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The nutritional strategy of European rabbits is affected by age and sex: Females eat more and have better nutrient optimisation

Pablo-Jesús Marín-García[1] | Lola Llobat[1] | Juan Antonio Aguayo-Adán[2] | Torben Larsen[3] | María Cambra-López[4] | Enrique Blas[4] | Juan José Pascual[4] | Carlos Rouco[5]

[1]Department of Animal Production and Health, Veterinary Public Health and Food Science and Technology (PASAPTA), Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain

[2]Departamento de Botánica, Ecología y Fisiología Vegetal, Ecology Area, Faculty of Science, University of Córdoba, Cordoba, Spain

[3]Department of Animal Science, Aarhus University, Tjele, Denmark

[4]Institute for Animal Science and Technology, Universitat Politècnica de València, Valencia, Spain

[5]Department of Plant Biology and Ecology, University of Seville, Seville, Spain

Correspondence
Pablo-Jesús Marín-García, Department of Animal Production and Health, Veterinary Public Health and Food Science and Technology (PASAPTA), Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, 46113 Valencia, Spain.
Email: Pablo.maringarcia@uchceu.es

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Abstract

The ecological interest in the European rabbit (*Oryctolagus cuniculus*) has grown since it was declared an endangered species. Nutrition is fundamental in its dispersion and the key to its success. This is the main reason why knowledge of their nutritional preferences/requirements could play a fundamental role in rabbit biology and, therefore, in their conservation. The objectives of the work will be to elucidate how age and sex affect both nutritional preferences/requirements, and the nutrient optimisation of European rabbits. To address this gap, 70 wild European rabbits were studied. The rabbits were divided according to their age: adult ($n = 43$) and young ($n = 27$). Two groups were differentiated according to the adult rabbits' sex: females ($n = 28$) and males ($n = 15$). We analysed the relative (%) and absolute (g) chemical composition of the rabbits' gastric contents: dry matter (DM), ashes (ASH), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin, hemicelluloses and celluloses, highly digestible nonnitrogenous nutrients (HDNN: fat, starch, and soluble fibre), well from the blood was analysed with plasmatic urea nitrogen (PUN), nonesterified fatty acid (NEFA) and glucose (GLU). As a nutrient optimisation measure, the following indices between blood metabolites and stomach content were calculated: PUN/CP total content, NEFA/DM total content and GLU/DM total content ratios. Our works showed that age and sex affected the nutritional strategy of rabbits. Regarding age: adults showed lower CP (−14%; $p = 0.0217$) and higher HDNN (+21%; $p = 0.0399$) relative content than young rabbits, and absolute amount of most nutrients: DM (+59%; $p = 0.001$), OM (+43%; $p = 0.0049$), ASH (+54%; $p = 0.0085$), Hemicelluloses (+73%; $p = 0.0084$), Cellulose (+27%; $p = 0.0452$), and HDNN (+63%; $p = 0.0012$). In addition, adults showed better nutrient optimisation. Sex did not affect the relative chemical composition of the gastric content, but it showed a clear higher gastric content by females (+85%; $p < 0.0001$) and higher intake of most of the absolute chemical components: DM (+64%; $p < 0.001$), CP (+56%; $p = 0.0005$), OM (+58%; $p = 0.0001$), ASH (+44%; $p = 0.0123$), HDNN (+39%; $p = 0.001$), NDF (+59%; $p = 0.001$), ADF (+64%; $p = 0.0003$), lignin (+82%; $p = 0.0036$) and cellulose (+58%; $p = 0.0002$). Finally, we observed that females had better nutrient optimisation than males. This works supports the idea that feeding ecology and nutrition are particularly relevant to reproductive success and fitness in wild animals.

KEYWORDS

dry matter, ecology nutrition, fitness, protein, nutrient optimisation

1 | INTRODUCTION

The ecological interest in the European rabbit (*Oryctolagus cuniculus*) has grown since it was declared an endangered species (Villafuerte & Delibes-Mateos, 2019). Furthermore is considered a keystone species in the Mediterranean ecosystem (Cortés-Avizanda et al., 2015; Ferreira & Alves, 2009). Nutrition is fundamental to its dispersion and the key to its success (Villafuerte et al., 1997).

Diet can be analysed from a quantitative and qualitative point of view. The first one is relatively simple, but the second one is more complicated since the abundance of each nutritional component must be determined. Regarding quantity, biomass available could be estimated by measuring the amount of dry matter (DM) in the pasture layer and then correcting for the pasture cover in each area (Lombardi et al., 2003). Concerning qualitative is more complicated, since previous study and the analysis of chemical components are needed (Lombardi et al., 2007). Diet quality has been identified as a key regulator of mammalian populations' survival rate and reproductive success (Parker et al., 2009), but overall, these studies are scarce, since most studies focus on analysing the protein content (Gil-Jiménez et al., 2015). Measuring the quality of the nutritional resources available to wild herbivores is critical to understanding trophic regulation processes. However, directly assessing dietary nutritional characteristics is usually difficult (Gil-Jiménez et al., 2015).

One of the qualitative diet study's main limitations is the measurement's difficulty. Traditionally it can be done by assessing the plants that animals have access to, but it may be that these are not ingested (Llobat & Marín-García, 2022). Another option is to study through microhistological analysis of faecal pellets (Marques & Mathias, 2001), but this work uses gastric content because there exists a correlation between feed intake and gastric content (Sanderson & Vanderweele, 1975). It is interesting to know all the nutritional components ingested and the degree of use relating it to blood parameters (Marín-García et al., 2022a, 2022b).

Nutritional ecology aims to unravel the extensive web of nutritional links that guide animals in interacting with their ecological and social environments (Raubenheimer et al., 2009, 2012). In this case, age and sex could affect nutritional ecology, as observed in other species (Vogel et al., 2017). Conservation physiology mainly aims to understand the physiological responses of organisms to changed environments and the factors that cause conservation problems (Seebacher & Franklin, 2012; Wikelski & Cooke, 2006). In this case, factors such as age and sex can be considered that affect both.

In this work, we hypothesise that both the nutritional requirements and the optimisation of resources are affected by sex and age. Thus, the work's main objectives were on knowing how age and sex affect nutritional preferences, their nutritional requirements, and the optimisation of resources for European rabbits.

2 | MATERIAL AND METHODS

2.1 | Animal ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required, as no animals were killed specifically for this study. Samples were collected from wild rabbits legally hunted during the official hunting season in full compliance with Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

2.2 | Animals and sampling

A total of 70 wild European rabbits were used in this experiment. In May 2021, hunters captured all of the animals from various preserves in the Valencian region of eastern Spain. At the same time of day, early in the morning, all samples were collected (approximately 08:00 a.m.) Classification and sampling were done for all animals (Figure 1). Regarding classification, the animals were divided according to age: adult ($n = 43$) and young ($n = 27$). The criteria for this classification were physical characteristics, weight (on av. 1.39 vs. 0.60 kg for adult and young animals, respectively) and length (on av. 40.6 vs. 30.3 cm for adult and young animals, respectively). The two groups of those classified as adults were differentiated according to sex: females ($n = 28$) and males ($n = 15$). The animals were sampled for digestive content and blood samples. Each digestive tract was removed and weighed to determine its gastric content weight (the

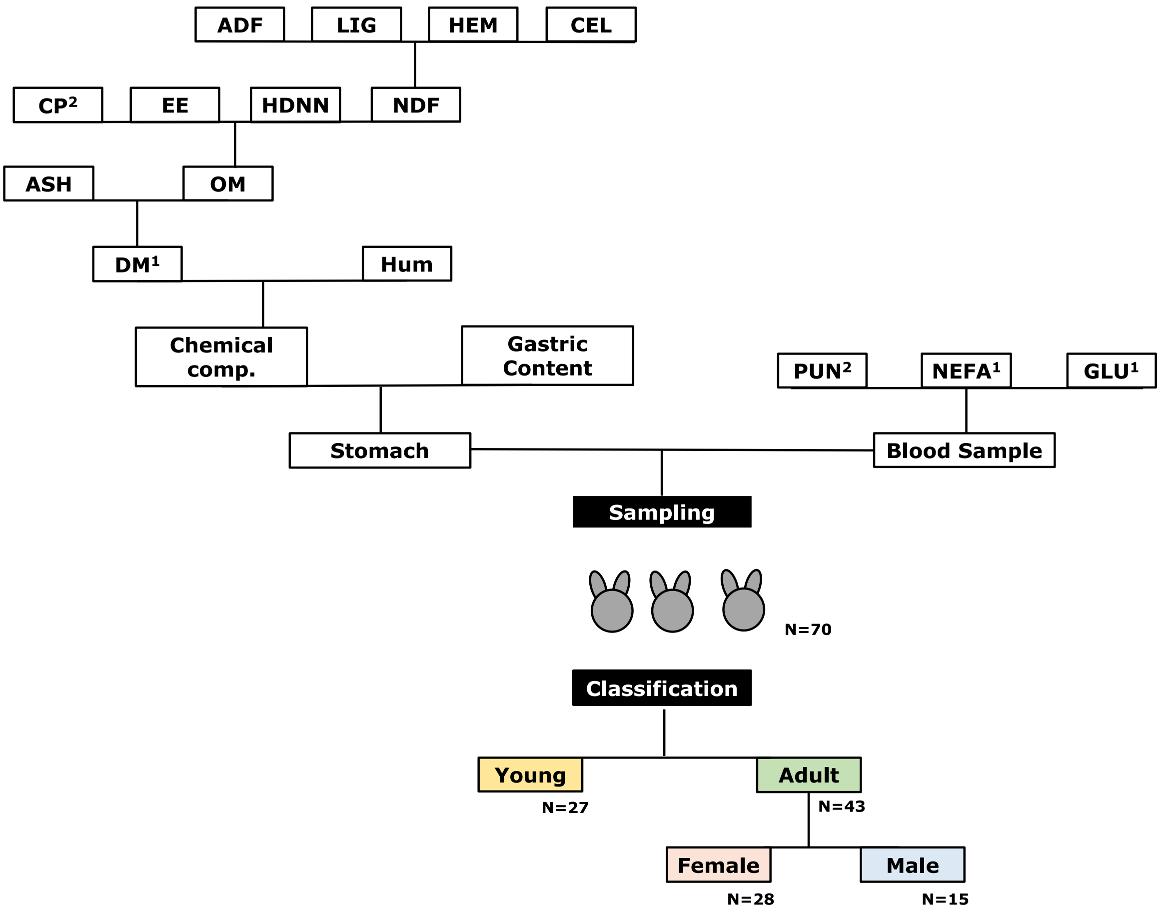

FIGURE 1 Experimental design: For each animal, sampling and classification. were carried out. Classification: Animals were divided according to their age: Adult (*n* = 43) and young (*n* = 27), of those classified as adults, the two groups were differentiated according to sex: Females (*n* = 28) and Males (*n* = 15). Sampling: Samples were collected from wild rabbits legally hunted during the official hunting season in full compliance with the Spanish regulations. Gastric content was weighed and analysed for relative: ADF, acid detergent fiber; ASH, ashes; CEL, cellulose; CP, crude protein; DM, dry matter; EE, ether extract; HDNN, highly digestible nonnitrogenous nutrients (fat, starch, and soluble fiber); HEM, hemicellulose; Hum, humidity; LIG, lignin; NDF, neutral detergent fiber; OM, organic matter. The absolute composition was calculated by multiplying the relative composition by the weight of the gastric content. Regarding blood samples, it was analysed: GLU, glucose; NEFA, nonesterified fatty acid; PUN, plasmatic urea nitrogen. [1,2]Ratios PUN/CP absolute content, NEFA/DM absolute content and GLU/DM absolute content were analyzed as nutrient optimisation measure. [Color figure can be viewed at wileyonlinelibrary.com]

stomach was weighed in its entirety, i.e., the weight of the stomach and contents). The supernatant plasma was retrieved after blood samples (1 mL in EDTA vials). It was obtained from the thoracic cavity and promptly centrifuged for 5 min at 700*g*. Gastric content and plasma were stored frozen (−20°C) until further analysis.

2.3 | Chemical analysis

2.3.1 | Chemical composition of the gastric content

Relative chemical composition of the gastric content was analysed for dry matter (DM), ashes (ASH), crude protein (CP), ether extract (EE), neutral detergent fibre (aNDFom), acid detergent fibre (ADFom) and lignin (sa), 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 sulphuric acid [sa]) were analysed sequentially (Van Soest et al., 1991).

2.3.2 | Blood samples

Utilising a commercial kit, Plasmatic Urea Nitrogen (PUN) determination was carried out (Urea/BUN-Color, BioSystems S.A.). After defrosting and tempering the samples, 1 µL was pipetted into test tubes (each batch included a standard and a blank). Each sample was then added to 1 mL of reagent A, which contains 500 U/mL of urease, 20 mmol/L of phosphate buffer, 62 mmol/L of sodium salicylate, and 3.4 mmol/L of sodium nitroprusside. Then, 1 mL of reactant B (sodium hypochlorite

7 mmol/L and sodium hydroxide 150 mmol/L) was added, properly mixed and incubated for another 5 min at 37°C. At 600 nm, each sample's absorbance was finally measured against a blank. Blood nonesterified fatty acid (NEFA) was determined using the Wako, NEFA C ACS-ACOD assay method. Analyses were performed using an ADVIA 1800 ®Chemistry System autoanalyser (Siemens Medical Solutions). The final step was to measure blood plasma glucose using Siemens Diagnostics® Clinical Methods for ADVIA 1800.

2.4 | Statistical analysis

Organic matter (OM) was calculated by subtracting the amount of ASH from the DM. Fat, starch and soluble fibre were calculated as the difference by subtracting the amount of ash, CP and NDF from the DM. These chemical components are considered as highly digestible nonnitrogenous nutrients (HDNN). Cellulose was calculated by subtracting the amount of ADF from the NDF and Hemicellulose was calculated by subtracting the amount of lignin from the ADF. The absolute chemical composition of the gastric contents was calculated from the relative composition and the total DM values observed in the gastric contents. The following indices between blood metabolites and stomach content were calculated as a nutrient optimisation measure: PUN/CP total content, NEFA/DM total content and GLU/DM total content.

Relative and absolute DM, ASH, OM, CP, EE, HDNN, NDF, ADF, Lignin, Hemicellulose, Cellulose, PUN/CP, NEFA/DM and GLU/DM were fitted to a normal distribution. After that, two independent analyses were performed. Using a GLM model from the Statistical Analysis System, both traits were examined as dependent variables (SAS, 2009), including age and sex as the main fixed effect. Least square mean comparisons were performed by *t*-test.

3 | RESULTS

3.1 | Age effect

Age affects both CP and HDNN relative content (Table 1). Adults showed lower CP (−14%; $p = 0.0217$) and higher HDNN (+21%; $p = 0.0399$) relative content than young rabbits. Adults showed higher Gastric content (+96%; $p<0.001$), and it provokes higher absolute chemical composition of several chemical compositions: DM (+59%; p=0.001), OM (+43%; p=0.0049), ASH (+54%; p=0.0085), Hemicelluloses (+73%; p=0.0084), Cellulose (+27%; p=0.0452) and HDNN (+63%; p=0.0012) than young animals (Table 2). Concerning nutrient optimisation, adults showed less PUN/CP (−45%; p=0.0039), NEFA/DM (−72%; p=0.0024) and Glucose/DM (−54%; p=0.0293) ratio than young animals (Figure 2).

TABLE 1 Effect of the age on relative chemical composition of the gastric content of European rabbit ($n = 70$).

	Adult	Young	*p* value
DM (%)	14.71 ± 3.25	17.69 ± 0.0.82	0.0053
ASH (%DM)	9.05 ± 0.34	8.35 ± 0.45	0.1561
OM (%DM)	90.95 ± 0.34	91.66 ± 0.45	0.2197
CP (%DM)	24.02 ± 0.96	27.86 ± 1.32	0.0217
NDF (%DM)	39.23 ± 1.16	40.76 ± 1.68	0.4587
ADF (%DM)	23.90 ± 0.77	24.42 ± 1.19	0.7017
Lignin (%DM)	4.82 ± 0.40	4.68 ± 0.59	0.7856
Hemicellulose (%DM)	15.33 ± 0.89	16.33 ± 1.29	0.5235
Cellulose (%DM)	19.41 ± 0.66	19.75 ± 0.96	0.7793
HDNN (%DM)	28.43 ± 1.36	23.46 ± 1.95	0.0399

Note: Least square mean ± standard errors.

Abbreviations: ADF, acid detergent fibre; ASH, ashes; CP, crude protein; DM, dry matter; HDNN, highly digestible nonnitrogenous nutrients (fat, starch, and soluble fibre); NDF, neutral detergent fibre; OM, organic matter.

TABLE 2 Effect of the age on gastric content and absolute chemical composition (g) of the gastric content of European rabbit ($n = 70$).

	Adult	Young	*p* value
Gastric content	46.46 ± 2.80	23.74 ± 3.46	<0.001
DM	6.63 ± 0.44	4.18 ± 0.55	0.0010
CP	1.58 ± 0.13	1.30 ± 018	0.2186
OM	6.29 ± 0.39	4.40 ± 0.52	0.0049
ASH	0.63 ± 0.05	0.41 ± 0.06	0.0085
NDF	2.67 ± 0.19	2.22 ± 0.27	0.1753
ADF	1.61 ± 0.11	1.31 ± 0.15	0.1043
Lignin	0.30 ± 0.04	0.28 ± 0.06	0.7208
Hemicellulose	0.26 ± 0.02	0.15 ± 0.03	0.0084
Cellulose	1.31 ± 0.08	1.03 ± 0.11	0.0452
HDNN	1.92 ± 0.13	1.18 ± 0.18	0.0012

Note: Least square means ± standard errors.

Abbreviations: ADF, acid detergent fibre; ASH, ashes; CP, crude protein; DM, dry matter; HDNN, highly digestible nonnitrogenous nutrients (fat, starch, and soluble fibre); NDF, neutral detergent fibre; OM, organic matter.

3.2 | Sex effect

Sex did not affect the relative chemical composition of the gastric content (Table 3). Nevertheless, it showed a clear higher Gastric content by Females (+85%; $p < 0.0001$), and it provokes that females showed a higher intake of the following chemical components: DM (+64%; $p < 0.001$), CP (+56%; $p = 0.0005$), OM (+58%; $p = 0.0001$), ASH (+44%;

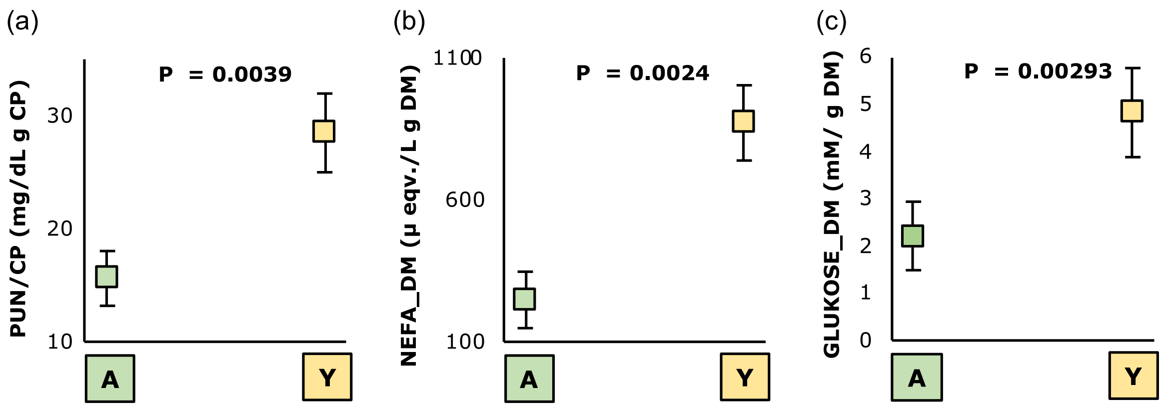

FIGURE 2 Effect of the age (A = adult; Y = young) on the ratios: PUN/CP (a) NEFA/DM (b) and Glucose/DM (c), on blood and gastric content, of European rabbit, *n* = 70. Least square means ± standard errors. CP, crude protein (*n* = 70); DM, dry matter; NEFA, nonesterified fatty acid; PUN, plasmatic urea nitrogen. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Effect of the sex on the relative chemical composition of the gastric content of the adults of European rabbit (*n* = 43).

	Female	Male	*p* value
DM (%)	14.40 ± 0.63	16.17 ± 0.79	0.0868
ASH (%DM)	8.77 ± 0.36	9.62 ± 0.47	0.1561
OM (%DM)	91.23 ± 0.36	90.38 ± 0.47	0.1561
CP (%DM)	25.09 ± 1.17	23.59 ± 1.48	0.4319
NDF (%DM)	39.85 ± 1.35	39.00 ± 1.82	0.7075
ADF (%DM)	24.25 ± 0.94	23.39 ± 1.27	0.5918
Lignin (%DM)	4.82 ± 0.51	4.36 ± 0.69	0.5970
Hemicellulose (%DM)	15.60 ± 1.09	15.60 ± 1.46	0.9998
Cellulose (%DM)	19.43 ± 0.77	19.03 ± 1.04	0.7596
HDNN (%DM)	26.89 ± 1.47	28.54 ± 1.98	0.5087

Note: Least square means ± standard errors.

Abbreviations: ADF, acid detergent fibre; ASH, ashes; CP, crude protein; DM, dry matter; HDNN, highly digestible nonnitrogenous nutrients (fat, starch, and soluble fibre); NDF, neutral detergent fibre; OM, organic matter.

TABLE 4 Effect of the sex on gastric content and absolute chemical composition (g) of the gastric content of the adults of the European rabbit (*n* = 43).

	Female	Male	*p* value
Gastric content	56.12 ± 2.54	30.38 ± 3.17	<0.0001
DM	8.01 ± 0.43	4.87 ± 0.54	<0.0001
CP	2.01 ± 0.15	1.29 ± 0.18	0.0005
OM	7.42 ± 0.38	4.71 ± 0.50	0.0001
ASH	0.72 ± 0.05	0.50 ± 0.07	0.0123
NDF	3.21 ± 0.20	2.02 ± 0.27	0.0010
ADF	1.93 ± 0.11	1.18 ± 0.15	0.0003
Lignin	0.40 ± 0.05	0.22 ± 0.06	0.0036
Hemicellulose	0.29 ± 0.03	0.19 ± 0.04	0.0718
Cellulose	1.53 ± 0.08	0.97 ± 0.11	0.0002
HDNN	2.09 ± 0.16	1.50 ± 0.19	0.0226

Note: Least square means ± standard errors.

Abbreviations: ADF, acid detergent fibre; ASH, ashes; CP, crude protein; DM, dry matter; HDNN, highly digestible nonnitrogenous nutrients (fat, starch, and soluble fibre); NDF, neutral detergent fibre; OM, organic matter.

$p = 0.0123$), HDNN (+39%; $p = 0.001$), NDF (+59%; $p = 0.001$), ADF (+64%; $p = 0.0003$), Lignin (+82%; $p = 0.0036$) and Cellulose (+58%; $p = 0.0002$) than males (Table 4). Regarding nutrient optimisation, females showed less PUN/CP (−48%; $p < 0.001$) and Glucose/DM (−68%; $p = 0.0261$) ratio than males (Figure 3). And finally, neither age nor sex affected EE levels, neither in absolute nor relative amounts. It was the mean values of 7.75% and 0.64 g, respectively.

4 | DISCUSSION

The main aim of this work was to elucidate how age and sex affect nutritional preferences/requirements and optimisation of resources of European rabbits. Nutritional preferences/requirements were measured through gastric content since there is a correlation between the level of ingestion and gastric filling (Bellier et al., 1995). Considering the sampling time (08:00 h) and the gastric content reflects the content ingested for the previous 3 and 6 h, this content would represent the ingestion produced between 02:00 and 05:00 h (Blas & de, Wiseman, 2010). According to the circadian pattern of feed intake, this moment would correspond to the maximum ingestion (29% of the total feed intake) and minimum incidence of caecotrophy, which occurs mainly during the light period (Carabaño et al., 2010). In this context, we can ensure that the work results refer to the food itself. Animals can select a diet that meets the requirements. Several trials on broilers and pig species show that

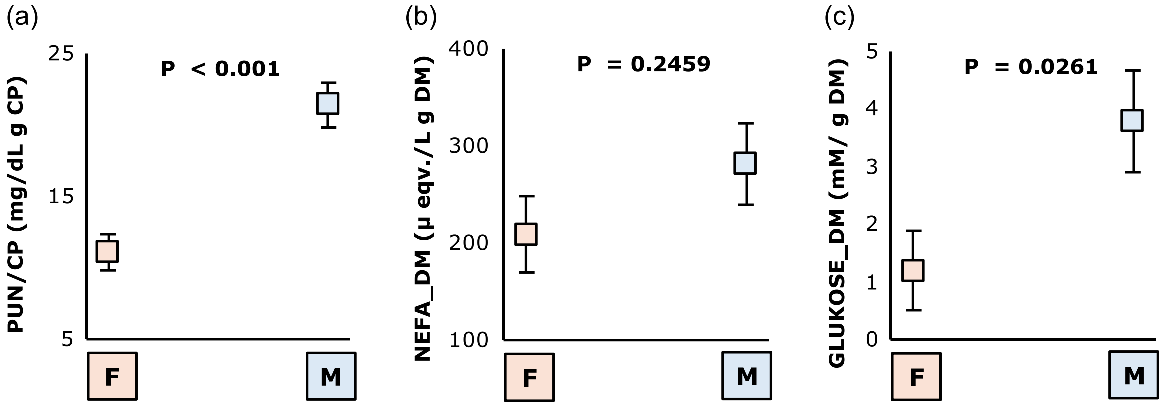

FIGURE 3 Effect of the sex (F = female; M = male) on the ratios: PUN/CP (a) NEFA/DM (b) and Glucose/DM) (c), on blood and gastric content, of European rabbit, *n* = 48 (*Oryctolagus cuniculus*). (Least square means±standard errors). CP, crude protein; DM, dry matter; NEFA, nonesterified fatty acid; PUN, plasmatic urea nitrogen. [Color figure can be viewed at wileyonlinelibrary.com]

animals can choose between different diets to obtain a nutrient intake that matches their requirements (Kyriazakis et al., 1990). This fact has also been studied in rabbits, where animals could choose their preferred diet, showing high intra-individual repeatability in their preference (Marín-García et al., 2021). For this reason, we jointly study the preferences and nutritional requirements.

Concerning the age effect, the relative chemical composition was similar among the groups. However, the CP proportion was higher in young animals. These results may be because lactating animals are in the group of young individuals. Protein output at rabbit's lactation peak is 4.3 and 2.4 higher than Holstein cows and hybrid sows (Maertens et al., 2010). Another possible explanation may be that young animals have higher protein requirements (Marín-García & Llobat, 2021). Because of this higher percentage of CP, young animals showed lower relative values of HDNN. This may be because young animals need less proportion of fat, starch and soluble fibre. Moreover, this, in turn, may be due to their less well-formed digestive system.

Regarding nutritional efficiency, it seems that young animals show less efficiency than adults, which could be explained by digestive system maturation. For example, Debray et al. (2003) showed that the enzyme activity of trypsin, chymotrypsin, lipase and amylase increased with age. Another plausible explanation is that it could be due to the natural selection of individuals that are more efficient in optimising resources (Moodie, 1972).

Regarding the effect of sex, a different relative preference between sexes was not verified, but a greater quantity of most of the nutrients was found (on av. +58%). This would indicate greater nutritional requirements by females.

This can be explained by the high reproductive needs of rabbit does. In fact, Fortun-Lamothe (2006), showed that an energy deficit occurring during lactation leads to a decrease in reproductive performance and the physiological mechanisms implicated in these effects. In the natural environment, these higher nutritional requirements by females have also been observed. Batzli (1986) suggests that nutritional factors control the timing of breeding in California voles, and assure that nutrition influences the population densities of lemmings and voles. These samples were obtained in a 'reproductive peak', and these differences were also observed (Cooke, 2014; Joseph, 1909). However, studies that analyse rabbit's chemical, and nutritional intake are scarce and difficult to compare with others. Females showed better nutrient optimisation; it is the first time that better energy and protein optimisation has been observed in the wild. These observations delve into the interaction between reproductive status and different resource acquisition, as observed in domestic animals (Pascual et al., 2013). It is interesting to mark as a weak point, the measurement of feed intake in the previous moment as an indicator of the food, after all, this is a specific point, and the ideal would be a longer study in time. Although, as previously stated, it is the most concrete way of measuring the chemical composition of the European rabbit's diet. It is interesting to mention that the dispersion of this very cosmopolitan species makes it, especially the study of the different responses in these Mediterranean ecosystems, so expanding this study to areas of the southern hemisphere would give a more global vision of the response of this animal (Huertas Herrera et al., 2022).

5 | CONCLUSIONS

Our work provides measured values of nutritional components never evaluated in gastric content, both in relative and absolute content. Our work's main conclusion is that European rabbits' nutritional strategy is affected by age and sex. Regarding age, Adults showed lower relative CP and higher HDNN levels. Due to increased intake, the absolute values of all components were higher in adults. Furthermore, adults showed better nutrient optimisation than young animals. Concerning sex, females showed clearly higher gastric content, which provoked higher intake of many chemical components. In addition, it is verified that females had better nutrient optimisation.

AUTHOR CONTRIBUTIONS

Pablo-Jesús Marín-García, Carlos Rouco, Lola Llobat, María Cambra-López, Enrique Blas and Juan José Pascual designed the study; Pablo-Jesús Marín-García, Carlos Rouco and Juan Antonio Aguayo-Adán collected rabbit samples, Pablo-Jesús Marín-García analysed rabbit samples, completed statistical analysis and wrote a first draft; Pablo-Jesús Marín-García led the writing of the final version of the manuscript with inputs from the rest of the authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Pablo-Jesús Marín-García http://orcid.org/0000-0001-9593-3476
Lola Llobat http://orcid.org/0000-0002-9806-9174

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