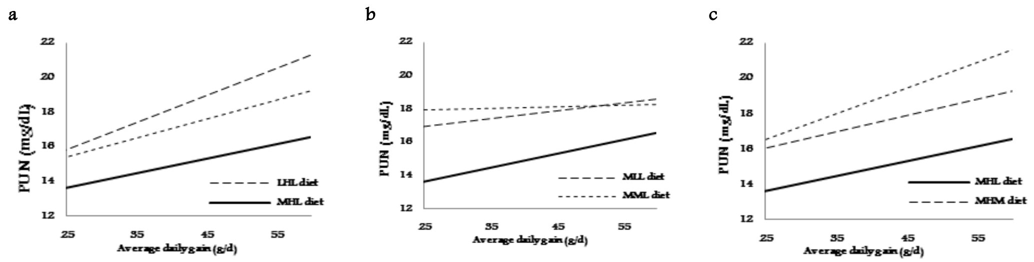

Figure 3.2: Relationship between plasmatic urea nitrogen (PUN) at 21:00h with average daily gain for MHL diet (bold line) compared with diets varying only in the level of one amino acid (a: lysine; b: sulphur amino acids; c: threonine). First, second and third letters indicate lysine, sulphur amino acids and threonine levels respectively (H: high; M: medium; L: low)

On the other hand, PUN at 21:00 h increased with average daily gain, probably as a consequence of increasing feed intake. Interestingly, MHL diet minimized PUN throughout the entire average daily gain range, compared with diets varying only in the level of one amino acid, suggesting that the requirements in the studied amino acids, expressed as a proportion of DM, would not be dependent on growth rate.

Taking into account than PUN could be an adequate indicator to detect amino acid imbalances in growing rabbit diets (Marín-García et al., 2019b), the results of the current study suggest that MHL diet could be the most adequate to meet lysine, sulphur amino acids and threonine requirements of growing rabbits, so that, in relation to the usual recommendations, the content of sulphur amino acids should be raised (to 6.6 g/kg DM) and that of threonine should be reduced (to 5.7 g/kg DM). Monteiro-Motta et al. (2013) obtained minimum urinary urea concentration with 6.6 g sulphur amino acids/kg DM.

Conclusions

Taking into account than PUN could be an adequate indicator to detect amino acid imbalances in growing rabbit diets , the total dietary amino acid combination that reduced PUN levels (with respect current recommendations) has 8.1, 6.6 and 5.7 g/kg on dry matter for lysine, sulphur amino acid and threonine, respectively. This combination could improve productive traits but in order to confirm this hypothesis, studies on the effects of MHL diet on growth performance and protein retention are necessary.

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PAPER 4

A new proposal for the lysine, sulphur amino acids and threonine recommendations for growing with high growth rate

Marín-García, P.J., Ródenas, L. Martínez-Paredes, E., Moya, V.J., Cambra-López M., Blas, E., Pascual, J.J.

Abstract

There are some evidences about the possible presence of some limiting amino acid in the diets for rabbits with high growth rates when current recommendations are used. In a previous trial, it was observed that certain combination for the first three limiting amino acids in rabbits minimized the levels of plasmatic urea nitrogen, which could indicate a better utilization of the dietary protein in growing rabbits. The main aim of this work was to evaluate if this new amino acid combination (called MHL) improves growth performance respect to current recommendations (called MMM) in growing rabbits with high growth rate. Both diets were formulated according with current recommendations for all nutrients except for lysine, sulphur amino acids and threonine. Diet MMM includes 8.1, 5.8 and 6.9 g/kg dry matter (DM) of total lysine, sulphur amino acids and threonine, respectively, while diet MHL includes 8.1, 6.6 and 5.7 g/kg DM, respectively. A total of 290 weaned rabbits (28 days old) from a paternal line selected for growth rate were used. . Apparent faecal digestibility of DM, crude protein (CP) and gross energy of the diets from 49 to 53 days of age, as well as the apparent ileal digestibility of DM, CP and amino acids at 63 days of age, were determined with 28 and 30 animals, respectively. Protein, energy and amino acids retained in the empty body during the growing period were also determined by slaughtering 15 weaning rabbits at 28 days of age and other 55 animals at 63 days of age. No significant differences in apparent faecal and ileal digestibility between diets were observed (except higher apparent ileal digestibility of threonine in diet MMM), but animals fed with diet MHL showed better global average daily gain (+2.3 g/d; P=0.0482) and feed conversion ratio (-0.10; P=0.0229) than those fed with the diet MMM. No significant differences between dietary treatments for nutrient retention per unit of empty body weight were observed. However, when animals were fed with diet MMM, the retained protein to retained energy ratio in the empty body weight gain decreased as daily empty body weight gain increased (P=0.0086), but this effect was not observed when fed with diet MHL. In conclusion, MHL combination for the first three limiting amino acids allows an improvement of performance in rabbits of high growth rate. For that reason, we propose as recommendations for rabbits of high growth rate 8.1, 6.6 and 5.7 g/kg DM of total and 5.3, 4.3 and 2.9 g/kg DM of apparent ileal digestible lysine, sulphur amino acids and threonine, respectively.

Keywords: Rabbit, growth rate, protein, amino acids, retention and digestibility.

Introduction

In growing rabbits, selection for feed efficiency has been indirectly done in paternal lines by selecting average daily gain (ADG), because there is a genetic negative correlation between these two traits (Blasco, 1989; Baselga, 2004). Considering the expected genetic progress in these paternal lines (0.45 g/d per generation and two generations per year; Piles and Blasco, 2003), in the last 20 years the ADG of these animals may have increased by 40% and there are currently animals with high ADG. In pararell, nutrients requirements of these high performing animals must have increased. However, since epizootic illness enteropathy onset in rabbits, the dietary protein content in commercial diets has been reduced (Trocino et al., 2013). The use of the current diets in growing rabbits from paternal lines could be affecting the expression of their genetic potential, and thus the correct elaboration of the rankings.

Recently, Marín-García et al. (2019a) observed that, although animals with ADG above 50 g/d showed a better feed conversion ratio (FCR), they had lower protein and higher energy retention to that expected. Carabaño et al. (2009) reported that dietary protein levels around 140 g/kg did not impair growth performance of growing rabbits with ADG up to 55 g per day, if DP/DE is around 9.5 and the amino acid supply is correct. However, the higher ADG of the current animals and their protein retention lower than expected could be suggesting the existence of some limiting amino acid when such diets are used in animals with high growth rates.

Subsequently, Marín-García et al. (2019b) observed that plasma urea nitrogen (PUN), which corresponds to the amount of nitrogen in form of urea circulating in the bloodstream, could be an adequate indicator to detect amino acid imbalances in growing rabbit diets and the adequate methodology to optimize its use for this aim was established. Lysine, sulphur amino acids and threonine have been considered the first three limiting amino acids in rabbits, and their requirements for growing rabbits are considered well-established (de Blas and González-Mateos, 2010). For this reason, using the PUN technique, Marín-García et al. (2019c) tested three dietary levels [M, medium; according with the current recommendations (de Blas and González-Mateos, 2010); H, high, + 15% of this recommendations; and L, low, −15%)] of these three limiting amino acids (lysine, sulphur amino acids and threonine) in a 3x3x3 factorial design. Their results established an amino acid combination that minimized the PUN levels in the growing rabbits (MHL for lysine, sulphur amino acids and threonine, respectively) with respect to the current recommendations (MMM).

On the other hand, feed formulation based on the amount of ileal digestible amino acids provide more precision compared to those based on total dietary or faecal digestible amino acids. Therefore, it would be advisable to have the ileal digestible amino acids content of the main raw materials as well as the ileal digestible amino acid requirements for the different vital and productive functions. In rabbits, information on the ileal digestible amino acids content of raw materials is available (Villamide et al., 2010a, 2010b), but there is scarce information on ileal digestible amino acid requirements (Carabaño et al., 2009), especially compared with other monogastric species. Dietary amino acids requirements were initially proposed by Lebas (1989), and updated by de Blas and González-Mateos (2010). Nevertheless, current recommendations are mainly expressed in total dietary or apparent faecal digestible amino acids.. Furthermore, rabbits have certain peculiarities as mainly the caecotrophy and itscontribution to protein supply, (Carabaño et al., 2000; Villamide et al., 2010a), which could hinder the proper evaluation of protein requirements of growing rabbits.

Table 4.1: Ingredients (g/kg) and chemical composition (g/kg dry matter) of the basal mixture and the experimental diets

Ingredients (g/kg)		Chemical composition (g/kg DM)	MMM	MHL
Wheat bran	300	Dry matter (DM; g/kg)	929	921
Straw	175	Ashes	70.7	71.2
Sunflower meal	167	Crude protein	155	157
Alfalfa hay	148	Gross Energy (MJ/kg DM)	18.01	18.23
Beet pulp	120	Ether extract	25.8	26.0
Barley	42	Neutral detergent fiber	456	460
Beet molasses	29	Acid detergent fiber	263	267
Palm oil	4.5	Acid detergent lignin	56.1	56.4
Sodium chloride	4.8	Digestible Energy[2] (MJ/kg DM)	10.85	10.98
Calcium carbonate	2.6	Digestible Protein[2]	113	115
L-Lysine HCL	2.4	Digestible Protein/ Digestible Energy (g/MJ)	10.41	10.47
Monocalcium phosphate	0.2	Alanine	5.61	5.62
Vitamin-trace element mixture[1]	5.2	Arginine	7.96	8.03
		Aspartic Acid	12.2	12.4
		Cystine	2.28	2.30
		Glutamic acid	24.8	26.3
		Glycine	7.27	7.23
		Histidine	2.91	2.94
		Isoleucine	5.32	5.27
		Leucine	9.25	9.33
		Lysine	8.10[3]	8.10[3]
		Methionine	3.50[4]	4.31[4]
		Phenylalanine	5.85	5.91
		Proline	8.14	8.21
		Serine	6.25	6.47
		Sulphur amino acids	5.78	6.61
		Threonine	6.90[5]	5.70[5]
		Tyrosine	2.88	2.99
		Valine	7.16	7.23

[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] Experimentally determined according to Perez et al. (1995). Using 14 helthy growing rabbits of 49 days of age per diet weaned at 28 days. Faeces were collected during 4 days after a period of 7 days of adaptation to diets. [345] Values were obtained by adding L-Lysine HCL, DL-Methionine and L-Threonine, respectively.

The objective of this work was to know if the amino acid combination for the three first limiting amino acids (MHL), which minimized PUN in Marín-García et al. (2019c), could improve the performance and protein retention of growing rabbits with high growth rate respect to a diet formulated with current recommendations (MMM). In addition, evaluating both diets at apparent ileal level, we will try to provide greater knowledge on protein requirements in growing rabbits.

Material and methods

The experimental procedure was approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València and carried out following the recommendations of the European Group on Rabbit Nutrition (Fernández-Carmona et al., 2005) and Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes (Boletín Oficial del Estado, 2013).

Experimental Diets

Table 4.1 shows the ingredients and chemical composition of the experimental diets used in this work. The basal mixture was formulated according with the current recommendations (de Blas and González-Mateos, 2010), with the exception of the three most limiting amino acids (lysine, sulphur amino acids and threonine) where their content were lower. The experimental diets were obtained by the addition of industrial amino acids (L-Lysine HCL, DL-Methionine and L-threonine) in the basal mixture to achieve the values of current recommendations (de Blas and González-Mateos,

2010) in MMM diet (8.10, 5.78 and 6.90 g/kg DM for lysine, sulphur amino acids and threonine, respectively), or the dietary levels which reduced PUN in a previous trial (Marín-García et al., 2019c) in MHL diet (8.10, 6.61 and 5.70 g/kg DM for lysine, sulphur amino acids and threonine, respectively). A version of the diets including 5 g/kg DM of alfalfa hay marked with ytterbium was also manufactured.

Animals

Two-hundred ninety weaned rabbits (28 days old) from the R line were used. Line R was obtained after two generations of randomly mating from a pool of animals of three commercial sire lines (Estany et al., 1992), and then selected by ADG in the growing period during 38 generations. This line is characterized by a high growth rate during the growing period, and it has been developed at the Institute of Animal Science and Technology of the Universitat Politècnica de València.

Experimental procedure

The experimental procedure was carried out during one year (in different batches), under a controlled environment (animals were kept at 15ºC to 22ºC, with a photoperiod of 12 hours of light and 12 hours of darkness). At 28 days of age, 15 weaned rabbits were slaughtered by intracardiac puncture with sodium thiopental [75 mg/kg of live weight (LW)] to determine the empty body characteristics at this age. The rest of the animals (n=275) were housed in individual cages and assigned to one of the two experimental diets, which were provided ad libitum received until 63 days of age. Mortality and morbidity (presence of diarrhea) was daily controlled, and feed intake and LW were weekly registered. Animals presenting any digestive anomaly, weight loss or low ingestion were discarded.

At 42 days of age, 28 randomly selected animals were housed in individual metabolic cages of 52x44x32 cm and, after a week of acclimatization, a faecal digestibility trial was conducted according Perez et al. (1995). From 49 to 53 days of age, feed consumption was controlled and faeces produced were collected. Faeces were stored in identified plastic bags and frozen at -20ºC until analysis. From 53 days of age, 30 randomly selected animals began to receive the same feed but marked with ytterbium until slaughtering at 63 days of age. At this age, 55 randomly animals (including the above mentioned 30 animals) were weighed and slaughtered by intracardiac injection of sodium thiopental (75 mg kg^{-1}LW) between 19:00 to 23:00 h, to minimise the influence of caecotrophy on the composition of digestive contents (Merino and Carabaño, 2003). Samples of ileal content were obtained from the distal part of the small intestine (around 20-30 cm before the ileo-caeco-colic valve) for each animal receiving the market feeds, frozen at -20ºC, freeze-dried and ground. The whole digestive tract was emptied and reintroduced into the body of the slaughtered animals. Empty bodies, obtained at 28 and 63 days of age (15 and 55 animals, respectively) were weighed and placed in plastic bags, identified and frozen at -40ºC. Frozen empty bodies were crushed and homogenized in a cutting machine

(Tecator, Abusson, France), and a sample per animal was freeze-dried and stored at -40ºC until analysis.

Chemical analysis

Feed was analysed for DM, ashes, CP, neutral detergent fibre (aNDFom), acid detergent fibre (ADFom), lignin (sa), starch, gross energy (GE) and amino acid content. Faeces were analysed for DM, CP and GE, while ileal samples for DM, CP and amino acid content. Finally, empty bodies samples were analysed for DM, CP, GE and amino acid content. Samples were analysed according to the methods of AOAC (2000): 934.01 for DM, 942.05 for ash and 976.06 for CP. The aNDFom (assayed with a thermo-stable amylase and expressed exclusive of residual ash), ADFom (expressed exclusive of residual ash) and lignin (determined by solubilisation of cellulose with sulfuric acid) were analysed sequentially (Van Soest et al., 1991). GE was determined by adiabatic bomb calorimetry (Gallenkamp Autobomb, Loughborough, UK).

The amino acid content was determined after acid hydrolysis with HCL 6N at 110ºC for 23 h as previously described by Bosch et al. (1995), using a Waters (Milford, Massachusetts, USA) HPLC system consisting of two pumps (Mod. 515, Waters), an autosampler (Mod. 717, Waters), a fluorescence detector (Mod. 474, Waters) and a temperature control mod- ule. Aminobutyric acid was added as internal standard after hydrolyzation. The amino acids were derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and separated with a C-18 reverse-phase column Waters AcQ Tag (150 mm × 3.9 mm). Methionine and cystine were determined separately as methionine sulphone and cysteic acid respectively after performic acid oxidation followed by acid hydrolysis (Alagón et al., 2016).

Calculations and statistical Analysis

From the information obtained from those weaned rabbits slaughtered at 28 days of age (empty body weight and CP, GE and amino acids contents in the empty body), there were fitted linear regression equations using LW at 28 days of age as independent variable for each of them. Values for all these variables at 28 days of age for the animals slaughtered at the end of retention trial (63 days of age) were estimated for each animal with these mentioned equations to properly determine nutrients retention during the growing period.

Data on performance [LW, ADG, daily feed intake (DFI) and FCR] were analysed using a MIXED model of SAS (SAS Institute, 2009) taking into account the lack of homoscedasticity of data. The model included the diet, the batch, the week and their interactions as fixed effects. Data from apparent digestibility coefficients (both faecal and ileal) and nutrients retained in the empty body of the animals (energy, protein and amino acids) were analysed using a GLM model of SAS, including the diet as the only fixed effect. Some comparisons between slopes were also made, using a SAS GLM procedure.

Results

The effect of the experimental diet on the main coefficients of apparent faecal and ileal digestibility are presented in Table 4.2. No significant differences in apparent faecal and ileal digestibility between diets were observed, with the exception of the coefficient of the apparent ileal digestibility of threonine, which was higher for the diet MMM than MHL (+0.14; P=0.0408).

Regarding the health status of the rabbits during the growing period, there were no significant differences on both mortality and morbidity of the rabbits between the dietary treatments, being on average 24.3 and 11.1%, respectively. Table 4.3 shows the data regarding the performance in healthy animals. Daily feed intake and live weight of animals were not significantly different between diets. Nevertheless, animals fed with the diet MHL showed better global ADG (+2.3 g/d; P=0.0482) and global FCR (-0.10; P=0.0229) than those fed with the diet MMM. These global differences are mainly due to the best ADG and FCR observed in the animals from 49 to 63 days of life (on av. +5.6 g/d and -0.29, respectively; P<0.05), with no significant differences being observed previously.

Energy, protein and amino acids retained per g of empty body weight gain during the growing period for each experimental diet are presented in Table 4.4. No significant differences between diets were observed.

Finally, Figure 4.1 shows the relationship between the daily empty body weight gain (DEBWG) and the retained protein to retained energy ratio obtained for each diet. In the case of animals fed with the diet MMM, an increase of DEBWG decreased this (-0.283 g/MJs per g of DEBWG; P=0.0086). However, no significant effect of

DEBWG on this ratio was observed (P=0.2692), when animals were fed with the diet MHL.

Table 4.2: Coefficients of apparent faecal (n=28) and ileal (n=30) digestibility in function of the experimental diet (Least square mean ± standard error).

	MMM	MHL	P-value
Faecal coefficients: (49 to 53 days old)			
Dry matter	0.547 ± 0.006	0.533 ± 0.005	0.0770
Crude protein	0.733 ± 0.007	0.731 ± 0.008	0.1122
Gross energy	0.606 ± 0.005	0.599 ± 0.005	0.2806
Ileal coefficients: (63 days old)			
Dry matter	0.395 ± 0.025	0.439 ± 0.028	0.2577
Crude protein	0.576 ± 0.036	0.556 ± 0.037	0.6957
Alanine	0.591 ± 0.046	0.552 ± 0.052	0.5612
Arginine	0.805 ± 0.027	0.753 ± 0.031	0.2373
Aspartic acid	0.618 ± 0.049	0.561 ± 0.057	0.4548
Cystine	0.416 ± 0.052	0.552 ± 0.061	0.1064
Glutamic acid	0.781 ± 0.033	0.731 ± 0.038	0.3151
Glycine	0.344 ± 0.052	0.339 ± 0.064	0.9575
Histidine	0.753 ± 0.033	0.684 ± 0.038	0.1761
Isoleucine	0.658 ± 0.049	0.596 ± 0.056	0.4048
Leucine	0.664 ± 0.049	0.606 ± 0.055	0.4234
Lysine	0.708 ± 0.050	0.600 ± 0.058	0.1541
Methionine	0.741 ± 0.027	0.740 ± 0.034	0.9795
Phenylalanine	0.691 ± 0.044	0.635 ± 0.051	0.4014
Proline	0.678 ± 0.029	0.690 ± 0.033	0.7828
Serine	0.521 ± 0.041	0.461 ± 0.060	0.4440
Threonine	0.645 ± 0.042	0.507 ± 0.048	0.0408
Tyrosine	0.549 ± 0.050	0.505 ± 0.057	0.5667
Valine	0.622 ± 0.045	0.578 ± 0.050	0.5106

Table 4.3: Performance of the growing rabbits in function of the experimental diet (n=161) (Least square mean ± standard error).

	MMM	MHL	P-value
Live weight (g)			
Day 28	587±37.0	606±36.8	0.6949
Day 35	901±36.8	922±36.5	0.6830
Day 42	1302±28.2	1325±27.4	0.5446
Day 49	1737±28.3	1749±27.4	0.7656
Day 56	2134±31.8	2168±31.2	0.4184
Day 63	2514±34.9	2592±34.5	0.0969
Feed intake (g/d)			
Week 1	78±2.61	83±1.92	0.1522
Week 2	120±3.29	122±3.28	0.5936
Week 3	164±2.94	162±2.90	0.6455
Week 4	193±3.78	194±3.78	0.8366
Week 5	212±3.89	215±3.90	0.6534
Global	151±2.56	151±2.36	0.9605
Average daily gain (g/d)			
Week 1	44.32±1.38	45.25±1.39	0.6277
Week 2	56.69±1.52	57.80±1.53	0.6015
Week 3	61.56±1.49	60.80±1.50	0.7115
Week 4	55.98±1.34	60.14±1.35	0.0255
Week 5	53.56±1.54	60.66±1.55	0.0010
Global	54.61±0.84	56.89±0.77	0.0482
Feed conversion ratio			
Week 1	1.75±0.04	1.86±0.04	0.0609
Week 2	2.13±0.03	2.12±0.03	0.9605
Week 3	2.70±0.06	2.71±0.06	0.9354
Week 4	3.48±0.07	3.26±0.07	0.0286
Week 5	3.99±0.08	3.64±0.08	0.0036
Global	2.75±0.03	2.65±0.03	0.0229

Table 4.4: Retained nergy (kJ, protein (g) and amino acids (g) per g of empty body weight gain during the growing period for each experimental diet (n=55) (Least square mean ± standard error).

	MMM	MHL	P-value
Energy	8.999 ±0.239	8.679 ±0.239	0.3493
Protein	0.2104 ±0.0043	0.2024 ±0.0043	0.1898
Alanine	0.0099 ±0.0003	0.0096 ±0.0003	0.3712
Arginine	0.0147 ±0.0005	0.0150 ±0.0005	0.7518
Aspartic acid	0.0150 ±0.0005	0.0148 ±0.0005	0.8158
Cystine	0.0036 ±0.0002	0.0035 ±0.0002	0.6560
Glutamic acid	0.0241 ±0.0008	0.0247 ±0.0008	0.6029
Glycine	0.0170 ±0.0004	0.0161 ±0.0004	0.1516
Histidine	0.0040 ±0.0002	0.0040 ±0.0003	0.9822
Isoleucine	0.0069 ±0.0001	0.0069 ±0.0001	0.9041
Leucine	0.0138 ±0.0003	0.0138 ±0.0003	0.8959
Lysine	0.0121 ±0.0004	0.0120 ±0.0004	0.9501
Methionine	0.0040 ±0.0001	0.0038 ±0.0001	0.1526
Phenylalanine	0.0069 ±0.0002	0.0069 ±0.0002	0.9542
Proline	0.0097 ±0.0003	0.0096 ±0.0003	0.7890
Serine	0.0086 ±0.0003	0.0089 ±0.0003	0.5548
Threonine	0.0074 ±0.0003	0.0077 ±0.0002	0.4507
Tyrosine	0.0055 ±0.0003	0.0057 ±0.0003	0.5733
Valine	0.0093 ±0.0002	0.0094 ±0.0002	0.8986

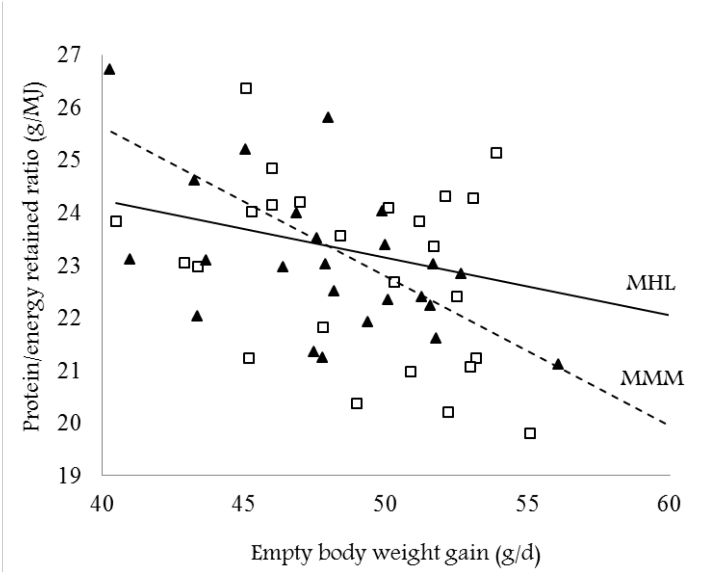

Figure 4.1: Relationship between daily empty body weight gain with the retained protein to retained energy ratio in the empty body weight gain in function of the experimental diet: ▲ MMM and □ MHL.

Discussion

Our initial hypothesis was that the combination of amino acids (diet MHL) that minimized PUN in growing rabbits in a previous study (Marín-García et al., 2019c) should also be the one that would optimize the dietary protein utilization and would improve the growth performance with respect to the current recommendations (diet MMM). As expected, average coefficients of both diets for apparent faecal and ileal digestibility were similar,. The only significant difference found between the diets was a higher apparent ileal digestibility coefficient for threonine in diet MMM (P=0.0408), which includes a greater amount of industrial L-threonine. This result may be a consequence of the high digestibility of the industrial amino acids added in the diet. Taboada et al. (1996) and de Blas et al. (1998) observed that faecal apparent digestibility of the supplemented DL-methionine and L-threonine were almost complete (102.9±0.9 and 93.0±6.0 %, respectively). In fact, Delgado et al. (2019), evaluating the effect of dietary supplementation with arginine and glutamic acid, observed an increase in the ileal digestibility of such amino acids when increasing the contribution of those from industrial origin. However, diet MHL did not show better digestibility of methionine, in spite of including a greater amount of industrial DL-methionine than the diet MMM..

On average, performance was similar to that obtained for this line in previous studies (García-Quirós et al., 2014; Mínguez et al., 2016; Marín-García et al., 2019a). As expected, growing rabbits from this paternal line presented high ADG (averaging almost 56 g/d) and low FCR (about 2.7), achieving about 2.55 kg of LW at 63 days of age. Thus, this population of animals can allow us to correctly test our initial hypothesis: could a diet, formulated to minimize PUN (−15%; Marín-García et al., 2019c), improve the performance in rabbits characterised by a high growth rate? The answer was yes. Growing rabbits fed with diet MHL showed better ADG and FCR during the last two weeks of growingperiod than those fed with a diet formulated followingthe current recommendations (diet MMM). A reduction of the PUN could be an indicator of a better fitting to the animal requirements, increasing both protein and energy available for growth (Bohinski, 1979; Ottaway and Apps, 1984). The reason why the differences in growth traits were mainly observed at the end of the fattening period may be due to different causes. It may be because the choice of diet that minimized PUN was made at 48 days of age (Marín-García et al., 2019c), this diet being more representative of the amino acid requirements at that age. But it could also be due to a greater impact of an adequate dietary amino acid content on growth at that age. Lean content and lean-to-bone ratio of rabbit carcass increases almost steadily from weeks 1 to 15 of age (Deltoro and López, 1985).

An increase in ADG is usually associated with an increase in the total amount of protein retained in the empty body (Gidenne and Perez, 2000; Birolo et al., 2016; Marín-García et al., 2019a). However, the content in energy, protein and amino acids per unit of empty body weight gain was similar with both diet, indicating that the composition of empty body remained unaltered. In fact, the content of sulphur amino acids and threonine relative to lysine in the empty body (0.619 and 0.620, respectively) was similar to that observed thirty years ago (0.615 and 0.640, respectively; Mouhgan et al., 1988). When slow and fast growth lines are compared in broiler chickens, the recommendations of lysine per kg of feed do not increase, only the daily requirements increase (Han and Baker, 1991; 1993). However, some other studies show that the recommendations of sulphur amino acids (especially cystine, very abundant in feathers) are higher in the fastest growing lines compared to those of slow growth (Kalinowski et al., 2003a; 2003b). In our work, animals fed with diet MMM reduced their retained protein to retained energy ratio in the empty body as growth rate increases (Figure 4.1), as previously observed by Marín-García et al. (2019a) with a diet with very similar in lysise, sulphur aminoacids and threonine content. This fact evidences the existence of some limiting amino acid, which would derive a greater amount of amino acids as energy source, increasing PUN. However, this effect is not significant when we use the diet MHL, being perhaps this new combination of amino acids the optimal one under current conditions.

In view of these results, it could be interesting to assess and compare the current amino acid recommendations (de Blas and González-Mateos, 2010) with a new proposal, at total as well as apparent ileal digestible content, to provide more accurate information, which allows us to formulate diets for growing rabbits more fitted to their current requirements. In the case of lysine, no change is proposed in the total content (8.1 g/kg DM). This value is quite similar to those recommended by other authors (Adamson and Fisher, 1973; Taboada et al., 1994), although higher to that proposed by the NRC in 1977 (6.7 g/kg DM). Assuming the average apparent ileal digestibility of lysine obtained in the curren work (0.65), a value of 5.3 g of apparent ileal digestible lysine/kg DM may be proposed. The improved growth performance obtained in our work with dietary sulphur amino acid content (6.6 g/kg DM) higher than current recommendation (5.8 g/kg DM) have already been observed by other authors (Adamson and Fisher, 1973; NRC, 1977; Berchiche and Lebas, 1994; Taboada et al., 1996), which obtained greater performance with sulphur amino acid content from 6.7 to 8.0 g/kg DM, that could be indicating that current recommendation possibly underestimates the sulphur amino acid requirements of growing rabbits. Therefore, a proposal of 6.6 g/kg DM can be made in the case of sulphur amino acids and, assuming the average apparent ileal digestibility of cystine and methionine obtained in the curren work (0.48 and 0.74. respectively), a value of 4.3 g of apparent ileal digestible sulphur amino acids/kg DM may be also proposed. Finally, in the case of threonine, the current recommendation is 6.9 g/kg DM, closer to those proposed by NRC (1977) and de Blas et al. (1988) (6.7 g/kg DM). However, Adamson and Fisher, (1973) proposed lower values for the threonine (5.6 g/kg DM), quite similar to our proposal of 5.7 g/kg DM and, assuming the apparent ileal digestibility of threonine in the diet MHL (0.51), 2.9 g of apparent ileal digestible threonine/kg DM. A possible explanation for discrepancies in the threonine recommendations may be both in the contribution of caecotrophy or in possible interactions with other amino acids in the diet, as glycine. We have to take into account that the contribution of soft faeces to the total threonine intake is the highest of all amino acids (Nicodemus et al., 1999), being able to alleviate its inclusion in the diet.

Conclusions

Compared to the current recommendations (de Blas and Mateos, 2010),the combination of the first three limiting amino acids that minimized the PUN level in the work of Marín-García et al. (2019c) allows an improvement of ADG and FCR in growing rabbits of high growth rate,without changes in the energy, protein and amino acid retention per unit of empty body weight gain. For that reason, we propose new recommendations for rabbits of high growth rate: 8.1, 6.6 and 5.7 g/kg DM of total lysine, sulphur amino acids and threonine, respectively; and 5.3, 4.3 and 2.9 g/kg DM of apparent ileal digestible lysine, sulphur amino acids and threonine, respectively.

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GENERAL DISCUSSION

At the end of the introduction it was exposed that the main objective of this thesis was to provide new amino acid recommendations for the current growing rabbits with high growth rate, using plasmatic urea nitrogen (PUN) as a tool to determine amino acid imbalances, aiming to improve protein utilization, and consequently, growth performance, health status and nitrogen excretion during the growing period. However, to achieve this goal, it was necessary to establish specific objectives. In this general discussion let's address each of them.

O.1. To assess the effect of a common diet on protein digestibility and retention in growing rabbits selected by growth rate, and to obtain some indices revealing the presence of limiting amino acids.

Prior to the development of this thesis, Carabaño et al. (2010) observed that current dietary protein level (around 140 g of crude protein/kg feed) are adequate for crossbred animals, and it did not impair growth performance up to 55 grams of average daily gain. However, the results from the Paper 1 revealed a new circumstance: when they were fed with a common moderate protein diets, animals with higher growth rate proportionally retained less protein than those with low growth rate , allocating the remaining amino acids as a source of energy (Figure 5.1a). This fact can be related to possible presence of some limiting amino acid in animals with high growth rates when fed with the current diets.

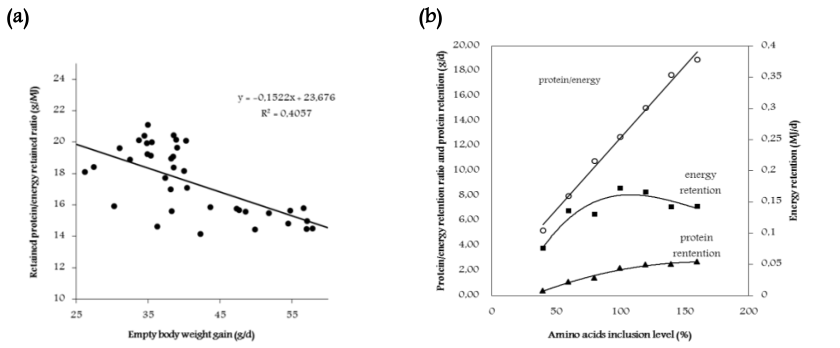

Figure 5.1: (a) Relationship between daily empty body weight gain (g/d) with the retained protein to retained energy ratio (g/MJ) in growing rabbits from 28 to 63 days of age. (b) Relationship between the amino acids inclusion level (% respect to recommendations) with the protein retention, energy retention and retained protein to retained energy ratio in chicken broilers from 8 to 21 days of age (Velu et al., 1971).

In fact, as early as 1971, Velu et al. observed that, when inducing an imbalance in the dietary level of amino acids in chicken broilers, although it led to a reduction of both the protein and the energy retained in the whole body of the animals, the retained protein to retained energy ratio fell significantly (Figure 5.1b), as in the present work in the case of animals with high growth rate (from line R, a paternal line). Therefore, our results can be compatible with the idea of the possible presence of some limiting amino acid in growing rabbits with high growth rates when fed with the current diets.

O.2. <u>To investigate the viability of PUN as an indicator of amino acid imbalance in rabbit feeds, and to establish the best methodology to use it.</u>

The potential interest of PUN as indicator of an amino acid imbalance has already been studied in other species such as pigs (Brown and Cline, 1974) or broilers (Donsbough et al., 2010).

Nevertheless, there exist enough peculiarities in rabbits' physiology (feeding behaviour and caecotrophy; Bellier et al., 1995; Villamide el at., 2010) that could affect its potential utilization. For this reason, it was necessary to check if PUN could be a metabolite useful to detect amino acid imbalances in growing rabbit diets. Paper 2 concluded that PUN could be used to detect lysine imbalances in growing rabbits. Blood samples obtained from animals fed with a balanced diet had clearly less PUN than when these same animals (latin square design) were fed with unbalanced diet. Furthermore, it was established the best moment to detect deficiencies as between 04:00h and 12:00h in growing rabbits when fed under ad libitum management, and at three hours after feeding reestablishment (21:00h) when a previous fasting period of 10h was applied (Figure 5.2).

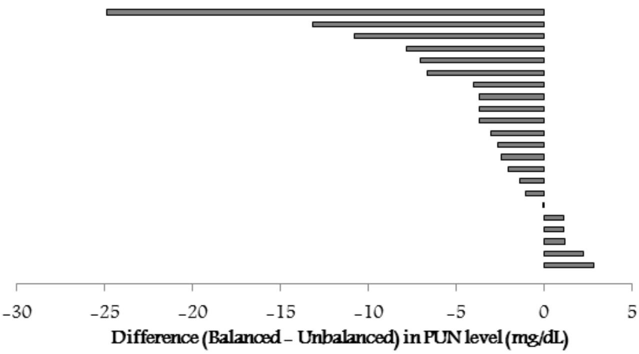

Figure 5.2: Individual differences in plasmatic urea nitrogen (PUN) obtained three hours after feeding reestablishment (21:00h) with balanced and unbalanced diets.

O.3. To determine the combination of the main limiting amino acids in growing rabbits (lysine, sulphur amino acids and threonine) able to reduce PUN.

Once determination of PUN was established as an adequate tool to detect possible amino acid imbalances, and with the evidences found that growing rabbits selected for growth rate could have different amino acid requirements than animals with a lower growth potential, it was the time to check what combination for the first three limiting amino acids allows the lowest PUN in the animals. Diet MHL lead animals to 13% less of PUN (Figure 5.3; P<0.05) that those with diet MMM (current recommendations). Diet MHL was formulated with the current total level of lysine (M), 15% more of sulphur amino acids (H) and 15% less of threonine (L). A priori, PUN reduction has some implications: more protein disposable and even more energy available (Ottaway and Apps, 1984; Bohinski, 1979; Leningher et al., 1983) and probably it would be an indicator of a better dietary protein utilization by animals. However, could this new combination to improve the performance of growing rabbits with high growth rate? An affirmative answer to this question would confirm your candidacy to overcome the current recommendations.

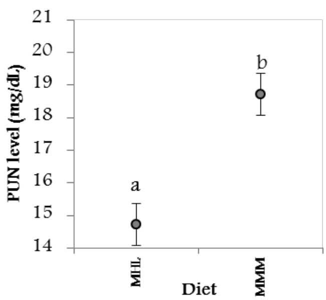

Figure 5.3: Mean and standard error of plasmatic urea nitrogen (PUN) of growing rabbits obtained with the current recommendations (Diet MMM) and with the choosed combination (Diet MHL) of lysine, sulphur amino acids and threonine. Means within different letter were significantly different (P<0.05).

O.4. To compare that combination obtained in O.3 with the current recommendations, regarding their effects on growth parameters.

Finally, to establish the new amino acid recommendations at ileal level, if aplicable.

In order to evaluate the possible effect on the growth traits and body protein retention of this new amino acid combination (diet MHL), a growing trial was performed in Paper 4. Animals fed with diet MHL showed better average daily gain and feed conversion ratio ($P<0.05$) than those fed with diet MMM, according to current recommendations (de Blas and González-Mateos, 2010). These results showed that current growing rabbits, characterized by high growth rates, could better express all their genetic potential with this new amino acid combination, being a candidate to be considered as new recommendations. In addition, in this work faecal and ileal digestibility trials were performed to determine the recommendations for these three amino acids at an apparent faecal and ileal digestible level. All values as total, apparent faecal and ileal apparent digestibible content obtained with the current and the new recommendations are summarize in Table 5.1.

Table 5.2 summarizes the comparison of total amino acid content between current recommendations and other studies in growing rabbits. In the case of lysine, literature values agreed, being similar current recommendations (de Blas and González-Mateos, 2010) to those published in other previous studies (Adamson and Fisher, 1973; Taboada et al., 1994). This suggest that lysine requirements (8.1 g/kg DM) are well established for growing rabbits, independently of their growth potential.

Table 5.1: Current and new recommendations (g/kg dry matter) for the first three limiting amino aci

	MMM (current recommendation)			MHL (new	
	Lysine	Sulphur amino acids	Threonine	Lysine	Sulph
Total	8.1[1]	5.8[1]	6.9[1]	8.1[2]	
Apparent faecal digestible	6.3[1]	4.4[1]	4.8[1]		
Aparent ileal digestible	5. 3[2]	3.7[2]	4.5[2]	5. 3[2]	

1 Current recommendations of de Blas y González-Mateos (2010). 2 Data obtained in the present work.

Table 5.2: Total dietary amino acid (Lysine = Lys; Sulphur Amino acid = sAA; Threonine = Thr) requirements (g/kg dry matter) different studies.

	De Blas and González-Mateos (2010)*	Taboada et al. (1994)	Taboada et al. (1996)	De Blas et al. (1998)	Adamson and Fisher (1973)	Berchich Lebas (1
Lys total	8.1	8.4 (+0.3)			7.8 (-0.3)	
sAA total	5.8		8.0 (+2.2)		6.7 (+0.9)	6.9 (+1
Thr total	6.9			6.7 (-0.2)	5.6 (-1.3)	

*: Current recommendations; (In parenthesis appears de difference between this work and current recommendations). Differe recommendations are expressed between brackets ().

In the case of sulphur amino acids, data seem suggest that current recommendations could underestimatd the requirements for growing rabbits with high growth rate. In fact, the results of this work shows that, an increment of 0.8 g/kg DM of sulphur amino acids improved growth performance. These higher requirements agrees with that observed in other previous studies (Adamson and Fisher, 1973; NRC, 1977; Berchiche and Lebas, 1994; Taboada et al., 1996).

In the case of threonine, the data are suggesting that it would be appropriate to reduce total levels on 1.2 g/Kg DM with respect current recommendations (de Blas and González-Mateos, 2010). These low threonine levels are similar with data provided by Adamson and Fisher (1973), where maximum performance was obtained with 5.6 (-1.3 g/kg DM).

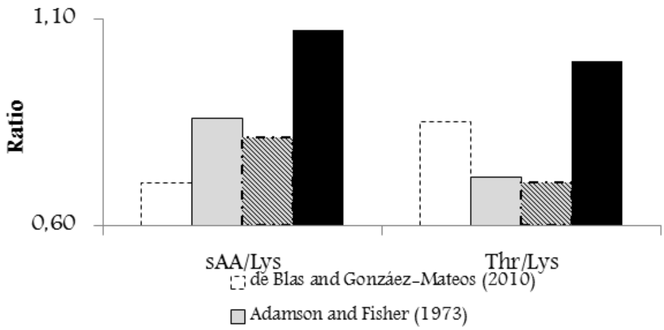

Figure 5.4. Recommended total dietary sulphur amino acids (sAA) and threonine (Thr) to lysine (Lys) ratios from different studies.

Finally, in Figure 5.4, the recommended total dietary sulphur amino acids and threonine to lysine ratios from different studies are represented. As it can be observed, Adamson and Fisher (1973), NRC

(1997) and the current work show a common recommendation, a sulphur amino acids to lysine ratio higher than the threonine to lysine ratio. Only current recommendations proposed the opposite. All of these data could be indicating a possible underestimation of the current sulphur amino acids requirements while, in the case of threonine, its requirements could have been overestimated (probably due to the great contribution of caecotrophy to threonine supply).

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CONCLUSSIONS

The main conclusions of this thesis are:

- Growing rabbits with a high growth rate did not show more digestive efficiency for protein. These animals retained less protein than expected when moderate protein diet is used. This fact seems to be caused by some limiting amino acid.
- Plasmatic Urea Nitrogen (PUN) could be an adequate indicator to detect deficiencies in limiting amino acids in growing rabbit diets.
- The dietary amino acid combination that minimized PUN was that including 8.1, 6.6 and 5.7 g/kg on dry matter (DM) for total lysine, sulphur amino acids and threonine, respectively.
- The previous combination that reduced the levels of PUN allowed an improvement of average daily gain and feed conversion ratio in growing rabbits of high growth rate, compared to the current recommendations.

Based on the main conclusions, we advise to change the current combination of the three typically limiting amino acids for rabbits characterized by high growth rate. This adjustment will directly improve protein utilization, and consequently, could contribute to optimize growth rate, feed conversion ratio and N excretion and genetic selection, without negative effects on health status. The new proposed levels are:

- 8.1, 6.6 and 5.7 g/kg DM of total lysine, sulphur amino acids and threonine, respectively.
- 5.3, 4.3 and 2.9 g/kg DM of apparent ileal digestible lysine, sulphur amino acids and threonine, respectively.

PERSPECTIVES

The perspectives of the thesis are:

- Plasmatic urea nitrogen (PUN) is a metabolite, which determination is fast and cheap. In this thesis has been demonstrated its viability as a tool to detect imbalances in growing rabbit diets. Hereinafter, we would like to spread its use in rabbit nutrition in order to optimize performance, nitrogen excretion, enteropathy incidence, etc.
- Although protein nutrition has been improved (providing more information on amino acid requirements), we thought it would be interesting to continue in this line to further refine the amino acid requirements according to the growth rate. In other words, go towards a precision protein nutrition.
- Assuming that the ideal amino acid profile could be achieved with this thesis; is the digestible protein to digestible energy ratio currenty recommended the correct one for the current growing rabbits?

It seems that this thesis provokes more questions than it answers, but this is the beauty of science.

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COURSES

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LPR: Teaching activity	2
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English B2.1	60
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Public administration procedure	20
Critical thinking	10
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XXXIII Animal nutrition	13
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Research results protection	20
Cientific divulgation strategies	20
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Effective time management	50
Environmental management in company	90
Management skills	35
Teams leadership and management	60
Social networks	75
Nutrition and dietetics	40
Power Point 2007 advance	90
Animal euthanasia	24

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- **Marín-García P.J.,** López M.C., Ródenas L., Martínez-Paredes E., Blas E., Pascual J.J., 2018. Effect of the levels of lysine, sulphur amino acids and threonine in diets for rabbits with high growth rates. 69th Annual Meeting of the European Federation of Animal Science. Dubvrovnik, Croatia. 1, 586.

- Moreno-Grúa E., Pérez-Fuentes S., Muñoz- Silvestre A., Viana D., Selva L., Arnau-Bonachera A., Martínez-Paredes E., **Marín-García P.,** Pascual J.J., Corpa J.M. 2019. Efecto de la selección genética y la vitrificación en los parámetros inmunológicos tras una infección con staphylococcus aureus. XVI congress of iberian society of cytometry. Salamanca, España. 1,4,

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CONFERENCES

- **Marín-García P.J.**, López M.C., Ródenas L., Martínez-Paredes E., Blas E., Pascual J.J., 2019. Dietary protein optimization using a rabbit model: towards a more sustainable production in nitrogen contamination. 44th Annual meeting of ASESCU. Aranda del Duero, España. 1, 47-50.

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- Moreno-Grúa E., Pérez-Fuentes S., Muñoz- Silvestre A., Viana D., Selva L., Arnau-Bonachera A., Martínez-Paredes E., **Marín-García P.**, Pascual J.J., Corpa J.M. 2019. How genetic selection affects growth rates in leukocyte populations of breeding females of a parental line. 44th Annual meeting of ASESCU. Aranda del Duero, España. 1, 75-79.

- **Marín-García P.J.**, López M.C., Ródenas L., Martínez-Paredes E., Blas E., Pascual J.J., 2019. Dietary protein optimization using a rabbit model: towards a more sustainable production in nitrogen contamination. 70th Annual Meeting of EAAP. Ghent, Belgium. 1, 493.

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- Arnau-Bonachera, A., **Marín-García, P.J.**, Blas, E., Pascual, J.J., 2016. Aditivos empleados en nutrición de conejos. Boletín de cunicultura. 179: 29-32.

- **Marín-García, P.J.**, Blas, E., Pascual, J.J., 2018. Nuevas recomendaciones en aminoácidos para mejorar el índice de conversión. Boletín de cunicultura. 189, 16-19.

- **Marín-García, P.J.**, Pascual, J.J., 2019. Is it possible to improve the gastrointestinal health of fattening rabbits through protein nutrition? CuniNews. 21, 15-20.

- **Marín-García, P.J.**, Ródenas, L., Martínez-Paredes, E., Blas, E., Pascual, J.J. (SENT). Effects of low protein levels in the diet on digestibility and protein retention in growing rabbits in function of growth rate. J. Anim. Feed. Sci. Techn..

- **Marín-García, P.J.**, Ródenas, L., Martínez-Paredes, E., Blas, E., Pascual, J.J. (SENT). Plasma urea nitrogen as an indicator of amino acid imbalance in rabbit feeds. Archives of Animal Nutr.

- **Marín-García, P.J.**, Ródenas, L., Martínez-Paredes, E., Blas, E., Pascual, J.J. (M Preparation). Determination of the new recommendations of lysine, sulphur amino acids and threonine in diets of growing rabbits using plasmatic urean nitrogen. J. Anim. Feed. Sci. Techn.. Preparation

- **Marín-García, P.J.**, Ródenas, L., Martínez-Paredes, E., Blas, E., Pascual, J.J. (M Preparation). Determination of the news amino acid recomendations for growing rabbits selected by growth rate. . J. Anim. Feed. Sci. Techn.. Preparation

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TEACHER FOCUS:

- **Marín-García, P.J.**, 2018. Oposiciones a profesor de secundaria especialidad en Biología y Geología. Volumen 1. Editorial Academia de Estudios Ados 2005, S.L. 978-84-92969-92-2.
- **Marín-García, P.J.**, 2018. Oposiciones a profesor de secundaria especialidad en Biología y Geología. Volumen 2. Editorial Academia de Estudios Ados 2005, S.L. 978-84-92969-92-5.
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RESEARCH PROJECTS

Role of smoking-releated hydroquinone in the development and progression of insulin resistance to diabetic retinopathy. Flight attendant medical research instate (famri) id: 072100_cia. Responsible: Maria E. Marín-Castano.

Rabbit Genetic improvement: Production, stress and longevity. MEC. (AGL 2008-030274). Responsible: Manuel Baselga Izquierdo .

Rabbit Genetic improvement: Selection response and its effect about reproduction, feed conversion and health using cryopreserved population as control. MEC. (AGL2014-53405-C2-1-P). Responsible: Francisco Marco Jiménez.

Rabbit Genetic improvement: : New strategies for to improve genetic response, feed conversion and health in paternal lines. MEC (AGL2014-53405-C2-1-P). Responsible: Francisco Marco Jiménez.

Factor driving the life spain of reproductive rabbit does. Faculty of agricultural sciences. AARHUS University. Responsible: Juan José Pascual Amorós

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UNIVERSITY TEACHING (85 CREDITS)				
YEAR	CREDITS	SUBJECT	COURSE	UNIVERSITY
19-20	16,5	ANIMAL NUTRITION	3^{th} DEGREE OF VETERINARY	CEU-UCH
19-20	21,0	MANAGEMENT AND HEALTH: RABBITS AND POULTRY	4^{th} DEGREE OF VETERINARY	CEU-UCH
18-19	31	ANIMAL NUTRITION	3^{th} DEGREE OF VETERINARY	CEU-UCH
18-19	3	MANAGEMENT AND HEALTH: PIGS	4^{th} DEGREE OF VETERINARY	CEU-UCH
18-19	2	MANAGEMENT AND HEALTH: RABBITS AND POULTRY	4^{th} DEGREE OF VETERINARY	CEU-UCH
18-19	1,5	STATISTICS	MSC IN ANIMAL SCIENCE	UPV
18-19	0,4	ANIMAL NUTRITION AND HEALTH	MSC IN ANIMAL SCIENCE	UPV
18-19	1,2	ANIMAL NUTRITION	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
17-18	3	MONOGASTRIC PRODUCTION	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
16-17	0,5	ANIMAL WELFARE	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
16-17	0,5	MONOGASTRIC PRODUCTION	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
16-17	2,4	ANIMAL NUTRITION	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
16-17	0,23	ANIMAL FUNCTION AND STRUCTURE	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
16-17	1,64	BASIS OF ANIMAL PRODUCTION	4^{th} DEGREE OF AGRICULTURAL & BIOL. ENG.	UPV
15-16		MIDDLE TEACHING(1 FULL COURSE) BIOLOGY & GEOLOGY PROFESSOR		

DIRECTION OF RESEARCH PROJECTS…			
YEAR	STUDENT	TITLE	UNIVERSITY
18	Arias-Quispe. J.C.	"Effect of lysine, sulphr amino acids and threonine levels in the diet on faecal and ileal digestibility of feed in rabbits selected by growth rate. Review of their amino acids requirements	UPV
17	Carnovale. F.	Improvement of genetic selection and feed conversion ratio in two different generations of rabbits meat	UPV
16	Boscolo. C.	Effect of dietary lysine, methionine and threonine level on plasma urea nitrogen as an indicator of amino acid metabolic imbalance in rabbit feed	UPV