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Effect of litter size on prepartum metabolic and amino acidic profile in rabbit does

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*The use of modern prolific lines of rabbit does in intensive production systems leads to an increase in productivity but also causes a rise in several problems related to the does' health status. Hence, the aim of this study was to investigate the effect of the litter size on the metabolic, inflammatory and plasma amino acid profile in rabbit does. The blood of 30 pregnant does was sampled on the 27th day of pregnancy. The does were retrospectively grouped according to the number of offspring into a high litter size group (**HI**, does with* $\geq$ *12 kits;* n = *16) and a low litter size group (**LO**, does with* $\leq$ *11 kits;* n = *14). Data were subjected to Pearson's correlation analysis. Further, data were analysed in agreement to a completely randomized design in which the main tested effect was litter size. The linear or quadratic trends of litter size on parameters of interests were* post hoc *compared by using orthogonal contrasts. In addition, compared with the LO group, the HI group had lower levels of glucose (−5%;* P < *0.01), zinc (−19%;* P < *0.05), albumin (−6%;* P < *0.05) and total cholesterol (−13%;* P < *0.07), but the total bilirubin level was higher in the HI group (+14%;* P < *0.05). Regarding the plasma amino acids, the HI group had lower concentrations of threonine (−15%), glycine (−16%), lysine (−16%) and tryptophan (−26%) and a higher level of glutamic acid (+43%;* P < *0.05) compared with the LO group. The exclusively ketogenic amount of amino acids was lower (*P < *0.06) in the HI (55.8 mg/100 ml) does compared with the LO does (56.8 mg/100 ml). These results show that a few days before delivery, rabbit does that gave birth to a higher number of offspring had a metabolic profile and an inflammatory status that was less favourable with respect to does who gave birth to a lower number of offspring. Moreover, the plasma amino acid profile points out that there was an enhanced catabolic condition in the rabbit does with a high number of gestated foetuses; it was likely related to the greater energy demand needed to support the pregnancy and an early inflammatory response.*

Keywords: rabbit doe, gestation, litter size, inflammometabolic condition, plasma amino acids

Implications

A better comprehension of the physiology of rabbit does around the time of parturition can help to ameliorate their welfare status. Four days before parturition, rabbit does with high number of gestated foetuses suffered metabolic stress and the liver responses could be imbalanced. The great energy demands by the foetuses is coped by glucose and amino acids. Hence, new nutritional strategies considering the amino acid requirements should be considered to improve the health, welfare and performance of the modern prolific lines of rabbit does.

Introduction

In the last decades, improvements in the rabbit breeding system allowed an increase in the efficiency and sustainability of the sector. At the same time, several problems related to the health status and welfare of does (Castellini *et al.*, 2010; Pascual *et al.*, 2013) led to high rates of mortality in their offspring, high rate of culling (due to infertility or disease) and a low rate of production (Rosell and de la Fuente, 2009; Sánchez *et al.*, 2012). In the production cycle of does, late pregnancy is the most critical phase (Xiccato, 1996; Minuti *et al.*, 2015). During this period, does undergo dramatic physiological challenges related to mobilization of body reserve and the consequent increase in circulating non-esterified fatty acids (Rebollar *et al.*, 2011;

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Martínez-Paredes *et al*., 2012; Savietto *et al*., 2016). The last week of gestation can be very stressful, especially if the number of gestated foetuses is high (Minuti *et al*., 2015). The physiological changes are of great importance because they are closely linked with factors that significantly affect the profitability of the system (e.g., the incidence of clinical and subclinical *postpartum* diseases, milk production, resumption of ovary activity) (Castellini *et al*., 2010). In our previous study, we observed does for a few days before parturition, who were suffering stress due to either metabolic or inflammatory disorders (Minuti *et al*., 2015). Interestingly, the disorders were affected by the number of offspring of the does (Minuti *et al*., 2015). The genetic selection for reproductive traits has mainly focused on the improvement in litter sizes, leading to highly prolific lines (Pascual *et al*., 2013). Consequently, the increasing number of foetuses in new breeds may exacerbate the condition of the does in the last week of pregnancy. New research is needed to study the main mechanisms involved in these phenomena and develop strategies to prevent or attenuate the adverse physiological conditions. The aim of the present study was to investigate the effect of the litter size on the metabolic and plasma amino acid profiles and inflammatory status before parturition in rabbit does who were pregnant with different numbers of foetuses.

Material and methods

Housing, animals and feeding

The study was carried out using 30 rabbit does (*Oryctolagus cuniculus*) from commercial hybrid lines (HYPLUS PS 19; Groupe Grimaud, Roussay, France). During the study period (November to December), the average daily temperatures ranged from 11.3°C to 18.4°C and the average relative humidity ranged from 65% to 75%, as maintained by a forced ventilation system. The does were 7 months old and were preparing for their third parturition. They were housed in individual flat-deck cages ($0.4 \times 0.6 \times 0.35$ m) equipped with a drinker and a manual feeder. The does were bred under a constant photoperiod of 16 h of light per day. They were given *ad libitum* access to water and to a commercial pelleted diet for lactating does (Fertilap; Purina-Cargill s.r.l., Spessa (PV), Italy; Supplementary Table S1) for the entire study, but neither feed intake nor water consumption was measured.

Blood sampling and analysis

On the 27th day of pregnancy, the blood samples were collected at around 0830 h. from the central auricular artery into 2-ml tubes containing sodium heparin as an anticoagulant (5 units of heparin per ml of blood). After bleeding, the BW was measured. Blood samples were immediately cooled in ice water. Within 1 to 2 h after bleeding, the plasma was obtained after centrifugation at 3500×**g** for 15 min at 4°C and stored at −20°C until analysed.

Samples were analysed for metabolic markers [glucose (mmol/l), total cholesterol (mmol/l), triglycerides (mmol/l), creatinine (mmol/l), urea (mmol/l), aspartate aminotransferase (U/l), γ-glutamyl transpeptidase (U/l), alkaline phosphatase (U/l), non-esterified fatty acids or **NEFAs** (mmol/l), β-hydroxybutyrate or **BHBA** (mmol/l)], minerals [Ca (mmol/l), P (mmol/l), Mg (mmol/l), K (mmol/l), Na (mmol/l), Cl (mmol/l), Zn (μmol/l)] and oxidative-inflammatory markers [total protein (g/l), albumin (g/l), globulin (g/l), total bilirubin (μmol/l), haptoglobin (g/l), ceruloplasmin (μmol/l), reactive oxygen metabolites (mg H_2O_2/100 ml), paraoxonase (U/ml), myeloperoxidase (U/l) and ferric reducing antioxidant power (μmol/l)]. Blood metabolites were analysed by an automated biochemistry analyzer (ILAB 650; Instrumentation Laboratory, Lexington, MA, USA), according to the methodology previously described (Calamari *et al*., 2016; Minuti *et al*., 2019).

Plasma (1 ml) was deproteinized by precipitation with picric acid (5 ml) using 0.5 ml of norleucine (2500 nm/ml) as an internal standard. Samples were centrifuged at 4500×**g** for 15 min and then 5 ml of supernatant was purified× with a Dowex 2 10 resin column with 100 to 200 meshes in Cl^- form. For three more times, HCl 0.02 N (5 ml) was added at the end of the process to wash the column. All materials exiting the column (sample and wash) were collected in a flask with a flat bottom and dried in a Rotavapor. Samples were then diluted with 5 ml of lithium buffer at a pH of 2.2 and filtered with a syringe filter of 0.22 μ. The amino acids were quantified using a postcolumn derivatization method that used ninhydrin as the analysis reagent and an amino acid analyzer (Jasco Inc., Easton, MD, USA), according to the official method (Moore and Stein, 1954). The amino acids were classified and grouped according to their metabolic destination following the grouping reported in Supplementary Table S2.

Animal grouping and statistical analysis

The average litter size at parturition for the 30 does was 11.6 ± 2.8 kits. In order to evaluate the effect of litter size on the blood metabolites and blood amino acid profile, does were retrospectively divided into two groups according to the number of their offspring, either a high litter size group (**HI**, does with ≥ 12 kits; $n = 16$) or a low litter size group (**LO**, does with ≤ 11 kits; $n = 14$). The criteria for grouping were the average litter size.

The normal distribution of data was assessed by the Proc univariate of SAS (release 8.0; SAS Inst. Inc., Cary, NC, USA) with the NORMAL statement. Data were subjected to ANOVA using the MIXED procedure of SAS. The statistical model included the fixed effect of the litter size group. The pairwise comparison was carried out using the LSD statement. The following model was applied:

$$Y_{ij} = \mu + LS_j + \varepsilon_{ij}$$

where Y_{ij} indicates the dependent variables (blood parameters and BW), μ is the overall mean, LS_j is the effect of the litter size ($_j$ = HI and LO) and ε_{ij} is the random error component.

Data were also analysed according to a completely randomized design. The model included the fixed effect of litter size and means were *post hoc* compared by orthogonal contrast to verify linear and quadratic effects of the main tested effect (litter size) on parameters of interest (blood parameters). Further, data were subjected to Pearson's correlation analysis using the CORR procedure of SAS. Significance was declared at $P < 0.05$.

Results

Animals

On average, does involved in this study delivered 11.6 kits. The HI group had on average 13.7 kits and the LO group had on average 9.4 kits. On the 27th day of pregnancy, the does weighed 4.73 and 4.60 kg in the HI and LO groups, respectively.

Metabolic profile

The effect of the litter size on the metabolic profile of the rabbit does on the 27th day of pregnancy is shown in Table 1. Regarding the energy and protein markers, significant differences were observed for the plasma glucose ($P < 0.01$) and total cholesterol ($P < 0.07$) concentrations; the results were lower in HI does compared with LO does. Regarding the plasma mineral concentrations, the Mg was higher ($P < 0.05$) and the Zn was lower ($P < 0.05$) in the HI does compared with the LO does. Significant differences were observed among inflammatory and oxidative stress markers. The HI group had a lower albumin concentration ($P < 0.05$) but a higher total bilirubin concentration ($P < 0.05$) compared with the LO group. Compared with the LO group, the HI does had numerically higher levels of ceruloplasmin, haptoglobin and myeloperoxidase, but the paraoxonase and cholesterol levels were lower.

Amino acid profile

Results of the plasma amino acid profile of does on the 27th day of pregnancy are reported in Table 2. Among the 26 identified amino acids, the litter size affected the levels of threonine, glycine, lysine and tryptophan ($P < 0.05$), causing the levels to be lower in the HI group compared with the LO

Table 1 *Effect of the litter size on plasma parameter on the 27th day of pregnancy in rabbit does classified as high litter size (HI = 16 does) and low litter size (LO = 14 does)*

Item	HI	LO	SEM	*P* value
Energy and protein markers				
Glucose (mmol/l)	7.03	7.41	0.09	0.006
Total cholesterol (mmol/l)	0.153	0.174	0.008	0.07
Triglycerides (mmol/l)	0.290	0.311	0.028	0.60
NEFAs (mmol/l)	0.126	0.123	0.015	0.99
BHBA (mmol/l)	0.093	0.083	0.013	0.65
Creatinine (μmol/l)	86.5	85.9	3.1	0.89
Urea (mmol/l)	6.51	6.81	0.26	0.40
Aspartate aminotransferase (U/l)	56.5	46.0	9.0	0.42
γ-Glutamyl transpeptidase (U/l)	5.05	4.63	0.40	0.47
Alkaline phosphatase (U/l)	22.3	21.2	1.3	0.55
Minerals				
Ca (mmol/l)	3.62	3.70	0.06	0.42
P (mmol/l)	1.43	1.41	0.08	0.91
Mg (mmol/l)	1.30	1.15	0.04	0.02
Na (mmol/l)	143.4	143.2	0.4	0.71
K (mmol/l)	5.16	5.06	0.16	0.67
Cl (mmol/l)	105.6	104.9	0.5	0.35
Zn (μmol/l)	14.7	18.3	1.1	0.03
Inflammatory and oxidative markers				
Total protein (g/l)	51.5	53.3	1.6	0.44
Albumin (g/l)	32.8	35.1	0.7	0.04
Globulin (g/l)	18.7	19.0	0.8	0.83
Total bilirubin (μmol/l)	2.03	1.78	0.09	0.05
Ceruloplasmin (μmol/l)	5.74	5.48	0.49	0.72
Haptoglobin (g/l)	1.17	1.04	0.12	0.45
Paraoxonase (U/ml)	198.9	213.9	14.2	0.46
Myeloperoxidase (U/l)	481.5	392.3	57.9	0.29
ROMs (mgH_2O_2/100 ml)	21.5	22.1	1.9	0.83
FRAP (μmol/l)	120.8	110.1	13.01	0.56

NEFAs = non-esterified fatty acids; BHBA = β-hydroxybutyrate; ROMs = reactive oxygen metabolites; FRAP = ferric reducing antioxidant power; HI = high litter size; LO = low litter size.

Table 2 *Effect of the litter size on plasma amino acid profile (mg/100 ml) on the 27th day of pregnancy in rabbit does classified as high litter size (HI = 16 does) and low litter size (LO = 14 does)*

Item	HI	LO	SEM	*P* value
	(mg/100 ml)	(mg/100 ml)		
Alanine	4.95	4.72	0.29	0.59
Arginine	3.46	3.88	0.24	0.21
Asparagine	4.41	4.46	0.31	0.92
Aspartic acid	0.46	0.45	0.04	0.90
Citrulline	1.37	1.49	0.06	0.18
Cystine	0.23	0.27	0.03	0.47
Cystathionine	0.53	0.53	0.03	0.86
Glutamic acid	2.18	1.53	0.22	0.02
Glutamine	16.75	16.07	1.07	0.66
Glycine	7.26	8.62	0.47	0.05
Histidine	3.46	3.59	0.11	0.42
1-Methylhistidine	0.27	0.30	0.04	0.61
3-Methylhistidine	0.30	0.27	0.03	0.42
Isoleucine	1.37	1.34	0.05	0.64
Leucine	1.62	1.63	0.06	0.97
Lysine	3.50	4.18	0.21	0.03
Methionine	0.92	0.94	0.05	0.82
Ornithine	1.38	1.57	0.09	0.15
Phenylalanine	2.38	2.22	0.09	0.21
Proline	4.95	5.21	0.27	0.50
Serine	3.75	4.01	0.18	0.31
Taurine	0.69	0.63	0.05	0.37
Threonine	2.87	3.37	0.17	0.04
Tryptophan	0.21	0.28	0.03	0.05
Tyrosine	1.24	1.21	0.05	0.77
Valine	3.26	3.36	0.12	0.58
Total AAs	69.23	71.31	2.43	0.55
Branched-chain AAs	6.25	6.32	0.22	0.83
Essential AAs	19.60	20.88	0.64	0.17
Non-essential AAs	49.40	50.16	1.95	0.79
Ketogenic AAs	13.19	14.21	0.49	0.15
Exclusively ketogenic AAs	5.12	5.81	0.26	0.07
Glucogenic AAs	63.88	65.23	2.26	0.68
Exclusively glucogenic AAs	55.80	56.82	2.09	0.73
Ketogenic and glucogenic AAs	8.07	8.40	0.27	0.39

AAs = amino acids; HI = high litter size; LO = low litter size.

group, and for glutamic acid, causing the levels to be higher in the HI group compared with the LO group ($P < 0.05$). For other amino acids, no significant differences were observed between the HI and LO groups. Based on the type of intermediates formed during the breakdown or catabolism of the exclusively ketogenic amount of amino acids, lower concentrations were found in the HI does compared with the LO does ($P < 0.06$).

Discussion

The use of modern prolific lines of rabbit does in intensive production systems leads to an increase in productivity but also causes a rise in several problems related to the does' health status. Better comprehension of the factors involved in the physiology of rabbit does around the time of parturition can help to ameliorate their adaptation and welfare as well as to revise the nutritional requirements of the animals to match their new physiological demands (Xiccato, 1996; Pascual *et al.*, 2013).

Effect of litter size

With the purpose to investigate the effect of the litter size on the metabolic status and amino acid profile in rabbit does, we retrospectively grouped does in HI and LO group. We focused on studying the does on the 27th day of pregnancy (4 days before parturition) because we had previously demonstrated that the most critical phase for periparturient rabbit does is the days immediately prior to parturition (Minuti *et al.*, 2015).

Data of the current study confirmed that before parturition, the HI rabbit does had a less favourable metabolic

conditions. Even though the HI does did not have a condition in which more fat mobilization occurred (i.e. the NEFAs and BHBA were similar in the HI and LO groups), as it was observed in the previous study (Minuti *et al.*, 2015), they did have a lower blood glucose concentration. This can be interpreted as a sign of the higher energy consumption necessary to satisfy the gestation requirements of more foetuses, especially in the last days of gestation (Gilbert *et al.*, 1984; Jean-Blain and Durix, 1985). It is confirmed by the negative relationship found between the glucose levels and the number of gestated foetuses (Figure 1).

Another aspect confirmed by the current study is the link of a higher number of offspring with a worse inflammatory condition in the days immediately before parturition. The greater concentration of bilirubin and the lower levels of albumin and zinc in the plasma are supportive of the assumption. These metabolites are considered inflammatory markers and related to the so-called acute-phase reaction (Trevisi and Minuti, 2018). During inflammation, the liver is activated and impaired at the same time. The synthesis of positive acute-phase markers (e.g. haptoglobin, ceruloplasmin) is increased for the purpose of protecting the organism against pathogens. Some positive acute-phase markers can capture minerals (e.g. Fe, Zn) from the blood to reduce the survival of pathogens. Other functions of the liver are impaired due to a reduced synthesis of common proteins,

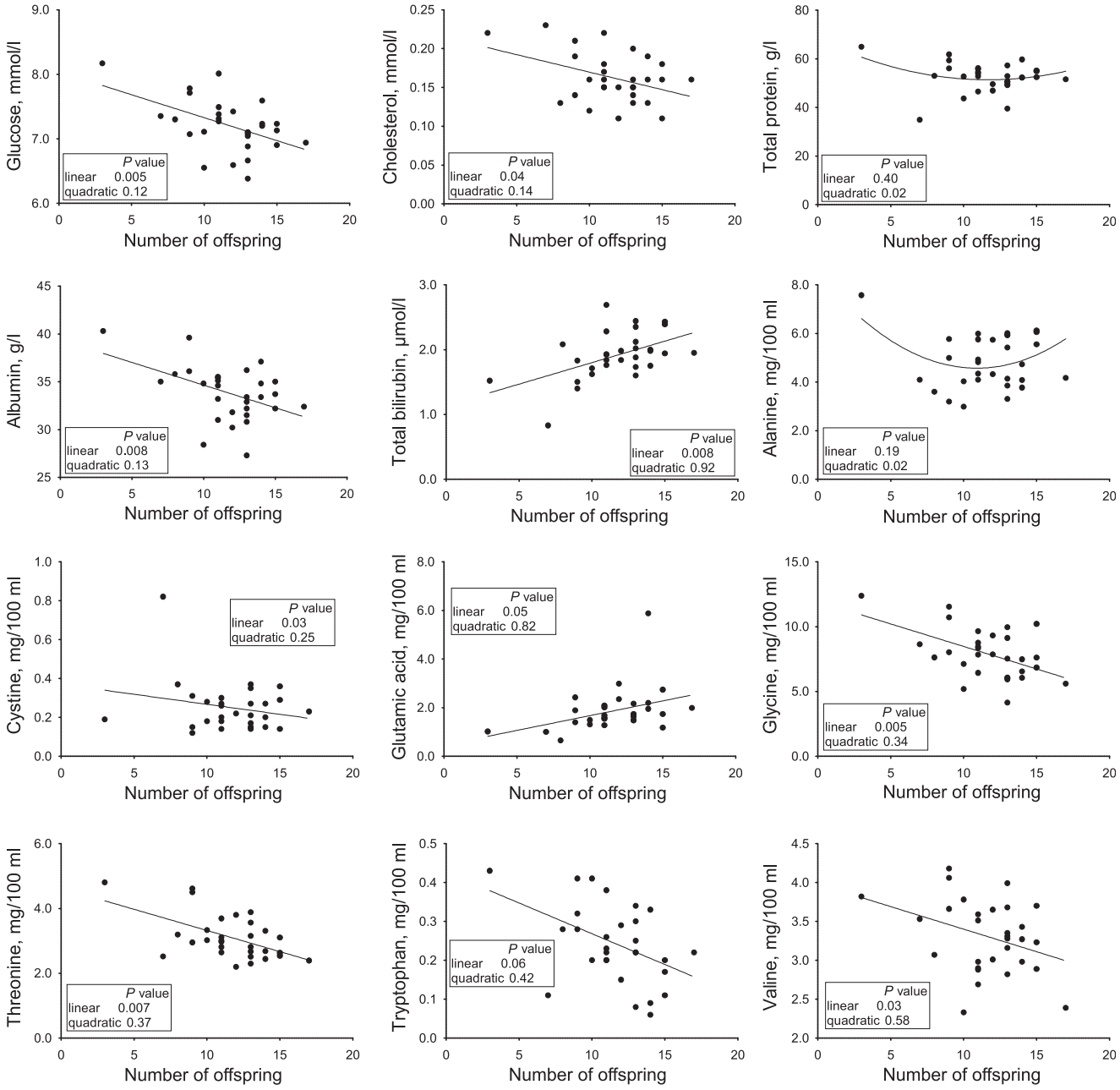

Figure 1 Plots of the linear or quadratic relationships between the number of the offspring and the concentration of blood parameters in 30 rabbit does on the 27th day of pregnancy.

likely because of the competition for substrates (Zhou *et al.*, 2016). These proteins are called negative acute-phase markers and include albumin, enzymes (i.e. enzymes for the clearance of bilirubin, so the concentration of this metabolite in blood increases), apolipoproteins and carriers of lipophilic molecules (i.e. cholesterol, hormones and vitamins) (Gruys *et al.*, 2005). It seems useful to note that both albumin and bilirubin had behaviours similar to those reported in our previous study on a smaller number of does. Both blood parameters showed a significant relation with the number of gestated foetuses, with a negative relation for albumin and a positive one for bilirubin (see Figure 1).

The unique aspect of this study is the amino acid profile data, which are very rare in studies focused on the peripartum period of mammals, particularly does. Data show interesting differences in the relationship to the size of the breed of rabbits. In this study, the feed intake as well as the circadian variability of the caecotrophic behaviour of the does was not measured, and this could represent a possible bias interpretation of the results, which should be attenuated by the high number of subjects involved in the study. However, of the nine essential amino acids, which the diet is the only source, only three showed significantly different values between the groups, and this may suggest that feed intake, as well as circadian variability of the caecotrophic behaviour of the does, did not affect the concentration of these amino acids in the plasma, and other mechanisms could be involved. The HI does showed lower plasma levels for three essential amino acids (threonine, lysine and tryptophan) and one non-essential amino acid (glycine). Glycine is an exclusively glucogenic amino acid; its catabolism generates pyruvate, which is used for the synthesis of glucose. Lysine is an exclusively ketogenic amino acid; its catabolism generates only acetyl-CoA, which is used in the ketogenesis pathway. Threonine and tryptophan are used in both the glucogenic and ketogenic metabolism; it produces both pyruvate and acetyl-CoA. It is not easy to interpret the lower concentrations of these amino acids in the plasma of does before parturition. To our knowledge, no literature related to the amino acid profile in rabbit plasma is available. The only study in which the amino acid profile was investigated involved male rabbits (Block and Hubbard, 1962). We hypothesized two possible mechanisms that alone or together could explain the phenomenon we observed. (1) The greater use as energy precursors of threonine, lysine and tryptophan can cope with the larger energy demand during the gestation of a greater number of foetuses, likely leading to a reduction in the concentrations of these three amino acids in the plasma of HI rabbits. The reason for this is not clear because only these amino acids and not others showed differences between the groups. The use of amino acid as energy source is also supported by their positive correlations with blood glucose (alanine, arginine, aspartic acid, glycine, lysine, ornithine and threonine) and negative correlations with NEFAs (alanine, arginine, asparagine, glutamine, glycine, isoleucine, leucine and lysine) and BHBA (arginine and glycine), all markers of negative energy condition

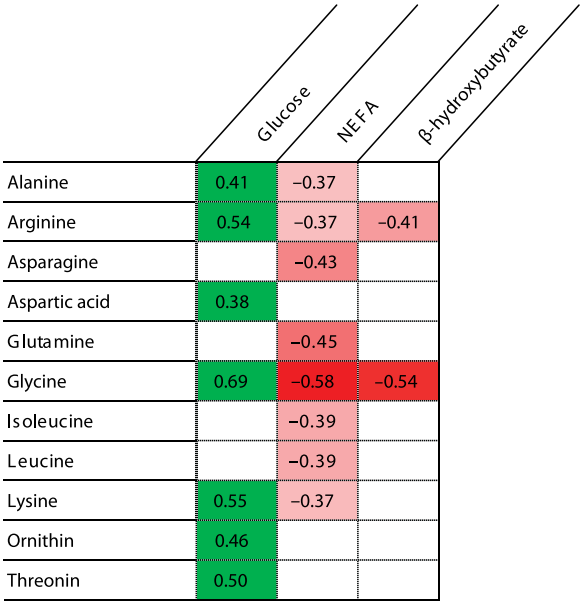

Figure 2 (colour online) Pearson's correlation coefficients (r) between plasma concentration of glucose, non-esterified fatty acids (NEFAs), β-hydroxybutyrate and plasmatic concentration of amino acids of 30 rabbit does on the 27th day of pregnancy. The red points out negative correlations, the green points out positive correlations. All the r values shown in the figure have a P value lower than 0.05.

(Figure 2). The higher plasma concentrations of glutamic acid in HI rabbits could be due to the greater muscle catabolism necessary to provide energy substrates during a situation of catabolism (Parigi-Bini *et al.*, 1990). Glutamic acid is, in fact, the most abundant amino acid in the protein of muscle tissue, and its higher blood level may be a sign of a greater mobilization of muscle tissue. This hypothesis could be supported by the greater reduction in BW observed after parturition in does with more offspring (Minuti *et al.*, 2015). (2) The reduction in plasma amino acids in HI does is likely due to their worst inflammatory condition (as already stated). The plasma amino acid concentrations during inflammation are reduced due to their increased extraction by the liver (Kurpad, 2006; Zhou *et al.*, 2016). Therefore, the lower amino acid levels in the HI does before parturition may be a result of the increased demand for hepatic synthesis. The fact that in our study no significantly higher values have been reached either for the positive markers of the acute phase (ceruloplasmin and haptoglobin) or for the markers of mobilization of the body's lipid reserves could indicate that few days before delivery does are in an initial phase of these phenomena, so that they are not yet detectable by markers in the blood. If this is true, the amino acid variations in the plasma would emerge as interesting predictive markers.

Conclusion

Few days before parturition, rabbit does with higher number of gestated foetuses have worse sub-clinical inflammatory status manifested by blood markers (lower concentration of albumin and zinc and higher concentration of bilirubin). From a

metabolic point of view, the gestation of a greater number of foetuses causes a greater energy requirement, which is coped with a higher consumption of glucose and the use of amino acids as energy source. On the whole, 4 days before parturition, the liver handled multiple requests at the same time and some responses could be imbalanced. Our results suggest that new nutritional strategies should be considered to improve the health, welfare and performance of rabbit doe breeds characterized by a high number of offspring.

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Declaration of interest

The authors declare no conflict of interest.

Ethics statement

This study complied with Italian laws on animal experimentation (DL n.116, 27/01/1992) and ethics (procedure's number ZN2013004). The experiment was carried out in the Università Cattolica del Sacro Cuore experimental barn (CERZOO, San Bonico, Piacenza).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731120000981

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