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Effects of feed restriction during pregnancy on maternal reproductive outcome, foetal hepatic IGF gene expression and offspring performance in the rabbit

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ABSTRACT

Primiparous female rabbits have high nutritional requirements and, while it is recommended that they are subjected to an extensive reproductive rhythm, this could lead to overweight, affecting reproductive outcomes. We hypothesised that restricting food intake during the less energetic period of gestation could improve reproductive outcome without impairing offspring viability. This study compares two groups of primiparous rabbit does in an extensive reproductive programme, one in which feed was restricted from Day 0 to Day 21 of gestation (**R021**), and another in which does were fed *ad libitum* (control) throughout pregnancy. The mother and offspring variables compared were (1) mother reproductive outcomes at the time points pre-implantation (Day 3 postartificial insemination [**AI**]), preterm (Day 28 post-AI) and birth; and (2) the prenatal offspring characteristic IGF system gene expression in foetal liver, liver fibrosis and foetus sex ratio, and postnatal factor viability and growth at birth, and survival and growth until weaning. Feed restriction did not affect the conception rate, embryo survival, or the number of morulae and blastocysts recovered at Day 3 post-AI. Preterm placenta size and efficiency were similar in the two groups. However, both implantation rate ($P < 0.001$) and the number of foetuses ($P = 0.05$) were higher in the R021 mothers than controls, while there was no difference in foetal viability. Foetal size and weight, the weights of most organs, organ weight/BW ratios and sex ratio were unaffected by feed restriction; these variables were only affected by uterine position ($P < 0.05$). Conversely, in the R021 does, foetal liver *IGBP1* and *IGF2* gene expression were dysregulated despite no liver fibrosis and a normal liver structure. No effects of restricted feed intake were produced on maternal fertility, prolificacy, or offspring birth weight, but control females weaned more kits. Litter weight and mortality rate during the lactation period were also unaffected. In conclusion, pre-implantation events and foetal development were unaffected by feed restriction. While some genes of the foetal hepatic IGF system were dysregulated during pregnancy, liver morphology appeared normal, and the growth of foetuses and kits until weaning was unmodified. This strategy of feed restriction in extensive reproductive rhythms seems to have no significant adverse effects on dam reproductive outcome or offspring growth and viability until weaning.

Implications

In many countries, rabbit meat will almost certainly be a good food source in the years to come. However, production systems need to adapt to new challenges including a fine balance between animal physiological demands and production costs. Feed restriction during the less demanding part of gestation could be an interesting strategy for rabbit farmers, as it avoids excessive fattening of inseminated females in extensive reproductive programmes and

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reduces feeding costs. This strategy was found here to have no detrimental effects on maternal reproductive outcome or the viability and health of offspring.

Introduction

Rabbits undergo their first artificial insemination (**AI**) shortly after puberty (16–18 weeks of age). From this time point, primiparous females spend lots of energy both in producing milk for their kits and for their own growth (Xiccato et al., 2005). When AI is performed under an intensive (4 days postpartum) or semi-intensive (11 days postpartum) reproductive rhythm, rabbit does are able to simultaneously maintain both pregnancy and lactation, yet primiparous does show reduced fertility (Rebollar et al., 2006). Accordingly, a more extensive reproductive programme is recommended for these young females to recover their body reserves, such as artificial insemination at 32 days postpartum (Arias-Álvarez et al., 2009). However, this extensive reproductive rhythm has several shortcomings such as the excessive build-up of fat, which may be further aggravated if does do not become pregnant and continue to gain weight (Rommers et al., 1999) which negatively affects their long-term reproductive function (Castellini et al., 2006). Thus, maternal food restriction (**MFR**) during a specific period after the second AI could modulate the energy status of mothers, and have fewer detrimental effects during the subsequent lactation and therefore enhance reproductive function (ovulation response and parturition-to-fertile AI interval). Further, this management strategy will help reduce feeding costs for rabbit farms (Manal et al., 2010), especially as they account for 60–70% of total farming costs (Maertens, 2010).

A study has shown that MFR during the first two weeks of pregnancy does not affect female reproductive performance (Nafeaa et al., 2011). However, when using this strategy, there could be nutritional mismatch between maternal dietary intake and the energy demands of the developing foetuses (Cleal et al., 2007) and this could lead to foetal reprogramming (Caton et al., 2019). During pregnancy, restricted energy intake may cause intrauterine growth restriction (**IUGR**) in several species such as rats (Herrera et al., 2006), humans (Desai and Hales, 1997), guinea pigs (Elias et al., 2016) and rabbits (López-Tello et al., 2017). In response to changes in maternal nutrition, there could be also a sex ratio adjustment (Tarrade et al., 2013). In such circumstances, growth alterations later in life have been also described (Fleming et al., 2018). The liver is one of the main organs involved in growth and metabolism. This critical organ integrates signals to control glucose and triglyceride production to thus provide other body organs with energy (Lee et al., 2016) and for growth (Vijayakumar et al., 2011). Compromised maternal energy intake can affect the liver and postnatal liver disease has been observed in the offspring of ewes (Hyatt et al., 2007) and humans (Wang et al., 2016) subjected to low nutrition levels during pregnancy. The consequence is usually liver fibrosis and the remodelling of this organ with life-threatening complications (Halász et al., 2016).

Liver is the main source of IGF system components, including *IGF1*, *IGF2* and their binding proteins (**IGFBPs**) (Hyatt et al., 2004). This system is important for several bodily functions (Brameld et al., 2000) including foetal growth (Gluckman and Pinal, 2003). *IGF1* and *IGF2* mainly bind to the *IGF1* receptor (***IGF1R***) activating the insulin receptor (***IR***). However, IGFBPs also modulate IGF activity by limiting the availability of free IGFs for the IGF receptors (Randhawa and Cohen, 2005). Intrauterine undernutrition could act by reprogramming the IGF system in rat foetuses (El Khattabi et al., 2003; Harel and Tannenbaum, 1995) as well as influencing pre- and postnatal growth. In fact, foetuses undergoing intrauterine growth restriction (IUGR) often show the upregulation of hepatic genes that are related to insulin signalling (Limesand et al., 2007). In the uterus of undernourished humans and rats, *IGF1* levels are usually reduced (Fowden 2003; Gao et al., 2015) as *IGFBP1* levels are often higher than normal (Crossey et al., 2002). In the present study, we examined whether any possible alterations caused by MFR could affect the offspring liver health and IGF system, both of which could impair the growth and viability of foetuses and offspring.

Our working hypothesis was that restricting food intake during the pregnancy period in which energy costs are at their lowest yet sufficient for embryogenesis and organogenesis (Anderson and Henck, 1994) could be a good strategy to avoid overfeeding without negatively affecting reproductive outcomes and offspring development. The aims of this study were to assess the impacts of MFR during the first three weeks of pregnancy on: (1) maternal reproductive outcome at pre-implantation (Day 3 post-AI), preterm (Day 28 post-AI), and birth; and (2) foetal liver expression of candidate IGF system genes, liver fibrosis, sex ratio, viability, birth weight and growth until weaning.

Material and methods

Animals and facilities

New Zealand White × California rabbits were kept in the animal house of the Polytechnic University of Madrid (**UPM**) under constant, automatically adjusted environmental conditions (light:-darkness 16:8, temperature: 20–25 °C, and relative humidity: 60–75% by forced ventilation). Animals were individually reared in flat-deck cages fitted with automatic drinkers for free access to water. All experimental procedures were approved by the Animal Ethics Committee of the UPM (PROEX 302/15) in compliance with Spanish guidelines for animal care and use in research and in line with European Union Regulation 2010/63/EU.

Experimental design

Maternal feed restriction was conducted according to a protocol previously designed by our group (García-García et al., 2021). To define a normal daily intake for the experimental animals, rabbit does were fed *ad libitum* with a commercial diet (2 400 kcal of digestible energy/kg, 35% neutral detergent fibre and 16% CP, NANTA, Madrid, Spain), and their feed intake was recorded during their first pregnancy. A mean of 175 g/animal/day was established as the daily intake for each female. Then, at 32 days postpartum, 151 primiparous rabbit does were injected with gonadorelin (20 μg/doe, i.m.; Inducel-GnRH, Ovejero, León, Spain) to induce ovulation and then artificially inseminated with fresh diluted semen (MA 24, Ovejero, León, Spain) (Day 0) (see Fig. 1). Does assigned to the control group (n = 76) were fed *ad libitum* during the whole gestation period with the commercial diet described above. Females in the experimental group (group **R021**; n = 75) were fed 60% of the above-mentioned daily feed intake (105 g/day) from Day 0 to Day 21 of gestation (gestation length is usually 31 days). All does finished their daily rations and were fed *ad libitum* from the last week of pregnancy to the end of the study.

Pre-implantation reproductive outcome

At Day 3 post-AI, 10 randomly chosen does per group were euthanised (30 mg/kg; Dolethal, Madrid, Spain). Embryo recovery through medial ventral laparotomy was performed by flushing the reproductive tract with buffered phosphate saline supplemented with 0.2% of bovine serum albumin (Sigma, USA). Embryos

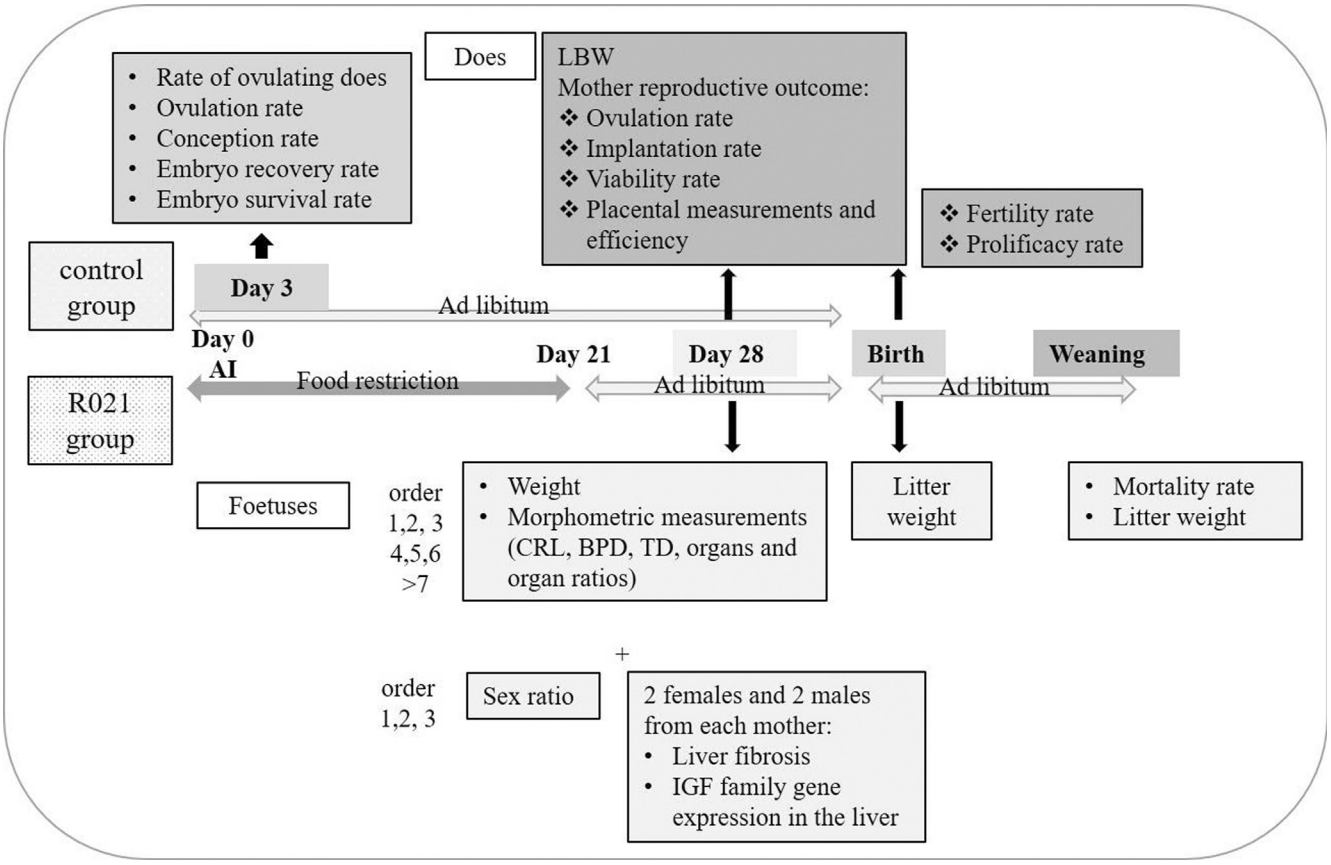

Fig. 1. Diagram of the experimental design. Rabbit females were feed restricted (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group) or fed *ad libitum* (control group) throughout gestation. Maternal and offspring measurements were taken at 3 days postinsemination, at 28 days of pregnancy and at birth, and kit measures were taken at weaning (4 weeks old). Gestation length was 31 days. CRL: crown-rump length; BPD: biparietal diameter; TD: transversal thoracic diameter; LWB: live body weight.

were classified based on conventional morphological criteria according to their developmental stage following the guidelines of the International Embryo Transfer Society. The following variables were recorded: rate of ovulating does ([number of does with corpora lutea (**CL**) in ovaries/number of euthanised does] × 100), ovulation rate (number of CL in the ovaries of ovulated does), conception rate ([number of does with recovered embryos/number of euthanised does] × 100), embryos recovered/female, and recovery rate ([total of recovered embryos/number of CL] × 100), number of morulae and blastocysts, and embryo viability ([number of viable morulae and blastocysts/number of CL] × 100) per female.

Preterm maternal reproductive outcome and foetal features

On pregnancy Day 28, five pregnant females from each group were randomly chosen after abdominal palpation, weighed to determine their BW, euthanised, and subjected to a medial ventral laparotomy. Ovaries were collected and the ovulation rate (number of CL on the ovarian surface per doe) was recorded. All foetuses from the gravid uterus were counted separated from their placentas, weighed, and classified as viable (when showing the typical morphology and weight for their gestational age) or non-viable (when dead in the uterus with signs of shrivelling and drying or reabsorption). Only viable foetuses were considered to calculate the implantation rate [(number of viable foetuses/number of CL) × 100] and viability rate [(number of viable foetuses/total number of foetuses) × 100]. Positions of foetuses recovered from each uterine horn were numbered beginning with the ones closest to the ovary (1, 2 and 3 = proximal; 4, 5 and 6 = mid-uterus; and 7 or above = distal). All viable foetuses were weighed and measured using scales and callipers to record crown–rump length (**CRL**, maximum distance from crown to tail), biparietal diameter (length from one parietal eminence to the other) and transversal thoracic diameter (length at the diaphragm insertion). The head, trunk, brain, liver, heart, lung, kidney and digestive tract of foetuses were removed and weighed. We also calculated ratios such as organ weight/foetal weight and brain weight/liver weight as indicators of IUGR. In addition, whole placentas from viable foetuses were weighed, and decidual and labyrinth sections were separated and weighed. Placenta efficiency was calculated as the foetal weight/-placental weight ratio.

For the liver study, six foetuses per doe located in proximal positions of the right and left uterine horns from the control (n = 30) and R021 (n = 30) groups were assessed. All foetuses were sexed by PCR, and sex ratios were calculated. Foetal liver fibrosis was assessed by Sirius red staining according to Huang et al. (2013). We also examined liver expression of the genes *IGF1*, *IGF1R*, *IR*, *IGFBP1* and *IGF2* gene by qPCR in two female and two male foetuses from each of the control (n = 10 females and 10 males) and R021 (n = 10 females and 10 males) dams.

Maternal reproductive outcome and offspring viability and growth at birth and at weaning

Remaining control (n = 61) and R021 (n = 60) females continued with their pregnancy until delivery. In these animals, we recorded fertility [(number of parturitions/number of AI) × 100] and prolificacy (total number of newborns, kits born alive and stillborn per litter). Litters (total born alive) were weighed at birth and then adjusted to 9–12 kits per doe in each experimental group.

At weaning (4 weeks old), litters were weighed, and the number of kits was recorded. Kits were checked once a day to determine mortality rate [(number of kits after litter size adjustment − number of weaned kits) * 100/number of kits after litter size adjustment].

PCR for foetus sex analysis

A piece of liver from the right lobe about 0.5–1.0 cm long was recovered, snap-frozen in liquid nitrogen and stored at −80 °C until PCR analysis for foetus sexing according to a previously described procedure (García-García et al., 2021). Briefly, after DNA extraction, the primers *OcSRY* and *OcGAPDH* were used (Table 1) for PCR and the products were confirmed by 2% agarose gel staining. The sex ratio was calculated as the number of females or males per total number of analysed animals in the experimental group.

mRNA extraction and RT-qPCR for relative gene expression of candidate IGF system genes

Total RNA was extracted from foetal livers using the TRIzol™ Plus RNA Purification Kit (Invitrogen – Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA quantification was performed with a NanoDrop ONE spectrophotometer, and quality was assessed in a 2100 Agilent Bioanalyzer (Agilent Technologies, CA, USA) using an RNA 6000 Nano Kit. Only A260/A280 absorbance ratios of 1.9–2.1 were accepted.

For cDNA synthesis, the High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions using 1 μg of total liver RNA in a total volume of 20 μL. Reverse transcriptase negative (RT−) controls were also prepared to check for genomic DNA contamination. Complementary DNA was stored at −20 °C until use.

Primers for RT-qPCR were designed using Probe Finder Assay Design Software (Roche Life Science, Germany) for the housekeeping genes *TBP* and *PGK1*, and synthesised by Metabion International AG (Planegg, Germany). For the candidate genes *IGF1*, *IGF1R*, *IR*, *IGFBP1* and *IGF2*, the Primer3 programme was used, and primers were synthesised by Sigma (USA) (Table 1). In a 10-μL volume of reaction mixture (PowerSYBR™ Green PCR Master-Mix, Thermo Fisher Scientific, Waltham, MA, USA), 2.5 μL of 1:2 diluted cDNAs was used. The working solution for forward and reverse primers was 300 nM. RT-qPCR conditions were as follows: 95 °C for 10 min and 40 cycles (95 °C for 15 s and then 60 °C for 1 min). For thermal cycling and fluorescence detection, we used QuantStudio™ 12 K Flex (Applied Biosystems, Foster City, CA, USA) with the corresponding software. Samples were analysed in duplicate in 384 well-plates (Applied Biosystems, Foster City, CA, USA). Analysis of RT- samples confirmed the absence of contaminating genomic DNA, and a duplicate non-template control (water instead of cDNA) in each plate ensured the absence of primer dimers. Threshold cycle (Ct) values were converted to relative expression values by the dCt method (Lucas et al., 2011).

Sirius red staining to detect foetal liver fibrosis

Another piece of liver adjacent to that recovered for PCR analysis was collected and fixed in 4% neutral buffered paraformaldehyde (PBS, pH 7.0) for 24 h at RT. The procedure was carried out as described previously (García-García et al., 2021). In brief, after a series of dehydration steps in ethanol and xylene, foetal livers were embedded in paraffin blocks and cut into 7-μm thick sections. Slides were stained with haematoxylin-eosin and Sirius red. For this purpose, we applied 1% Picro-Sirius Red (Sigma, Aldrich, Saint Louis, MO, USA) to dewaxed and hydrated sections for 1 h after several washes. Dehydrated slides were cleared, sealed and observed under a light microscope (Zeiss Axioplan-2, Oberkochen, Germany). Collagen fibres appearing as red staining on a pale-yellow background were taken as evidence of hepatic fibrosis based on correlation with collagen content according to López-De León and Rojkind (1985). For the analysis, five fields randomly selected from each liver section were analysed using a Plan Neofluar10x/30 lens and MetaMorph 6.0 software (Molecular Devices LLC, San Jose, CA, USA). The total collagen area and the percentage of fibrosis in the liver (expressed as a percentage relative to the total analysed area) were measured in the binary image using Image J (https://imagej.nih.gov).

Table 1
Gene primer sequences used for quantitative RT-PCR in foetal rabbit liver.

Gene	Primer sequence (5′–3′)	Accession number
OcSRY	Forward: AGCGGCCAGGAACGGGTCAAG Reverse: CTTCCGGCGAGGTCTGTACTTG	AY785433
OcGAPDH	Forward: TGAACGGATTTGGCCGCATTG Reverse: ATGCCGAAGTGGTCGTGGATG	L23961
TBP	Forward: TACCCTTCCCCCATGACC Reverse: TTGCAGCTGTGGTACAATCC	XM_002723497.3
PGK1	Forward: TGCTCGACAAAGTCAATGAGA Reverse: TCCATGTTGTTGAGCACCTT	XM_002720132.3
IGF1	Forward: CTTTTATTTCAACAAGCCCACAG Reverse: CTCCAGCCTCCTCAGATCAC	XM_008256716.2
IGF1R	Forward: AACCGCTGCCAGAAAATGTG Reverse: GCCGTGTCGTCGTCAGG	XM_017337784.1
IGFBP1	Forward: GCACAAAGCGGAAGGGG Reverse: GCTCCAGCACTCGGTAGAG	XM_002724212.3
IR	Forward: ACCGACTACCTGCTGCTGTT Reverse: TGACCAGCGCATAGTTGAAG	AY339877.1
IGF2	Forward: TGGAAGAACTTGCCCACGGAG Reverse: GCTGCATTGCTGCTTACCGC	JN825734.1

Abbreviations. SRY: sex-determining region Y protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; *TBP*: TATA-box binding protein; *PGK1*: phosphoglycerate kinase; *IGF1R*: IGF I receptor; *IGFBP1*: IGF binding protein; *IR*: insulin receptor; *RT*: Reverse Transcription.

Statistical analysis

Statistical analysis was performed using SAS software (SAS Inst. Inc., Cary, NC, USA). All data were first tested for normality via the univariate procedure. The rate of ovulating females, ovulation rate, conception rate, embryo survival and recovery rates on Day 3 post-AI; and foetal implantation rate, viability rate and sex ratio on Day 28, as well as mortality rate at 4 weeks of age were analysed by the Chi-square test using the CATMOD procedure. Treatment was considered as the main effect and the mother as the experimental unit.

Mean number of CL, total number of embryos, morulae and blastocysts recovered, BW of does, number of foetuses at 28 days of pregnancy, prolificacy, number of kits, and litter weights at 4 weeks of age were analysed by ANOVA using the GLM procedure. The model also included treatment as the main effect and mother as the experimental unit.

The effects of feed restriction, intrauterine position (proximal, mid-uterus and distal), as well as their interactions on foetal factors (weight, CRL, biparietal diameter, thoracic diameter, organ weight and organ weight/BW ratios) and placenta parameters were assessed by ANOVA (GLM procedure). The doe was considered as the experimental unit, and the number of viable foetuses present in the corresponding horn was used as a covariate.

The sex ratio of foetuses was analysed by the Chi-square test (CATMOD procedure) according to their positions (1, 2 or 3) in the uterine horns, treatment, and their interactions as the main effects in the model. Lastly, treatment, sex and positions (1, 2 and 3) were examined as main effects on fibrosis area, percentage of collagen fibres in the liver, and relative IGF system gene expression, as well as interactions between them. Foetal sex and position

Table 2
Reproductive parameters recorded at 28 days of pregnancy in *ad libitum*-fed rabbit females (control group) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group).

Item	Control (n = 5)	R021 (n = 5)	SEM	P-value
Live BW (g)	4 690	4 573	97.00	0.42
Total number of foetuses	11.00	14.00	0.92	0.05
Implantation rate (%)[1]	88.91	96.21	3.77	0.0001
Viability rate (%)[2]	94.64	90.00	12.14	0.34

Each pregnant doe (n = 5) was considered an experimental unit for analysis.
[1] (Number of viable foetuses/number of CL) × 100. CL: corpora lutea.
[2] (Number of viable foetuses/total number of foetuses) × 100.

Table 3
Effects of maternal food restriction (MFR) and intrauterine position on foetal development at 28 days of pregnancy in *ad libitum*-fed rabbit females (control group) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group).

	MFR		Position				P-value		
Item	Control	R021	Proximal	Mid-uterus	Distal	SEM	$P_{restriction}$	$P_{position}$	$P_{restrictionxposition}$
Foetal weight (g)	38.40	37.57	40.77[a]	36.50[b]	36.70[ab]	2.33	0.53	0.0002	0.41
Crown-rump length (mm)	99.72	97.92	101.1[a]	96.84[b]	98.47[ab]	2.43	0.19	0.0006	0.99
Biparietal diameter (mm)	19.23	19.14	19.47[a]	18.82[b]	19.26[ab]	0.47	0.74	0.007	0.99
Thoracic diameter (mm)	20.55	20.61	21.51[a]	20.68[b]	19.55[ab]	0.88	0.90	0.008	0.60
Head weight (g)	9.25	8.91	9.45[a]	8.87[b]	8.92[ab]	0.42	0.15	0.008	0.56
Trunk weight (g)	28.08	27.34	29.60[a]	26.22[b]	27.31[ab]	1.88	0.49	0.0005	0.20
Brain weight (g)	0.96	0.92	0.95	0.93	0.93	0.05	0.19	0.75	0.44
Brain ratio	0.02	0.03	0.02	0.03	0.03	0.00	0.13	0.09	0.51
Liver weight (g)	2.47	2.49	2.73[a]	2.34[b]	2.34[b]	0.05	0.92	0.009	0.39
Liver ratio	0.06	0.07	0.07	0.06	0.06	0.05	0.59	0.59	0.42
Brain:liver ratio	0.40	0.41	0.38	0.41	0.43	0.05	0.62	0.32	0.45
Heart weight (g)	0.22	0.22	0.23	0.21	0.22	0.02	0.67	0.11	0.49
Heart ratio	0.0058	0.0058	0.0056	0.0057	0.0061	0.00	0.92	0.30	0.09
Lung weight (g)	1.32	1.37	1.48[a]	1.28[b]	1.29[b]	0.10	0.40	0.0001	0.76
Lung ratio	0.037	0.034	0.04	0.04	0.04	0.00	0.08	0.40	0.92
Kidney weight (g)	0.35	0.32	0.356[a]	0.322[b]	0.336[ab]	0.03	0.16	0.04	0.75
Kidney ratio	0.009	0.009	0.009	0.009	0.009	0.00	0.15	0.57	0.97
Digestive tract (g)	1.82	1.68	1.77	1.75	1.73	0.15	0.1	0.92	0.04
Digestive tract ratio	0.048	0.045	0.043[a]	0.048[b]	0.048[b]	0.00	0.13	0.01	0.20

Each pregnant doe (n = 5) was considered an experimental unit. The number of viable foetuses present in the corresponding horn was used as a covariate.
Foetuses recovered from each uterine horn were numbered beginning at the end closest to the oviduct as follows: foetuses 1, 2, 3 (proximal); 4, 5, and 6 (mid-uterus); 7 and above (distal).
Foetal brain, liver, heart, lung, kidney, and digestive tract to weight ratios were calculated by dividing organ weight by foetal weight.
[a,b] Indicates significant differences in each row.

were only kept in the model when they emerged as significant ($P < 0.05$).

Significance was set at $P \leq 0.05$ and a statistical trend was considered when $0.05 < P \leq 0.1$. Results are presented as least square means and root standard error of the mean (RSEM).

Results

Prenatal reproductive outcomes in dams

In the control and R021 groups, respectively, rates of ovulating females (100 and 90%) and numbers of CL on the ovarian surface (10.7 ± 1.09 and 7.42 ± 1.19 CL) were similar, as were conception rates (90 and 80%), mean numbers of recovered embryos/female (8.67 ± 1.07 and 6.50 ± 1.14 embryos) and recovery rates (79.4 and 74.5%). Neither were differences detected in numbers of morulae and blastocysts recovered (morulae: 2.78 ± 0.88 and 2.38 ± 0.94; blastocysts: 4.11 ± 1.10 and 2.25 ± 1.18), or in embryo viability (75.4 ± 13.6 and 70.1 ± 14.4 %).

Results obtained on Day 28 of pregnancy are shown in Tables 2 and 3. Ovulation rates were similar in all does at a mean of 13.1 ± 1.02 CL/doe. BW was also similar in the two groups. However, R021 does had two more foetuses (P = 0.05) and a higher implantation rate ($P < 0.0001$) than control does, yet a similar viability rate. Litter size ranged from 9 to 17 foetuses.

Foetal BW, CRL, biparietal diameter and thoracic diameter, as well as the weight of all organs and their corresponding ratios, were not affected by treatment (Table 3). Nonetheless, foetal position had a significant effect ($P < 0.05$) on most of the parameters studied with highest values of these factors recorded for foetuses located proximally in the uterine horn.

The weight of the whole placenta (4.98 ± 0.19 and 4.97 ± 0.16 g), decidua (1.50 ± 0.06 and 1.42 ± 0.07 g) and labyrinth zone (3.46 ± 0.14 and 3.56 ± 0.17 g), as well as placenta efficiency (7.75 ± 0.27 and 7.40 ± 0.23) were similar in the control and R021 does, respectively. In both groups, the labyrinth zones of foetuses in proximal and mid-uterus positions were heavier (3.92 ± 0.19 and 3.49 ± 0.11 g, respectively) than those occupying distal positions (3.11 ± 0.25 g; P = 0.03).

Foetal sex ratio

The sex ratio of foetuses located proximally (1, 2 and 3) was unaffected by MFR (43.1 and 42.6% of females and 57.9 and 56.4% of males in the control and R021 groups, respectively). Nonetheless, as shown in Fig. 2, the percentage of females tended to be higher (P = 0.07) for position 1 (77.8% and 50% of females for the control and R021 groups, respectively), compared to the other positions (control does 33.3 and 18.2%, and R021 does 33.3 and 44.4% for positions 2 and 3, respectively).

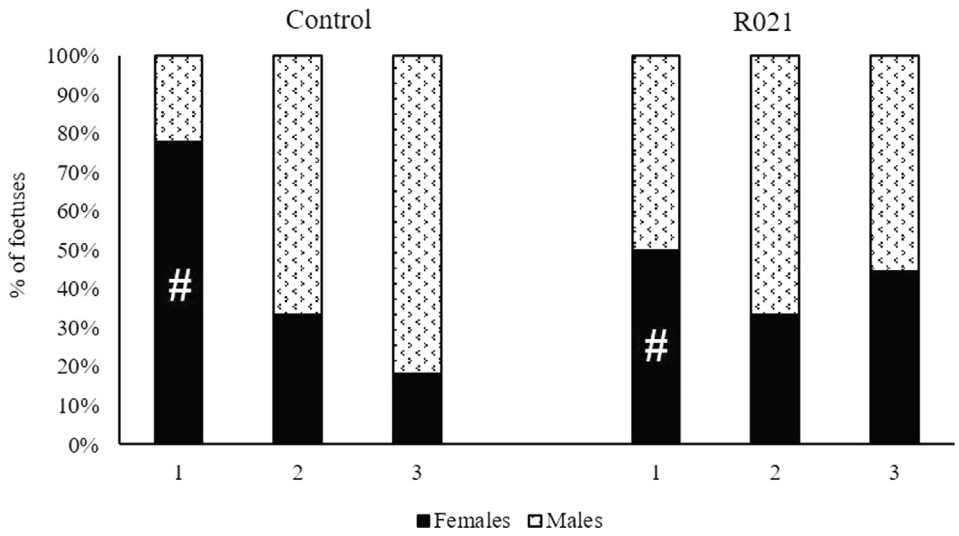

Fig. 2. Sex ratio of 28-day gestation foetuses located in a proximal position in the right and left horns of *ad libitum*-fed rabbit females (control group; n = 30) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group; n = 30). #P < 0.08 between position 1 and the rest of positions for female:male ratio.

Relative gene expression of candidate IGF system genes in the liver of foetuses

Our mRNA expression data for the genes examined in female and male foetuses located proximally in the uterine horns indicated downregulation of hepatic *IGFBP1* (P = 0.02) and upregulated *IGF2* (P = 0.04) in the liver of foetuses recovered from R021 does compared to control foetuses. As shown in Fig. 3, R021 females showed lower hepatic *IGFBP1* expression (P = 0.05), and a trend towards greater *IGF2* signalling (P = 0.09) compared to control females. Abundances of *IGF1*, *IGF1R* and *IR* mRNA remained constant in both the control and R021 groups. No interaction was observed between treatment and sex.

Foetal liver fibrosis

The livers of all analysed foetuses showed the typical lobular structure with normal central veins and portal tracts; hepatocytes showed normal cytological structures with haematopoietic cells (Fig. 4a and b). Collagen fibres were observed mainly around the portal tract and the central veins in both groups, and scarcely in the hepatic parenchyma (Fig. 4c and d). Fibrosis area (Fig. 4e) and collagen fibre percentage (Fig. 4f) did not differ between groups. No effects were observed of gender or uterine position on these parameters.

Postnatal reproductive outcome

At birth, fertility and prolificacy did not differ between groups, with similar rates detected of total number of newborns born alive or still (Table 4). Litter weight was unaffected by MFR.

Among the factors recorded at weaning, more kits were weaned by control than R021 females (10.4 ± 0.15 and 9.86 ± 0.15 kits; P = 0.01) yet litter weights were similar (6 122 ± 189 and 5 787 ± 183 g). Mortality rate during lactation was comparable in both groups (4.3 ± 1.14 control and 4.6 ± 1.11% R021).

Discussion

This study examines the effects of MFR starting on the day of AI. All does in the control (normal feed intake) and treatment (reduced feed intake) groups showed a similar number of CL in their ovaries, probably because follicles had developed and oocytes matured before MFR was started. In agreement with our results, no differences in ovulation rates were detected by others in response to feed restriction in early gestation (Almeida et al., 2000; Novak et al., 2003). Further, while it has been established that the oviduct environment could be modified by food restriction (Ashworth et al., 2009), we here observed no effects of MFR on early embryo development in terms of numbers of recovered embryos, embryo viability and morula/blastocyst rates as well as conception rates.

Throughout pregnancy, dams have different energy needs, the last week being the most energetically demanding owing to the exponential growth of the foetus (Robinson, 1986). In our experimental design, MFR was implemented during the first three weeks of pregnancy, when embryo development, implantation and early foetal growth take place. Both implantation rates and number of viable foetuses were higher for females in the R021 group at the end of pregnancy, although viability rates were similar. Other authors have detected less viable foetuses in undernourished rabbit does depending on MFR intensity, duration and time of implementation (Cappon et al., 2005; Matsuzawa et al., 1981; Naturil-Alfonso et al., 2016). It has been suggested that females could adopt this strategy of increasing the implantation rate to cope with nutrition restriction in an effort to preserve the species and maintain litter size (Sirotkin et al., 2017). In effect, the recently proposed "maternal buffering model" (Thayer et al., 2020) argues that pregnant females have multiple ways of ensuring the foetus is buffered from temporary deficits in maternal food intake. We noted that placenta efficiency was unaffected by MFR and no foetal signs of IUGR or asymmetry were observed, as overall no substantial changes in foetal size, organ weight or organ weight/BW ratios were produced. Further, MFR had no impact on the normal relationship between foetus weight and its position in the uterine horn, as early described in rabbits (Duncan, 1969). The largest foetuses were those found close to the ovarian end of the uterus, decreasing in size towards the cervical end, where foetuses showed the lowest average weight due to a smaller placenta size, consistent with the findings of others (Bautista et al., 2015; García et al., 2021).

Adaptive changes in sex ratio are produced in response to different insults in pregnant mothers (Rosenfeld and Roberts, 2004). In rabbits, sex is determined at around Day 15 postcoitus (Daniel-Carlier et al., 2013). However, we detected no MFR effect on sex ratio. Thus, it could be that our reduced intake treatment was not sufficiently severe to induce a sex-specific response, as it

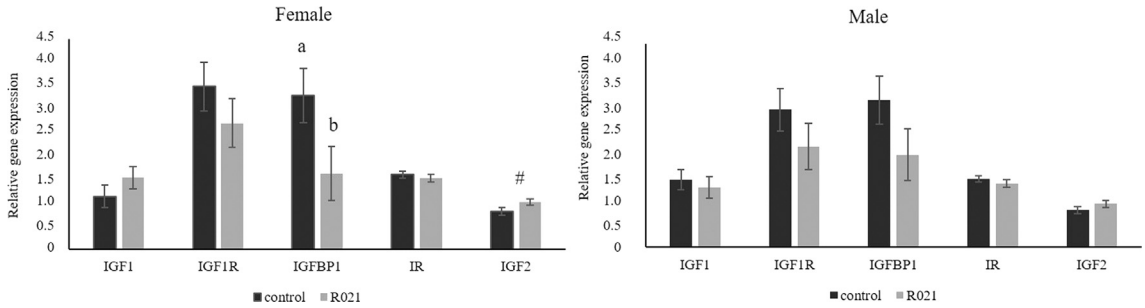

Fig. 3. Relative gene expression levels of *IGF1*, *IGF1R*, *IGFBP1*, *IR* and *IGF2* recorded in the livers of female and male rabbit foetuses on Day 28 of pregnancy in *ad libitum*-fed females (control group; n = 30) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group: n = 30). $^{ab}P \leq 0.05$; # Indicates trend ($P < 0.1$).

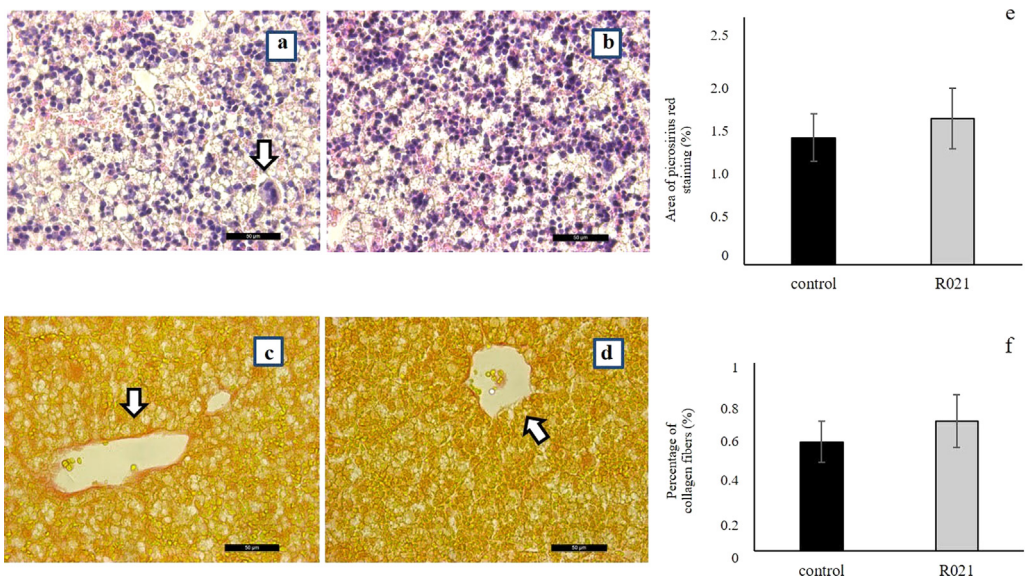

Fig. 4. Liver sections of rabbit foetuses recovered at 28 days of gestation from *ad libitum*-fed females (control group; n = 30) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group; n = 30). Histological images taken in the control (a) and R021 (b) groups (arrow indicates a monocyte to illustrate the haematopoietic function of foetal liver). Picro-Sirius red staining in control (c) and R021 (d) females. Note the presence of red-stained collagen fibres near the vein area (arrows) in both control (c) and R021 (d) sections. Collagen stained area (e) and percentage of interstitial collagen deposition in the whole area determined by Picro-Sirius red staining (f). Scale bar on representative images = 50 µm (magnification 40×).

Table 4
Reproductive outcomes recorded at birth in *ad libitum*-fed rabbit females (control group) and feed-restricted females (105 g/day) from Day 0 to Day 21 of pregnancy (R021 group).

Item	Control (n = 61)	R021 (n = 60)	SEM	P-value
Fertility (%)[1]	73.78	76.67		0.83
Prolificacy				
Total no. newborns	11.55	10.82	0.42	0.33
Born alive	11.22	10.58	0.43	0.41
Stillborn	0.33	0.24	0.10	0.59
Litter weight (g)	607.44	637.14	121.3	0.31

[1] (Number of parturitions/number of AI) × 100. AI: artificial insemination.

seems that not even strict MFR affects the sex ratio in this species (Petrere, 1993).

Undernourishment during foetal development can lead to a smaller liver size (Gao et al., 2014) and to fibrous liver (Campisano et al., 2017). In the present study, foetal liver weight and liver to BW ratio were not affected by MFR. During intrauterine development, the liver switches from acting as a haematopoietic organ to a metabolic one at mid-pregnancy (Vassy et al., 1988) and a collagen network appears late in the foetal liver (Ayres-Silva et al., 2011). Fibrosis has been seen in the foetal liver of feed-restricted sheep during the late gestation period (Gao et al., 2014), disrupting the typical hepatic architecture and inducing liver dysfunction (Jiao et al., 2009). However, consistent with the findings of Lu et al. (2014), we only observed evidence of mild fibrosis around the portal area in livers of both groups of foetuses along with preserved liver cytoarchitecture.

The present study reveals the modified expression of some *IGF* family genes, such as *IGFBP1* downregulation and *IGF2* upregulation in the livers of R021 foetuses. While this lower *IGFBP1* expression was contrary to what was expected, it could be a way of increasing *IGF1* availability (Rebouças et al., 2014) acting as a compensatory mechanism (Lu et al., 2017) to ensure foetal growth at

the end of pregnancy. In addition, *IGFBP1* expression could be directly inhibited by hyperinsulinemia (Clemmons, 2004), which has been associated with lower liver expression of *IGFBP1* mRNA (Smith et al., 2014). This is consistent with the results of a previous study in which we detected hyperinsulinemia in the foetuses of mothers subjected to the same feed restriction regimen as implemented here (García-García et al., 2021). While no differences were noted here in *IGF1* transcript levels in the foetal liver, mRNA expression may not reflect serum *IGF1* concentrations. In addition, *IR* and *IGF1R* expression were not affected by MFR. These data are in line with reported observations (Shaikh et al., 2005). Increased *IGF2* expression has been associated with foetal undernourishment (Gong et al., 2010). In sheep, reduced feed intake in late gestation was found to upregulate *IGF2* expression in the liver of preterm foetuses (Brameld et al., 2000). Contrarily, the expression of this growth factor was downregulated when feed was restricted in early pregnancy (Hyatt et al., 2004).

The female offspring of the R021 mothers often featured downregulated liver *IGBP1* and upregulated liver *IGF2* expression compared to control females. Placental *IGF2* has been also found to be increased in the daughters of feed-restricted dams (Börzsönyi et al., 2011). In control does, uterine position 1 was mostly occupied by female offspring, which have a higher likelihood of survival (Bautista et al., 2015) as foetuses located near the ovarian end of the uterus show a greater weight (Poigner et al., 2000) and viability (Flake et al., 1987).

In the postnatal period, fertility and prolificacy were unaltered by MFR, although control mothers weaned more kits despite a similar litter weight. According to Nafeaa et al. (2011), 60% MFR in the first half of pregnancy in nulliparous rabbit does did not affect the viability or weight of young kits at birth. Indeed, MFR followed by *ad libitum* feeding at the end of pregnancy may be beneficial, as feed intake is augmented when does return to *ad libitum* feeding (Manal et al., 2010; García-García et al., 2021). However, during the postnatal period, no differences in litter and mortality rate were produced between groups, probably because litter sizes were artificially equalised at birth. Consistently, Symeon et al. (2015) restricted maintenance requirements by 50% in rabbits over Days 7–19 of gestation or Days 20–27 of gestation with no effects detected on offspring BW during fattening.

Conclusions

In primiparous rabbit does, MFR during gestation (40% of their feed intake in the previous pregnancy) did not severely impact their reproductive behaviour at the time points pre-implantation, preterm, birth or weaning. Although foetal liver morphology was unaffected, some alterations were detected in the expression of some *IGF* family genes (*IGFBP1* and *IGF2*), mainly in foetuses of MFR females. These reprogramming events were, nevertheless, not observed in foetal growth or in the postnatal period. Offspring were able to compensate for in utero growth restriction, and showed similar BWs and organ development as non-growth-restricted progeny at preterm, while the viability and weight of offspring until weaning appeared normal. On rabbit farms, this could be a useful strategy, with no apparent detrimental consequences for the reproduction of does and development of kits until 4 weeks of life.

Ethics approval

Experimental work was assessed by the CEEA (Comité Ético de Experimentación Animal [Ethics Committee on Animal Research]) of the Polytechnic University of Madrid, Spain. Once approved by the CEEA, the Dirección General de Agricultura, Ganadería, Pesca y Acuicultura (Directorate-General for Agriculture, Livestock Farming, Fishing and Aquaculture) of the Madrid Community authorised the animal experiments (PROEX 302/15).

Data and model availability statement

None of the data were deposited in an official repository. All the data presented in this article were generated and analysed in this study. For additional information, please contact the corresponding author: rosa.garcia@vet.ucm.es.

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RMGG, **MAA**, **PGR** and **PLL** conceived and designed the study, and took part in fundraising.

RMGG, **MAA**, **PGR**, **MR**, **NFR** and **ASR** contributed to experimental data collection.

RMGG and **PGR** drafted the manuscript.

PGR performed the statistical analysis of data. All authors have critically reviewed the manuscript.

All authors have read and agreed to the published version of the manuscript.

Declaration of Interest

None.

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