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RESEARCH ARTICLE

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Targeted and untargeted metabolomic profiles in wild rabbit does (*Oryctolagus cuniculus*) of different breeding states (pregnant and lactating)

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Abstract

Ecological nutrition aims to unravel the extensive web of nutritional links that drives animals in their interactions with their ecological environments. Nutrition plays a key role in the success of European wild rabbit (*Oryctolagus cuniculus*) and could be affected by the breeding status of the animals and reflected in the metabolome of this species. As nutritional needs are considerably increased during pregnancy and lactation, the main objective of this work was to determine how the breeding status (pregnant and lactating) of European wild rabbit does affects nutritional requirements and their metabolome (using targeted and untargeted metabolomics), aiming to find a useful biomarker of breeding status and for monitoring nutritional requirements. To address this gap, 60 wild European rabbits were studied. Animals were divided according to their breeding status and only pregnant (n = 18) and lactating (n = 11) rabbit does were used (n = 29 in total). The body weight and length of each animal were analyzed. The relative and absolute chemical composition of the gastric content and whole blood sample were taken, and targeted and untargeted metabolomics were analyzed. As a main result, there were no differences in biometric measurements, gastric content, and targeted metabolomics, except for live weight and nonesterified fatty acids (NEFA), as pregnant animals showed higher live weight (+12%; $p = 0.0234$) and lower NEFA acid levels (−46%; $p = 0.0262$) than lactating females. Regarding untargeted metabolomics, a good differentiation of the metabolome of the two breeding groups was confirmed, and it was proven that pregnant animals showed higher plasmatic levels of succinic anhydride (3.48 more times; $p = 0.0236$), succinic acid (succinate) (3.1 more times; $p = 0.0068$) and propionic acid (3.98 more times; $p = 0.0121$) than lactating animals. However, lactating animals showed higher levels of N-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfoxide) cholan-24-yl]-Glycine (cholestadien) (2.4 more times; $p < 0.0420$), 4-maleyl-acetoacetate (MAA) (3.2 more times; $p < 0.0364$) and irilone (2.2 more times; $p = 0.0451$) than pregnant animals, any of these metabolites could be used as a

potential biomarker. From these results, it can be concluded that the most notable changes were observed in the metabolome of individuals, with most of the changes observed being due to energy and protein mobilisation.

KEYWORDS
Glycine, irilone, lactating, nonesterified fatty acids, pregnant, succinic acid

1 | INTRODUCTION

Rabbit meat production has been widely studied. For this reason, a high number of trials have been carried out in controlled environments of this animal species aiming to establish its nutritional requirements, as well as the interaction between nutrition and metabolic phenotype of these animals (Marín-García et al., 2020a, 2020b, 2023). In terms of biodiversity, European wild rabbit (*Oryctolagus cuniculus*) has been defined as a keystone species (Cortés-Avizanda et al., 2015) because it plays a relevant ecological role in the Mediterranean ecosystem (Malo et al., 1995). Due to different causes, this species has recently been classified as "endangered" by the International Union for Conservation of Nature (IUCN) (Anon). Due to everything explained above, the ecological interest in European wild rabbit conservation has grown in recent years (Llobat & Marín-García, 2022) and fields such as ecological nutrition and conservation physiology applied to this species have begun to be studied.

The main aim of ecological nutrition is to unravel the extensive web of nutritional links that drives animals in their interactions with their ecological environments (Parker, 2003; Raubenheimer et al., 2009) and metabolomics is a powerful tool to study it. Ecological nutrition can potentially contribute to multiple research fields such as conservation physiology (Raubenheimer et al., 2012), which 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 as breeding status -understood in this work as the possibility of females being pregnant or lactating- could have an impact on the fitness of this species, its nutrition requirements, and its metabolome. This impact has been extensively studied in rabbit production, wherein lactating animals exhibit higher nutritional requirements compared to pregnant rabbit does (Fortun-Lamothe, 2006). In recent years, studies in this species combining issues of molecular ecology with the fitness of the species have started to appear (Marín-García et al., 2022b, 2022b).

Reproductive status is one of the most important adaptive successes of European wild rabbit, which shows a clearly seasonal breeding (Bullough, 1961), and it has been proven that social interaction and climatology could affect reproduction of rabbit does (Boyd, 1986), but the most relevant factor affecting breeding status is the quality of the diet (Marín-García & Llobat, 2021), which has been identified as a key factor in mammalian populations (Parker et al., 2009). This is why nutritional resources available to wild herbivores are critical to understanding trophic regulation processes and thus their conservation. Diet studies on wild rabbits are scarce, as most studies focus on analysing just one nutrient (Gil-Jiménez et al., 2015). Recent research has explored the influence of sex and age on the dietary habits of wild rabbits, utilising gastric content analysis (Marín-García et al., 2023). However, a notable gap remains in the knowledge regarding the comparison of different breeding statuses using a metabolomics approach.

In this work, it is hypothesized that nutritional requirements and the metabolome are affected by breeding status. Thus, the work's main objectives were to determine how breeding status (pregnant and lactating) of European wild rabbits does affects nutritional requirements and their metabolome (using targeted and untargeted metabolomics), aiming to find a useful biomarker for monitoring nutritional requirements for these animals.

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 the Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

2.2 | Animals and sampling

A total of 29 wild European rabbit were used in this experiment. All animals were obtained as a product of hunting from different preserves located in the Valencian community (eastern Spain) during May 2021. All samples were obtained during morning hours, at the same time of day (approximately 08:00 a.m.) For all animals, classification—Animals were categorized as "pregnant" when fetuses were visually observed, and as "lactating" when the presence of milk in the breasts was detected and sampling were performed (Figure 1). Regarding classification, animals were divided according to their breeding status based on its internal traits (Pregnant and n = 18 and

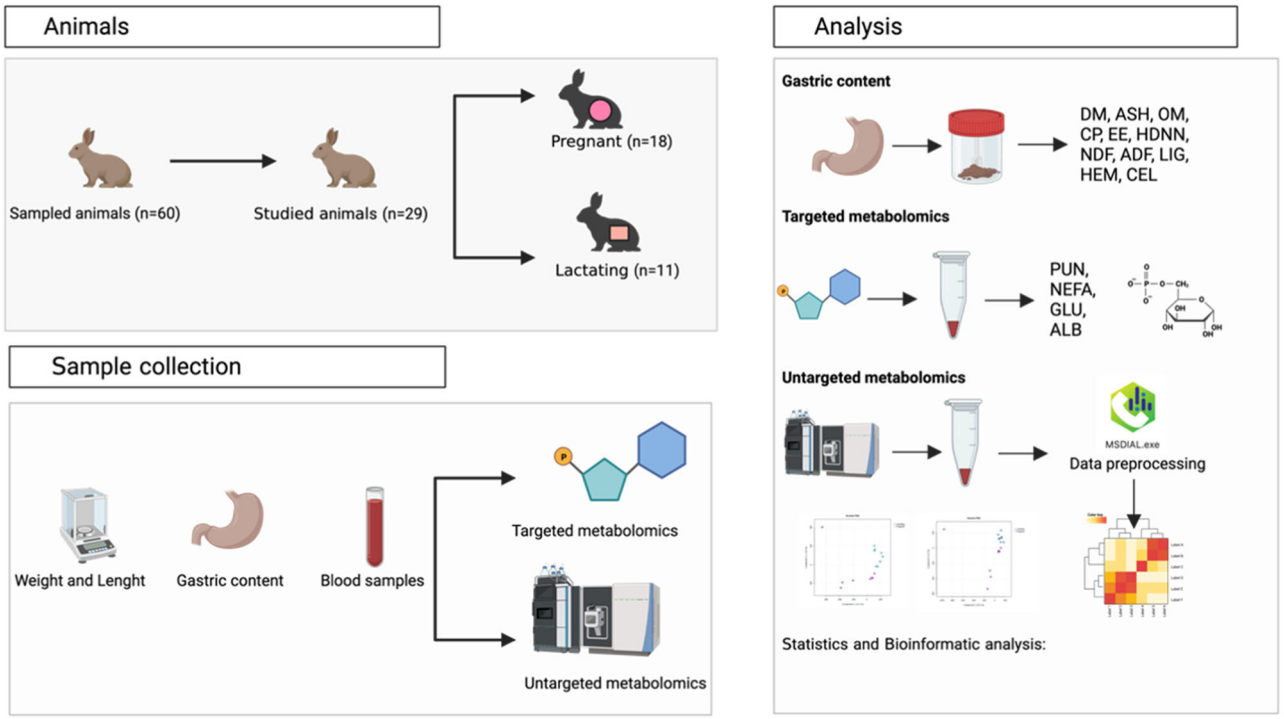


FIGURE 1 Experimental design. For each animal, sampling and classification were carried out. Classification: Animals (n = 60) were divided according to their breeding status: ● Pregnant (n = 18) and ■ Lactating (n = 11) wild rabbit does. Sampling: Samples were collected from wild rabbits legally hunted (for other proposes) during the official hunting season in full compliance with the Spanish regulations. Biometric measurements, gastric content and blood samples were taken from each animal. 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; HEM, hemicellulose; LIG, lignin; OM, organic matter; nonnitrogenous nutrients (fat, starch, and soluble fiber); NDF, neutral detergent fiber. Absolute composition was calculated by multiplying the relative composition by the weight of the gastric content. Blood samples were analyzed by both targeted and untargeted metabolomics. Regarding targeted metabolomics of blood samples, it was analyzed: ALB, albumin; GLU, glucose; NEFA, nonesterified fatty acid; PUN, plasmatic urea nitrogen; T. PRO, total protein and glutamate. Created with BioRender. com.

Lactating; n = 11) and only these animals were used in this work. As biometric measures, animal's body weight and length were monitored. The digestive content of each animal was extracted and weighed to calculate the gastric content weight (the stomach was weighed in its entirety, i.e., the weight of the stomach and its contents). Blood samples were taken from the thoracic cavity (1 mL in EDTA vials) and immediately centrifuged for 5 min at 700 G, and the plasma was extracted. Gastric content and plasma were stored frozen (−20°C) until further analysis.

2.3 | Chemical and statistical analysis

2.3.1 | Gastric content

The gastric content was analysed for dry matter (DM), ash (ASH), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin. Samples were analysed according to the methods of AOAC (Anon, 2000): 934.01 for DM, 942.05 for ash and 976.06 for CP. The NDF (assayed with a thermostable amylase and expressed exclusive of residual ash), ADF (expressed exclusive of residual ash) and lignin (determined by solubilisation of cellulose with sulphuric acid, SA) were analysed sequentially (Van Soest et al., 1991).

Organic matter (OM) was calculated by subtracting the amount of ASH from the DM. Highly digestible nonnitrogenous nutrients (HDNN) (starch plus soluble fibre) was calculated by subtracting the amount of ash, CP and NDF from the DM. Cellulose was calculated by subtracting the amount of ADF from the NDF and hemicellulose was calculated by subtracting the amount of lignin from 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.

2.3.2 | Targeted metabolomics analysis of plasma

Targeted metabolomics (n = 29) analysed were: plasmatic urea nitrogen (PUN), nonesterified fatty acids (NEFA), glucose, albumin and total protein, since they had been studied previously in wild rabbits (Marín-García et al., 2022a, 2022b; Marín-García et al., 2023).

The PUN determination was performed using a commercial kit (Urea/BUN-Color, BioSystems S.A., Barcelona, Spain).

NEFAs (nonesterified fatty acids) were determined using the Wako, NEFA C ACS-ACOD assay method. Analyses were performed

using an ADVIA 1800 ®Chemistry System autoanalyzer (Siemens Medical Solutions, Tarrytown, NY 10591, USA).

Plasma glucose, albumin, and total protein were determined according to standard procedures (Siemens Diagnostics®, Erlangen, Germany, Clinical Methods for ADVIA 1800).

2.3.3 | Untargeted liquid chromatography–mass spectrometry (LC-MS) metabolomics analysis of plasma

A total of 16 blood samples (8 for pregnant and 8 for lactating) were randomly selected. Blood plasma was prepared by deproteinization of a 150 µL sample with 450 µL ice-cold acetonitrile (100% ACN) containing an internal standard mix of glycocholic acid (glycine-1-13C) and p-chlorophenylalanine to a final concentration of 0.01 mg/mL. Samples were prepared in 96-well plates with 1 mL wells. Plates were mixed for 1 min, incubated at 4°C for 10 min, and centrifuged for 25 min at 2250× g and 4°C. Approximatively 400 µL supernatant was transferred to Sirocco Protein precipitation plates (Waters Corporation, Milford, MA). The filtered supernatant was transferred to two 200 µL 96-well plates (65 µL per well), and plates were vacuum centrifuged to dryness (ca. 2.5 h, 805× g and 30°C). Resuspension of the samples was done in a mix of H_2O:ACN:FA (95:5:0.1) using the same volume before evaporation. A protective film was welded onto the plate using a heat sealer, and the plates were centrifuged at 3700 rpm, 4°C for 25 min before the LC-MS analysis.

The samples were analysed by UHPLC using a Nexera X2 LC coupled to an LCMS-9030 Q-TOF MS system (Shimadzu Corporation, Kyoto, Japan) using both positive and negative electrospray ionisation (ESI). Chromatographic separations were performed using an Acquity HSS T3 column (1.7 µm 100 ×2.1 mm, Waters Ltd., Elstree). The column temperature was set to 40°C, the samples were maintained at 10°C and 3 µl aliquots were injected onto the column. The chromatographic system used a binary gradient of Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile with 0.1% formic acid) with a flow rate of 0.4 mL/min. A linear gradient was used from 5% B to 100% B over 12 min, and 1 min hold at 100% before returning to the initial conditions of 5%B for 3 min for column re-equilibration. This resulted in a total analysis time per sample of 16 min. MS detection was performed using a data-independent acquisition (DIA) method for MS and MS/MS analyses. The method acquired a single time-of-flight (TOF) MS scan (m/z 50–900) followed by 33 MS/MS mass scans over a mass range of m/z 40–900; each MS/MS mass scan had a precursor isolation width of 25.2 Da and a collision energy spread of 10–30 V, resulting in a cycle time of 0.9 s. This allowed collection of fragmentation data of all masses in the spectra across the entire LC gradient. The following MS parameters were used: ion-source temperature, 300°C; heated capillary temperature, 250°C; heat block temperature, 400°C; electrospray voltage 4.5 kV (ESI +) or −3.5 kV (ESI-); electrospray nebulisation gas flow, 3 L/min; drying gas flow, 10 L/min; detector voltage, 2.02 kV. Mass calibration was performed externally using a sodium iodide solution (400 ppm in methanol) from m/z 50–1000. Data acquisition was performed using LabSolutions software version 5.114 (Shimadzu Corporation, Kyoto, Japan).

Sample quality control and metabolomics data pre-processing

The quality of the chromatographic runs, the UPLC system stability and the accuracy of sample preparation were monitored using quality control samples (QCs). Serum QCs were prepared by pooling an aliquot of all samples and subjecting this pooled sample to the same sample preparation protocol as the samples. The QCs were injected multiple times throughout the analysis as well as at the beginning and end of the analysis and used in the data pre-processing for signal drift correction. Blanks were injected during the chromatographic analysis to monitor any external contaminants from solvents, eluents and carry-over effects. The sample order was randomized for the chromatographic analysis to eliminate biases in the results and to ensure that each sample group was affected equally.

MS-DIAL software (Tsugawa et al., 2015) was used to perform peak detection, alignment and gap filling for the data files. MS-DIAL parameters are provided in Supplementary Table 1. The MS-DIAL generated data matrix was exported to Excel and filtered to eliminate peaks present in blanks, and retention time was truncated to contain only portions of the chromatographic peaks, while masses higher than 700 m/z were discarded.

Initial principal component analysis (PCA) was performed using LatentiX 2.12 (LATENTIX Aps., Gilleleje, Denmark) to check the quality of the data set and eliminate potential outliers. Partial least-squares discriminant analysis (PLS-DA) models were built to determine the metabolites responsible for the differences between pregnant and lactating wild rabbit does. Validation of the models was performed using repeated random subsampling validation.

Chemical solvents and standards for metabolomics analysis

High-performance liquid chromatography (HPLC)-grade solvents and eluents were used for the untargeted metabolomics analysis as follows: HPLC-grade acetonitrile (VWR, West Chester, PA), formic acid (FA, Fluka, Merck KGaA, Darmstadt), and MilliQ grade water (MilliporeSigma, Burlington, MA). Internal standards included during the sample preparation were glycocholic acid (Glycine-1-13C), and 4-chloro-DL-phenylalanine (Sigma, Merck KGaA, Darmstadt) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt) and Cayman Chemical (Ann Arbor, MI).

Multivariate data analysis

Before any analysis, all metabolomics datasets were pretreated by Autoscaling and preliminary data mining for an overview was performed using principal component analysis (CPCA) (Wold et al., 1987).

Initial PCA was performed to check the quality of the data sets and eliminate potential outliers. Partial least-squares discriminant analysis (PLS-DA) models were built to determine the metabolites responsible for the differences between breeding status (Pregnant vs. lactating) as a continuous variable. Validation of the models was performed using full cross-validation (leave-one-out). Outliers were

detected based on the residual variance and the Hotelling's T2 plot. Models were assessed using the explained variation in Y, plots depicted actual and predicted values and the proportion of variation explained (R2). Variable selection was done by excluding low-importance variables based on the VIP scores. Variables for identification were selected using VIP scores and scaled regression coefficients (Van Der Peet-Schwering et al., 2021).

Metabolite identification

Metabolites were identified based on queries in the Human Metabolome Database (http://www.hmdb.ca) online database to obtain possible chemical structures using accurate mass and mass spectrometric fragmentation patterns. Annotated features were classified on different levels of identification according to Sumner et al., (Sumner et al.,, 2007). The metabolites were identified for the whole metabolome, which was considered as the entire complement of small molecules (Mr < 1000 Da) in a biological system, in this case, plasma.

2.3.4 | General statistics

Models were assessed using the explained variation in Y, plots depicted actual and predicted values, and the proportion of variation explained (R2). Variables for identification were selected using variable importance in projection (VIP) scores. No outliers were found. Body weight, length, nutritional metabolites -PUN, NEFAs, glucose, albumin and total protein-, relative and absolute chemical composition of the gastric content -DM, ASH, OM, CP, EE, HDNN, NDF, ADF, lignin, hemicellulose and cellulose- and the identified metabolites of untargeted metabolomics assay, were fitted to a normal distribution. All traits were analysed as dependent variables using a GLM model from the Statistical Analysis System (SAS.), including breeding status as a main fixed effect. Least square mean comparisons were performed by *t*-test. No relation between gastric content and nutritional metabolites was evaluated due to small sample size and the absence of significant differences between experimental groups.

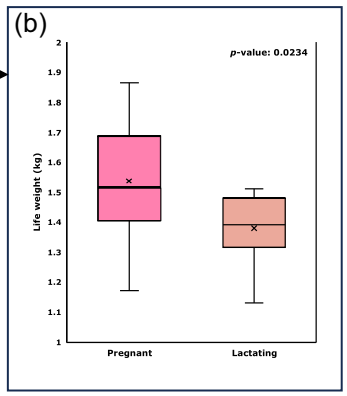
3 | RESULTS

Figure 2 represents the summary of the effects of the breeding status on the biometric, gastric content and targeted metabolomics. Regarding body measurement, only live weight was affected by breeding status. Pregnant animals showed higher (+ 12%; $p = 0.0234$) live weight than lactating (Figure 2a,b, respectively). Concerning gastric content, breeding status did not affect either relative or absolute chemical composition of the gastric content. Finally, in the

(a)

	Pregnant (n=18)			Lactating (n=11)			*p*-value
Body measurement:							
Life weight (kg)	1.54	±	0.04	1.38	±	0.05	0.0234
Length (cm)	41.89	±	0.41	40.55	±	0.53	0.0540
Gastric content:							
Gastric content (g)	55.81	±	3.86	57.81	±	4.56	0.7411
Stomach weight (g)	22.29	±	0.82	21.03	±	1.01	0.3404
Relative chemical composition of the gastric content:							
DM (%)	13.60	±	0.91	15.20	±	1.08	0.2043
ASH (% DM)	8.65	±	0.48	9.36	±	0.67	0.3493
OM (% DM)	91.35	±	0.48	90.64	±	0.67	0.3493
CP (% DM)	26.13	±	1.63	22.63	±	1.93	0.2577
NDF (%DM)	36.88	±	2.16	41.59	±	2.56	0.4276
ADF (%DM)	24.75	±	1.33	24.33	±	1.57	0.8383
Lignin (%DM)	5.10	±	0.84	4.58	±	1.00	0.6945
Hemicellulose (%DM)	14.13	±	1.38	17.26	±	1.63	0.1575
Cellulose (%DM)	19.65	±	0.96	19.75	±	1.39	0.9492
HDNN (%DM)	26.34	±	2.15	26.43	±	2.54	0.9798
Absolute chemical composition of the gastric content:							
DM (g)	7.40	±	0.61	8.59	±	0.73	0.2256
ASH (g)	0.64	±	0.08	0.83	±	0.10	0.1551
OM (g)	6.76	±	0.54	7.76	±	0.64	0.2482
CP (g)	1.93	±	0.22	1.96	±	0.26	0.9174
NDF (g)	2.89	±	0.33	3.61	±	0.39	0.1695
ADF (g)	1.83	±	0.18	2.08	±	0.21	0.3701
Lignin (g)	0.39	±	0.08	0.41	±	0.10	0.8922
Hemicellulose (g)	0.25	±	0.04	0.30	±	0.05	0.4339
Cellulose (g)	1.44	±	0.12	1.68	±	0.14	0.2366
HDNN (g)	1.94	±	0.24	2.19	±	0.27	0.4932
Targeted analyzed metabolites							
PUN (mg/dL)	18.37	±	1.03	19.17	±	1.31	0.6372
NEFA (μ eqv./L)	798	±	347	1488	±	439	0.0262
Glucose (mM)	5.61	±	1.91	11.19	±	2.34	0.0783
Albumin (g/L)	43.31	±	4.46	37.08	±	7.72	0.4959
Total Protein (g/L)	58.97	±	3.26	50.20	±	5.64	0.2000
Glutamate (microM)	329	±	37.7	156	±	92.24	0.1069

PUN: Plasma urea nitrogen; NEFA: Non-esterified fatty acids; DM: Dry matter; ASH: Ashes; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; HDNN: Highly digestible non-nitrogenous nutrients.



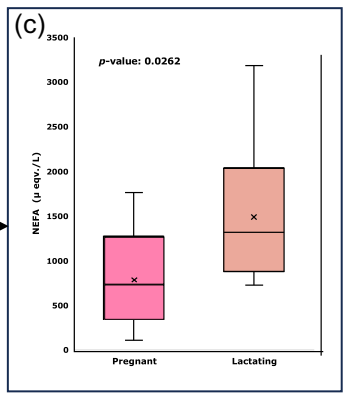


FIGURE 2 Summary of the effects of the breeding status on the results obtained by biometric, gastric content and targeted metabolomics test. (a) Effects of the breeding status on the parameters measured in wild rabbit does (n = 29). (b,c) Effect of the breeding status on life weight and NEFA, respectively. ○: Pregnant and □: Lactating. "x" stands the means.

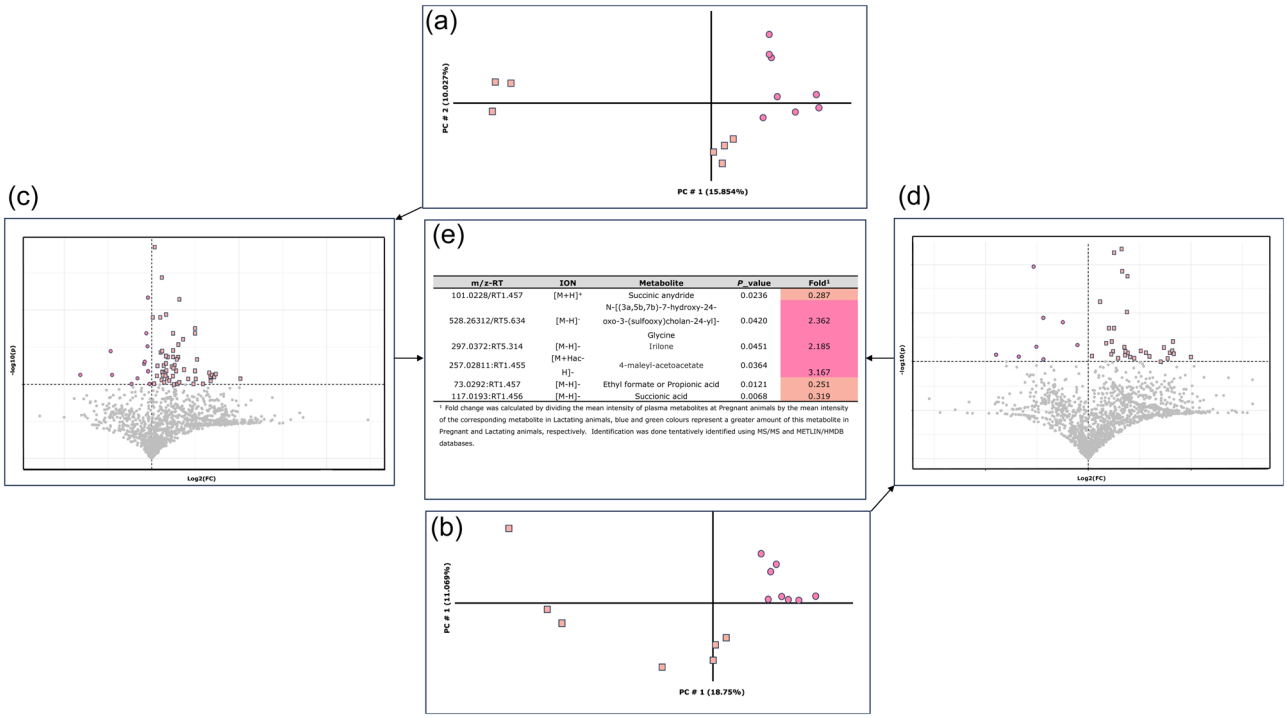
case of targeted metabolomics, breeding status did not affect some of the plasmatic targeted metabolites analysed, such as PUN, albumin, total protein, and glutamate. Nevertheless, lactating animals showed higher NEFA levels (+ 186%; $p = 0.0262$) than pregnant ones (Figure 2a,c). Also, a nonsignificant difference can be shown in the case of glucose where lactating animals showed higher glucose levels than pregnant ones (+ 199%; $p = 0.0783$).

Figure 3 represents the summary of the results obtained by untargeted metabolomics. Figure 3a,b represent the first two principal components obtained by sMBPLSR of untargeted metabolomics data in positive and negative mode, respectively. As shown, the variability associated with these principal components obtained from the metabolic profile (26% and 30%, respectively of the total) can be used to differentiate breeding status. In the volcano graphs (Figure 3c,d for positive and negative mode, respectively), the metabolites responsible for the discrimination between the breeding status could be observed. After the identification, Figure 3e summarises the identify metabolites that explain the highest differences between groups. Pregnant females showed higher plasmatic levels of succinic anhydride (3.48 more times; $p = 0.0236$), succinate (3.1 more times; $p = 0.0068$) and propionic acid (3.98 more times; $p = 0.0121$) than lactating animals. However, lactating animals showed higher levels of N-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfoxide) cholan-24-yl]-Glycine (cholestadien) (2.4 more times; $p < 0.0420$), 4-maleyl-acetoacetate (MAA) (3.2 more times; $p < 0.0364$) and irilone (2.2 more times; $p = 0.0451$) than pregnant animals.

4 | DISCUSSION

Metabolomics technologies can be used to define phenotypic patterns of small molecules in blood in different metabolic status. This work shows that, despite not observing differences in chemical composition of the gastric content, the breeding status (pregnant and lactating) of European wild rabbits does affects nutritional requirements and their metabolome (using targeted and untargeted metabolomics) and useful biomarkers for this identification are provided. Next, we proceed to develop the main results obtained.

Regarding biometric measurements, absolute and relative gastric content and targeted metabolomics, there were no significant differences in breeding status except for the weight and NEFA. As depicted in Figure 2a, none of the analysed parameters of digestive content showed significant differences, whether in relative or absolute value. There is limited research available on the feed intake capacity of wild rabbits. While studies have indicated that females tend to consume more than males (Marín-García et al., 2023), there is a gap in the existing literature regarding the impact of breeding



m/z-RT	ION	Metabolite	P_value	Fold[1]
101.0228/RT1.457	[M+H]+	Succinic anydride	0.0236	0.287
528.26312/RT5.634	[M-H]-	N-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfooxy)cholan-24-yl]-Glycine	0.0420	2.362
297.0372:RT5.314	[M-H]-	Irilone	0.0451	2.185
257.02811:RT1.455	[M+Hac-H]-	4-maleyl-acetoacetate	0.0364	3.167
73.0292:RT1.457	[M-H]-	Ethyl formate or Propionic acid	0.0121	0.251
117.0193:RT1.456	[M-H]-	Succinic acid	0.0068	0.319

[1] Fold change was calculated by dividing the mean intensity of plasma metabolites at Pregnant animals by the mean intensity of the corresponding metabolite in Lactating animals, blue and green colours represent a greater amount of this metabolite in Pregnant and Lactating animals, respectively. Identification was done tentatively identified using MS/MS and METLIN/HMDB databases.

FIGURE 3 Summary of the results obtained by the untargeted metabolomics test. (a,b) PLS models of plasma in positive and negative mode, respectively. The colours correspond to the two breeding status: ◯ Pregnant and □: Lactating. (c,d) Volcano plot showing significant differentially abundant metabolites between two breeding status (Two-sided Wilcoxon rank tests with the value adjusted by false discovery rate, FDR < 0.05) are shown; ◯ (Fold change > 1.169) □ (fold change < 0.95) in the volcano plot. Volcano plots are in positive and negative mode, respectively (r = 0.5765 and r = 0.6808, respectively). (e) List of plasmatic metabolites discriminating among breeding status (Pregnant vs. Lactating). Metabolites were tentatively identified using MS/MS and METLIN/HMDB databases. m/z-RT corresponds with query mases and its retention time.

stages on feed intake. However, this effect has been studied in controlled conditions where a greater digestive content was observed in lactating females than in pregnant ones (Xiccato et al., 1999). In this trial feed intake has been studied as the gastric content since there exist a correlationship between feed intake and gastric content (Sanderson & Vanderweele, 1975). The lack of significant differences in feed intake could stem from the observation that under wild conditions, all females tend to consume and maximize their ingestion. This hypothesis is supported by the visual observation of substantial digestive contents in all individuals, suggesting a high intake across the board. In this context, we should infer that any disparities observed in the metabolome, if linked to nutritional metabolites, are likely attributable to variations in their utilization rather than differences in ingestion levels. It is well known that pregnant animals have greater weight due to increased pregnancy (Partridge et al., 1986; Xiccato et al., 1995) and this can also be seen in this work. NEFA is indicating a short-time mobilisation of adipose tissue (Calle et al., 2017; Fortun-Lamothe, 2006; Gross et al., 2011). Low NEFA levels of pregnant does could be indicating a lower expenditure of body reserves compared with lactating animals, which would be being subjected to a clear nutritional challenge. It is well known that lactation is very costly in terms of energy (Fortun-Lamothe, 2006), which is why the increase in the NEFA levels would be related to these greater energy requirements. This trend has also been observed in other species, such as sows (Verheyen et al., 2007) and dairy cows (González et al., 2011), where the high levels of NEFA have been correlated to milk production (Mohebbi-Fani et al., 2019). In summary, our results will be suggesting that NEFA could be used as blood indicator of lipomobilisation during lactation in wild rabbit does. In accordance with the higher NEFA levels found in lactating females, these animals also presented higher levels of succinic anhydride, succinate and propionic acid. Succinic molecules are related to glucose metabolism and to lipid mobilisation. In fact, succinate is an important intermediate of the mitochondrial citric acid cycle (CAC) and contributes to lipolysis (Xiao et al., 2017). Furthermore, succinate has been demonstrated as a relevant controller of leptin expression (Villanueva-Carmona et al., 2023), which prevents the effects of fasting on reproductive process (Schneider et al., 2000). Related to fat accumulation, succinate controls the activation of adipose tissue (Mills et al., 2018), according to the anabolic phase of pregnancy where the female body stores nutrients (Meo & Hassain, 2016). Propionic acid, with a similar effect to other short-chain fatty acids, such as acetic or butyric, reduces food intakes (Al-Lahham et al., 2010), and has a gluconeogenic potential (Blaak et al., 2020). So, the elevated levels of succinic anhydride, succinate and propionic acid found in pregnant females respond to a high mobilisation of reserves in these animals, to assume the greater energy expenditure that pregnancy entails.

On the contrary, levels of cholestadien, MAA and irilone were higher in lactating females than pregnant ones. Cholestadien is an acyl glycine, a product of microbial enzymes metabolism. This metabolite is an uncommon sulphate bile acid (Goto et al., 2007), and was detected for the first time in urine samples of pregnant women (Meng et al., 1997). Related to cholesterol metabolism, an increase in the levels of this metabolite during lactation has been previously described in other species, such as cows (Schlegel et al., 2012) or rats (Smith et al., 1998), probably due to an increase in the expressions of genes involved in cholesterol metabolism during lactation.

MAA is a medium-chain keto acid and is an intermediate in the phenylalanine/tyrosine catabolism (Shroads et al., 2015). The isomerisation of MAA to fumarylacetoacetate is catalysed by the enzyme glutathione-S-transferase-zeta (GST-zeta) (Schultz et al., 2002) and an inhibition of this enzyme produces an increase of MAA and other tyrosine catabolites levels (Ammini et al., 2003). In summary, our data could be suggesting that the higher levels of MAA observed in lactating females could be a consequence of a greater need to produce proteins by these females. In fact it has been shown that a production of tyrosine increases during lactation due to prolactin effect (Voogt et al., 2001).

Finally, irilone is a red clover (*Trifolium pratense*) isoflavone (Lee et al., 2018). Red clover is one of the plants in the diet of hares (Freschi et al., 2014) and European wild rabbit (Delibes-Mateos et al., 2008) in the Mediterranean basin. According to our data, food consumption is similar in pregnant and lactating females, so the higher levels of irilone in the lactating does could be a consequence of a lower use of this compound by lactating females (increasing its excretion). In this context, more interesting is the oestrogenic potential (Lutter et al., 2014) and recent progestogenic activity found of this metabolite, which can enhance the progesterone signalling (Austin et al., 2021). It is suggested that the irilone has a synergic effect on progesterone and is capable of binding to the receptors of this hormone, enhancing its effects. Levels of progesterone and its receptors diminish during the pregnancy (Leavitt & Blaha, 1970), being practically undetectable during lactation in rabbits (Marongiu & Dimauro, 2013). This lack of progesterone receptors to bind to could explain the higher levels of irilone in the blood of lactating rabbits. Through untargeted metabolomics, this work has identified disparities in the metabolomes of pregnant and lactating animals. This phenomenon was also recently documented were it was observed alterations in the gut metabolomic profile due to genetic selection in captive rabbits (Casto-Rebollo et al., 2023). This work presents in a novel way an approach that combines information on digestive content, targeted and untargeted metabolomics in wild rabbits, being, as far as we have been able to find, the first work of its kind.

5 | CONCLUSIONS

This work aimed to determine how breeding status of European wild rabbits does affects nutritional requirements and their metabolome. In this sense, the main conclusions drawn from the work would be: (i) Targeted and untargeted metabolomics are more sensitive to breeding status than gastric content. (ii) Breeding status causes notable changes in the metabolome of individuals, as most of the observed changes are due to energy and protein metabolism.

Pregnant animals showed higher plasmatic levels of succinic anhydride, succinate, and propionic acid than lactating animals. However, lactating animals showed higher levels of NEFA, cholestadien, MAA and irilone than pregnant ones. These high levels in lactating females could be a consequence of an activation of cholesterol and protein catabolism, necessary for milk production in the case of cholestadien and MAA, and of a decrease of progesterone receptors during lactation period in the case of irilone. These metabolisms along with their associated metabolic pathways could be utilized to detect necessary changes in the reproductive status of animals, linking nutrition with the fitness of this species.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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