Naturil Alfonso, C.; Peñaranda, D.; Vicente Antón, JS.; Marco-Jiménez, F. (2017). Feed restriction regime in a rabbit line selected for growth rate alters oocyte maturation manifested by alteration in msy2 gene expression. Reproduction in Domestic Animals. 52(6):976-984. https://doi.org/10.1111/rda.13006

Feed restriction regime in a rabbit line selected for growth rate alters oocyte maturation manifested by alteration in msy2 gene expression

C. Naturil-Alfonso, D.S. Peñaranda, J.S. Vicente, F. Marco-Jiménez*

[1]Institute of Science and Animal Technology, Laboratorio de Biotecnología de la Reproducción, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

Running head: Feed restriction alters oocyte gene expression

Corresponding author:

*Prof. PhD Francisco Marco Jiménez, Reproductive Biotechnology Laboratory. Institute of Science and Animal Technology (ICTA) at the Polytechnic University of Valencia, C/Camino de Vera s/n, 46022 Valencia, Spain.

Email address: fmarco@dca.upv.es.

Tel.: +34 96 3879435, fax: +34 96 3877439.

Abstract

Young rabbit females selected for growth rate may have nutritional needs which may not be met with the common practice of feed restriction during rearing in commercial rabbit production. The aim of this study was to analyse whether two different feeding programmes: ad libitum or restricted (130g/day) feeding, applied in young rabbit females for one month at the end of rearing, could modulate the origin of ovulation process and the quality of the oocytes. At 16 weeks of age, 34 females were randomly assigned to restricted or ad libitum feeding, maintaining these conditions for a month. Then, in an initial experiment, transcriptional profiling of hypothalamus-hypophysis tissue was performed to assess failure to ovulate. In the second experiment, the gene expression analysis of some candidate genes related with oocytes quality was performed. Our results demonstrated that neither of the two feeding programmes modified the transcription of hypothalamus-hypophysis tissue, while the only differences in MSY2 expression were found in *in vivo* mature oocytes ready for successful fertilization. Specifically, MSY2 was over-expressed in oocytes from females fed ad libitum. MSY2 is one of the most abundant proteins in the oocyte and has proven to be a key regulator of maternal RNA transcription and translation. This finding suggests that MSY2 gene is a promising gene in our understanding of the relationship between high growth rate and reproductive performance decline.

Key words: feed restriction, hypothalamus-hypophysis axis, oocyte quality, growth rate, rabbit

Introduction

Management of young rabbit females during the rearing period aims to develop adequate body growth in order to increase the long-term productivity and lifespan of rabbit females, avoiding problems related with over-conditioning during their future reproductive life (Martínez-Paredes et al., 2015). Different feeding programmes, such as feed to appetite (ad libitum) or feed restriction, are common practice in commercial rabbit production (Szendrő et al., 2012). An ad libitum diet provides better sexual development in terms of receptivity, ovulation rate, blastocyst size and implantation rate (Ashworth et al., 1999a, b; Rommers et al., 2004). However, overfeeding young females before first mating can lead to conditions of fattiness (Szendrő et al., 2012). In contrast, feed restriction improves reproductive performance in farm animals (Eiben et al., 2001; Rommers et al., 2004).

Several studies have demonstrated that variations in feed intake (energy) may have a direct or indirect effect on female reproduction through alterations in GnRH secretion, inhibition or delay of LH surge and fluctuations of estradiol-17β and progesterone concentrations (Boland et al., 2001; Kiyma et al., 2004; Ferguson et al., 2003). Likewise, they have been shown to affect ovarian follicle development (Alexander et al., 2007) and follicular characteristics (Armstrong et al., 2001), ovulation (Yan et al., 2008), oocyte morphology and developmental potential (McEvoy et al., 1995; O'Callaghan et al., 2000; Ferguson et al., 2003), embryo development (García-García et al., 2011) and fertility rates (Brecchia et al., 2006), resulting in reduced oocyte quality in terms of in vitro fertilization rates and embryonic development (Papadopoulos et al., 2001).

In this sense, several studies in females from a rabbit synthetic line selected for growth rate over 36 generations demonstrated a significant reduction in the ovulation frequency (30%) in comparison to New Zealand white (Vicente et al., 2012; Naturil-Alfonso et al., 2016). These ovulation failures have been related to reduced LH plasma concentrations (Viudes-de-Castro et al., 1995; Vicente et al., 2003; 2012; Naturil-Alfonso et al., 2016). It has been shown that fasting modifies the responses of the anterior pituitary gland to GnRH activity, leading to a reduced synthesis of LH by pituitary cells (Brecchia et al., 2006; Ferguson et al., 2003). Moreover, Labreque et al. (2014) reported that the suppression of LH pulses may have an impact either on follicle development or on the oocyte quality. In the same way, similar results in oocyte and/or embryo quality of this rabbit synthetic line selected for increased growth have recently been suggested when females are submitted to different feeding regimens (Naturil-Alfonso et al., 2015).

Hence, the aim of this study was to analyse whether two different feeding programmes, consisting of ad libitum or restricted feeding applied in young rabbit females for one month at the end of rearing, could modulate the origin of the ovulation process and the quality of the *in vivo* mature oocytes ready for successful fertilization. Our hypothesis was that these young females with 36 generations of selection for growth rate, when subjected to a restrictive regime to avoid over-thickening, could not cope with their needs at first conception, causing long-term nutrient deficiency which leads to the subsequent reproductive problems characteristics of this synthetic line.

Material and methods

All chemicals used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) unless stated otherwise.

1. Ethical Statement

The experiment was performed in accordance with the principles of animal care published by Spanish Royal Decree 53/2013 (BOE 2013). The animal studies were approved by the Committee of Ethics and animal Welfare of the Universidad Politécnica de Valencia (procedure 2015/vsc/PEA/00061). Researchers involved in work with the animals held an animal experimentation licence issued by the Spanish authorities.

2. Animals

A total of 34 does from a synthetic line selected for growth rate between weaning and slaughter time (9th wk of life) for 36 generations (named Line R, Instituto de Ciencia y Tecnología Animal, Estany et al., 1992) were used in this study. Young females were maintained under a 16 h light/8 h dark photoperiod across the experiment. All females were fed ad libitum until 12 weeks of age with a commercial diet (on dry matter basis: 17.5% crude protein, 3.5% ether extract, 16.7% crude fibre, 2938 kcal/kg). Until 9 weeks of age, young females were caged collectively, and subsequently housed individually. At 12 weeks of age, all females were feed restricted, as is common practice in commercial rabbit production. Animals received 130 g/day to cover energy requirements for maintenance (340 kJ day-1 kg-1 LW0.75, Xiccato and Trocino, 2010). At 16 weeks of age, females were then randomly assigned to feed restriction (n = 17) or feeding ad libitum (n = 17). The feeding regime was maintained for 4 weeks

3. Experiment 1. Hypothalamus-pituitary microarray

At 20 weeks, 22 receptive females (10 feed ad libitum and 12 feed restricted) were euthanized by intravenous injection of 200 mg/kg of pentobarbital sodium (Dolethal, Vétoquinol, Madrid, Spain). Hypothalamus-pituitary was recovered and samples of approximately 15-20 mg from each female were frozen individually in the liquid nitrogen before storing at -80ºC for later RNA extraction.

At the moment of sacrifice, body weight and body composition were determined. The perirenal fat thickness (PFT) was measured by ultrasound to evaluate body condition, as described by Pascual et al. (2000). Briefly, images were obtained by a portable colour Doppler ultrasound device (Esaote, Spain) with 7.5 MHz linear probe (4-12 MHz range). PFT measures were indirectly obtained using the software of the ultrasound unit.

3.1. RNA extraction

PolyA RNA was extracted from hypothalamus-pituitary. Total RNA was isolated from each female using traditional phenol/chloroform extraction by sonication in the Trizol reagent (Invitrogen) as described by Llobat et al. (2012). Then, RNA was purified by RNA Clean-up columns (Nucleospin, Madrid, Spain) and the concentration, quality and integrity of RNA were evaluated by Bioanalyzer 2100 (Agilent Technologies).

3.2. Microarray analysis

For the two-colour microarray analysis, four biological replicates were used for ad libitum hypothalamus-pituitary, including two dye swaps to compensate dye-bias. For

restricted hypothalamus-pituitary, four biological replicates were used, including one dye-swap.

Total RNA (100 ng) was amplified using QuickAmp Labelling Kit (Agilent Technologies, Madrid, Spain), following the manufacturer's instructions. The complementary RNA (cRNA) generated was purified and labelled with Cyanine 3 dye (Cy3) and Cyanine 5 dye (Cy5). Excess dye was removed with the QIAquick PCR purification kit (Quiagen Iberia S.L., Madrid, Spain) and dye incorporation and concentration were determined using the microarray setting on the Nanodrop 1000.

Equal amounts of Cy3 and Cy5 labelled samples (825 ng) were mixed with 10X Blocking Agent and Fragmentation Buffer, then 55 μL of the mixture were hybridised into the Rabbit 44x oligonucleotide array G2519F (Agilent Technologies, Madrid, Spain). After 17 hours at 65ºC, hybridized slides were washed and scanned using the Agilent DNA Microarray Scanner G2565B (Agilent Technologies, Madrid, Spain). The resulting images were processed using Feature Extraction v.10 Software (Agilent Technologies, Madrid, Spain with default parameters. Normalization with the locally weighted linear regression (LOWESS) algorithm and identification of differentially expressed transcripts was achieved using the Limma package in R (www.r-project.org). P-values were adjusted for multiple testing using Benjamini and Hochberg false discovery rate (FDR) and differences of $p<0.05$ were considered significant.

3.3. Real-time PCR

To validate the microarray results obtained, seven genes (LRP2: LDL-related protein; EMP1: Epithelial membrane protein 1; ANXA3: Annexin A3; RBP4: Retinol-binding protein 4; CITED1: Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain; TNFAIP6: Tumour necrosis factor alpha-induced protein 6;

EGFLAM: EGF-like fibronectin type III and laminin G domains) were carried out in twenty-two independent pool samples (microarray samples plus additional pools).

To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L, Madrid, Spain) was performed from total RNA (1000 ng). Afterwards, reverse transcription was carried out using the qScriptTM cDNA Synthesis kit (Quanta BioSciences Inc., Gaithersburg, USA) according to the manufacturer's instructions. Real-time PCR (RT-PCR) reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every PCR was performed with 5 µL of 1:20 diluted cDNA template (except for TNFAIP6 and EGFLAM which was 1:10 diluted cDNA), 250nM of forward and reverse primers (Table 1) and 10µL of PowerSYBR Green PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 40 cycles of 95ºC (1 sec) and 60ºC (30 sec). After RT-PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. Serial dilutions of cDNA pool made from several samples were run in triplicate to assess PCR efficiency and determine the sample dilution. The analysed samples were run in duplicate. Non-template controls (cDNA was replaced by water) for each primer pair were run in all plates. A ΔΔCt method adjusted for PCR efficiency was used, employing the geometric average of H2AFZ (H2A histone family member Z) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference gene (Llobat et al., 2012). Relative expression of cDNA pool from various samples was used as the calibrator to normalize all samples within one RT-PCR run or between several runs. The products of RT-PCR were confirmed by ethidium bromide-stained 2% agarose gel electrophoresis in 1x Bionic buffer (Sigma-Aldrich Química S.S, Alcobendas, Madrid, Spain).

4. Experiment 2. Oocyte gene expression

4.1. Oocyte recovery

At 20 weeks, 12 receptive females (7 feed ad libitum and 5 feed restricted) were induced to ovulate by an intramuscular injection of 1 μg of Busereline Acetate (Suprefact, Hoechst Marion Rousell, S.A., Madrid, Spain). Sixteen hours post-ovulation, females were euthanized as described above and reproductive tracts were recovered. Oocytes were recovered by perfusion of each oviduct with 10 mL of pre-warmed DPBS supplemented with 0.2% of BSA. For each female, the ovulation rate was noted counting ovulation scars (number of *corpora lutea)* and ovulation recovery rate was calculated as the number of oocytes recovered in each female from the total of *corpora lutea* in both ovaries. Finally, oocytes were pooled to randomize female effect and stored at -80ºC for later RNA extraction.

4.2. RNA extraction

As the amount of RNA present in a single oocyte is rather limited, for each experimental group (ad libitum and restricted derived oocytes) six pools of 6–10 oocytes were generated.

A pool of oocytes was homogenized after two freezing steps in Liquid Nitrogen. Afterwards, RNA isolation was performed using Dynabeads® mRNA Purification Kit (Invitrogen) according to the manufacturer's instructions. Finally, RNA amplification, DNAse step and purification was performed using MasterPure™ RNA Purification Kit, according to manufacturer's instructions (Epicentre, Barcelona, Spain).

RNA isolated from oocytes was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen S.A., Barcelona, Spain) in a total volume of 20 μL, according to the manufacturer's instructions. The RT reaction was carried out at 25 °C for 5 min and 50 °C for 60 min, followed by an inactivation step at 75 °C for 15 min.

4.3. Primer design

Two genes, Histone (H2afz) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were selected as reference genes in the quantitative RT-PCR, in accordance with Llobat et al. (2011). Furthermore, gene specific primers were designed exon-exon and assayed to evaluate MSY2: Y-box binding protein 2; MATER: NLR family, pyrin domain containing 5, IPTR1: inositol 1,4,5-triphosphate receptor 1; IPTR2: inositol 1,4,5-triphosphate receptor 2; IPTR3: inositol 1,4,5-triphosphate receptor 3; eIF4E: eukaryotic translation initiation factor 4E; PARN: poly(A)-specific ribonuclease; PAPOL-A: poly(A) polymerase alpha; PAPOL-G: poly(A) polymerase gamma; GDF9: growth differentiation factor 9; ZAR1: zygote arrest 1; YY1: ubiquitously distributed transcription factor expression (Table 2). To avoid DNA contamination, DNA and RNA samples were run to check possible amplification.

4.4. Real-time PCR

The mRNA expression was analysed by real-time PCR in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA) with 5 μL of 1:10 diluted cDNA template, and as previously described.

5. Statistical analysis

Body weight and PFT were analysed by student t-test. Ovulation rate and oocyte recovery rate were analysed by a GLM including feeding regimen (ad libitum or

restricted) as fixed factor. For mRNA expression, data on relative mRNA abundance among different groups in each comparison were analysed after data normalization by Napierian logarithm transformation and evaluated using GLM.

Data were analysed using the SPSS software package, version 16.0 (SPSS Inc, Chicago Illinois, USA 2002). Results are reported as least mean squares ± SEM. Means were considered statistically different at $P<0.05$.

Results

Ad libitum females showed significantly increased ingestion of 55% compared to the restricted group (235.5 ± 6.87 g/day vs. 130.0 g/day). In addition, ad libitum females showed to be heavier than restricted ones (5899.09 ± 133.187 kg vs. 5329.00 ± 139.687 kg, for ad libitum and restricted females, respectively, P=0.008). As expected, the same tendency was reported for PFT (9.63 ± 0.231 mm vs. 8.89 ± 0.243 mm, for ad libitum and restricted females, respectively, P=0.040).

1. Experiment 1. Hypothalamus-pituitary microarray

Limma analysis after normalization did not reveal any significant changes in gene expression. A total of 7 genes represented on the microarray (LPR2, EMP1, ANXA3, RBP4, CITED1, TNFAIP6, EGFLAM) were selected and tested using RT-PCR. EMP1 is thought to be involved in the regulation of different processes such as cell cycle or cell-cell recognition, and high levels of EMP1 expression have been related with cell differentiation and arrest (Wulf and Suter, 1999). ANXA3, EGFLAM and TNAIP6 genes were selected because they are mainly involved in cellular growth, differentiation and proliferation, including angiogenesis and membrane fusion (Gerke et al., 2005;

Haider & Knöfler, 2009; Han & Townes-Anderson, 2012; Leali et al., 2012). The genes TNF-alpha, LRP2 and cited1 were chosen for action directly within the hypothalamus on modulation of food intake (TNF-alpha [Romanatto et al., 2007], LRP2 [Gil et al., 2013]) and diurnal activity rhythms (cited1, [Gerstner & Landry, 2006]). In the case of RBP4 gene, it is associated with adiposity and insulin resistance (Jung & Choi, 2014). However, according to the microarray results, no significant differences were observed between groups using RT-PCR (Figure 1).

2. Experiment 2. Oocyte gene expression

2.1 Oocyte recovery

No differences were found either in ovulation rate or in recovery rate. Ovulation rate was 12.2 ± 1.60 and 13.2 ± 1.13 for ad libitum and restricted donor females, respectively. The oocyte recovery rate was 0.87 ± 0.043 vs. 0.92 ± 0.024, for ad libitum and restricted donor females respectively.

2.2. Oocyte gene expression

Differences in relative transcript abundance between groups are shown in Figure 2. These genes were selected because they represent essential stages of oocyte maturation and development, such as oocyte activation, mRNA transcription and oocyte-embryo transition. From the total of 12 genes analysed, only MSY2 gene expression was shown to be different between both groups of oocytes. Oocytes from females fed ad libitum showed a higher MSY2 gene expression levels than oocytes from restricted females.

Discussion

Previous studies have shown that rabbit females selected for growth rate have severe reproductive problems, which start with ovulation failures (Vicente et al., 2012; Naturil-Alfonso et al., 2016a). These females have been shown to weigh 26% more than maternal or other lines/breeds not used in meat production (De la Fuente & Rosell, 2012). Our hypothesis was that the restricted nutritional regime in the last pubertal period prior to onset of the reproductive period is not enough to cope with the needs during reproduction of these females with higher growth rate and conversion index, causing a long-term deficiency which leads to the subsequent reproductive problems. Indeed, females fed ad libitum have shown reduced gestational losses and higher fetal growth than restricted does (Naturil-Alfonso et al., 2016b).

Balasubramanian et al. (2012) showed that body weight and diet could suppress reproductive function by acting on the hypophysis or the ovary, causing a reduction in ovulatory function. Diet and food ingestion modify different metabolic hormones, such as leptin, insulin and neuropeptide Y, which can eventually alter the reproductive function. Specifically, leptin controls GnRH functionally through its ability to activate kisspeptin-positive neurons (Smith et al., 2006). Kisspeptin has been shown to stimulate GnRH neurons in several species (Martin et al., 2008), which finally affects the reproductive capacity in animals by disturbing LH and FSH secretion, and hence estradiol and testosterone release.

A previous study showed that naturally mated females presented higher failures in ovulation rate due to a decreased LH release (Naturil-Alfonso et al., 2016a). These authors hypothesized the existence of an insufficient neuroendocrine reflex in the hypothalamus-hypophysis system to trigger adequate GnRH secretion. However, our results revealed no differences in the neuroendocrine system, as no differences in gene expression were detected between the hypothalamus-hypophysis system from ad libitum

and restricted females. The absence of differences leads us to conclude that ovulation failures may be caused by peripheral mechanisms rather than central mechanisms, such as failure in the nervous stimuli to the hypothalamus-hypophysis system.

On the other hand, nutritional support has been identified as one of the relevant factors affecting embryo developmental competence of oocytes and embryos (Moussa et al., 2015). Indeed, following our hypothesis, maternal nutrition is essential to provide the follicular cells with enough energy to grow and complete developmental programmes (Moussa et al., 2105). Nevertheless, despite the evidence, discrepancies are still found in the literature regarding the impact of nutrition on oocyte and embryonic quality.

In rabbit, Mahmoud et al. (2006) described the same oocyte recovery rate between feed restricted (60% of unrestricted) during one/two months and unrestricted animals. In contrast, Dadoud et al. (2012) reported higher oocyte recovery rate from control fed females (150 g/day) than those fed 70% of control diet. Our results here showed no differences in either ovulation rate or recovery rate between oocytes from ad libitum and restricted females, in accordance with those published by Mahmoud et al. (2006).

Likewise, Dadoud et al. (2012) observed reduced oocyte quality in restricted females with lower gene expression of GDF-9 in oocytes from restricted females from the high range of quality classification. Moreover, García-García et al. (2011) reported a drop in embryo development and speed of development in embryos from 72h post-conception fasted females, while no differences in blastocyst gene expression were detected.

In this study, we observed no significant effects between both groups of oocytes in most of the chosen candidate genes related to oocyte activation (ITPR1, ITPR2, ITPR3, PAPOL-A, PAPOL-G, GDF9, ZAR1), mRNA translation and transcription (MSY2, eIF4E, PARN1, YY1) and oocyte-embryo transition (MATER). The only gene from the

group selected that proved to be differentially expressed between both groups of oocytes was MSY2. MSY2 is a Y-box protein involved in relevant processes of oocyte maturation and growth such as stability, transcription and translation regulation of maternal mRNAs. Thus, it is responsible for alterations in the pattern of protein synthesis during oocyte maturation (Gu et al., 1998; Yu et al., 2001). MSY2 relevance became evident with the finding that this protein is one of the most abundant oocyte proteins identified to date, accounting for roughly 2% of the total protein in the fully grown oocyte (Yu et al., 2001). The results of this study revealed a decreased MSY2 gene expression in oocytes derived from restricted females when compared with oocytes from females fed ad libitum. A reduced MSY2 expression in the oocyte has been identified with reduction in oocyte maturation and activation, probably due to abnormal meiotic spindle and also with a reduction in both protein synthesis and total amount of mRNA in fully growth oocytes (Yu et al., 2004). The effects of these altered processes are a drop in fertility, litter size and developmental arrest during pre- or post-implantatory development. Indeed, we previously reported that embryos from females fed ad libitum after rearing period for one month until reproduction presented lower gestational losses and higher litter size than those from restricted females (Naturil-Alfonso et al., 2016b).

Conclusion

In conclusion, the feed restriction in young females selected for growth rate at the end of rearing did not seem alter the hypothalamus-hypophysis axis, but is sufficient to provoke an upregulation of MSY2 gene in mature oocytes ready for successful fertilization. This finding suggests that MSY2 gene is a promising gene in our understanding of the relationship between high growth rate and reproductive performance decline. Nevertheless, further studies should be carried out on dietary

needs of females with high growth rate, but also at other transcriptional levels, with techniques such as microarray analysis, massive sequencing or proteomic analysis for in-depth study of the mechanisms underlying the oocyte alterations observed.

Acknowledgements

This work was supported by the Spanish Research Projects AGL2014-53405-C2-1-P and AGL2011-30170-C02-01 (CICYT). Carmen Naturil was supported by a research grant from the Education Ministry of the Valencian Regional Government (programme VALi+d. ACIF / 2013 / 296). We wish to thank the technical support of R. Lavara. English text version was revised by N. Macowan English Language Service.

Author contributions

C. Naturil-Alfonso, J. S. Vicente and F. Marco-Jiménez designed and contributed to the acquisition, analysis and interpretation of data. They also drafted and critically revised the manuscript. D. S. Peñaranda provided molecular technical assistance.

Conflict of interest

None of the authors have any conflict of interest to declare.

References

Alexander, B.M., Kiyma Z., McFarland, M., Van Krik, E.A., Hallford, D.M., Hawkins, D.E., Kane, K.K., & Moss, G.E. (2007). Influence of short-term fasting during the luteal

phase of the oestrous cycle on ovarian follicular development during the ensuing proestrous of ewes. *Anim Reprod Sci*, 97, 356-363.

Armstrong, D.G., McEvoy, T.G., Baxter, G., Robinson, J.J., Hogg, C.O., Woad, K.J., Webb, R., & Sinclair, K.D. (2001). Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biol Reprod*, 64, 1624-1632.

Ashworth, C.J., Antipatis, C., & Beattie, L. (1999a). Effects of pre- and post-mating nutritional status on hepatic function, progesterone concentration, uterine protein secretion and embryo survival in Meishan pigs. *Reprod Fertil Dev*, 11, 67-73.

Ashworth, C.J., Beattie, L., Antipatis, C., & Vallet, J. (1999b). Effects of pre- and post-mating feed intake on blastocyst size, secretory function and glucose metabolism in Meishan gilts. *Reprod Fertil Dev*, 11, 323-327.

Balasubramanian, P., Jagannathan, L., Subramanian, M., Gilbreath, E.T., MohanKumar, P.S., & MohanKumar, S.M.J. (2012). High fat diet affects reproductive functions in female diet-induced obese and dietary resistant rats. *J Neurondocrinol*, 24, 748-755.

Baselga, M. (2004). Genetic improvement of meat rabbits. Programmes and diffusion. In: Proc. 8th World Rabbit Congress, Puebla, Mexico, 1-13.

Blasco, A. (1989). Genética y nutrición del conejo. In C. de Blas Alimentación del conejo. Ediciones Mundi, Prensa, Madrid.

Boland, M.P., Lonergan, P., & O'Callaghan, D. (2001). Effect of nutrtion on endocrine parameters, ovarian physiology and oocyte and embryo development. *Theriogenology*, 55, 1323-1340.

Brecchia, G., Bonanno, A., Galeati, G., Federici, C., Maranesi, M., Gobbetti, A., Zerani, M., & Boiti, C. (2006). Hormonal and metabolic adaptation to fasting: Effects on the hypothalamic-pituitary-ovarian axis and reproductive performance of rabbit does. *Dom Anim Endocrinol*, 31, 105-122.

Daoud, N.M., Mahrous, K.F., & Ezzo, O.H. (2012). Feed restriction as a biostimulant of the production of oocyte, their quality and GDF-9 gene expression in rabbit oocytes. *Anim Reprod Sci*, 136, 121-127.

De la Fuente, L.F. & Rosell, J.M. (2012). Body weight and body condition of breeding rabbits in commercial units. *J Anim Sci*, 90, 3252-3258.

Estany, J., Camacho, J., Baselga, M., & Blasco, A. (1992). Selection response of growth rate in rabbits for meat production. *Genet Sel Evol*, 24, 527-537.

Ferguson, E.M., Ashworth, C.J., Edwards, S.A., Hawkins, N., Hepburn, N., & Hunter, M.G. (2003). Effect of different nutritional regimens before ovulation on plama concentrations of metabolic and reproductive hormones and oocyte maturation in gilts. *Reproduction*, 126, 61-71.

García-García, R.M., Rebollar, P.G., Arias-Álvarez, M., Sakr, O.G., Bermejo-Álvarez, P., Brecchia, G., Gutierrez-Adan, A., Zerani, M., Boiti, C., & Lorenzo, P.L. (2011). Acute fasting before conception affects metabolic and endocrine status without impacting follicle and oocyte development and embryo gene expression in the rabbit. *Reprod Fertil Dev*, 23, 759-768

Gerke, V., Creutz, C.E. & Moss, S.E. (2005). Annexins: linking Ca2+ signalling to membrane dynamics. *Nat Rev Mol Cell Biol,* 6: 449–461.

Gerstner, J.R. & Landry, C.F. (2007). Expression of the transcriptional coactivator CITED1 in the adult and developing murine brain. *Dev Neurosci,* 29, 203-12.

Gil, S.Y., Youn, B.S., Byun, K., Huang, H., Namkoong, C., Jang, P.G., Lee, J.Y., Jo, Y.H., Kang, G.M., Kim, H.K., Shin, M.S., Pietrzik, C.U., Lee, B., Kim, Y.B. & Kim, M.S. (2013). Clusterin and LRP2 are critical components of the hypothalamic feeding regulatory pathway. *Nat Commun*, 4, 1862.

Gu, W., Tekur, S., Reinbold, R., Eppig, J.J., Choi, Y.C., Zheng, J.Z., Murray, M.T., & Hecht, N.B. (1998). Mammalian male and female germ cells express germ cell-specific Y-box protein, MSY2. *Biol Reprod*, 59, 1266-1274.

Haider, S. & Knöfler, M. (2009). Human Tumour Necrosis Factor: Physiological and Pathological Roles in Placenta and Endometrium. *Placenta*, 30, 111-123.

Han, J. & Townes-Anderson, E. (2012). Cell Specific Post-Translational Processingof Pikachurin, A Protein Involved in Retinal Synaptogenesis. *PLoS ONE,* 7, e50552.

Jung, U.J. & Choi, M.S. (2014) Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci*, 15, 6184-223.

Kiyma, Z., Alexander, B.M., Van Krik, E.A., Murdoch, W.J., Hallford, D.M., & Moss, G.E. (2004). Effects of feed restriction on reproductive and metabolic hormones in ewes. *J Anim Sci*, 82, 2548-2557.

Labreque, R., Vigneault, C., Blondin, P., & Sirard, M.A. (2014). Gene expression analysis of bovine oocytes at optimal coasting time combined with GnRH antagonist during the no-FSH period. *Theriogenology*, 81, 1092-1100.

Leali, D., Inforzato, A., Ronca, R., Bianchi, R., Belleri, M., Coltrini, D., Di Salle, E., Sironi, M., Norata, G.D., Bottazzi, B., Garlanda, C., Day, A.J. & Presta, M. (2012) Long pentraxin 3/tumor necrosis factor-stimulated gene-6 interaction: a biological rheostat for fibroblast growth factor 2-mediated angiogenesis. *Arterioscler Thromb Vasc Biol*, 32, 696-703.

Llobat, L., Marco-Jiménez, F., Peñaranda, DS, Saenz-de-Juano MD, Vicente JS. Effect of embryonic genotype on reference gene selection for RT-qPCR normalization. Reprod Domest Anim. 2012 Aug;47(4):629-34.

Luvoni, G.C. (2000). Current progress on assisted reproduction in dogs and cats: in vitro embryo production. *Reprod Nutr Dev*, 40, 505-512.

Mahmoud, K.G.H., Mobarak, M.S., Farghaly, A.A., Shahein, Y.E., & Ezzo, O.H. 2006. Effect of dietary restriction on genetic material and reproductive performance in rabbit. *Egypt J Genet Cytol*, 35, 129-143.

Martin, B., Golden, E., Carlson, O.D., Egan, J.M., Mattson, M.P., & Maudsley, S. (2008). Caloric restriction: Impact upon pituitary function and reproduction. *Ageing Res Rev*, 7, 209-224.

Martínez-Paredes, E., Ródenas, L., Pascual, J.J., Blas, E., Brecchia, G., Boiti, C., Cervera, C. (2015). Effects of rearing feeding programme on the young rabbit females' behaviour and welfare indicators. *World Rabbit Sci*, 23, 197-205.

McEvoy, T.G., Robinson, J.J., Atiken, R.P., Findlay, P.A., Palmer, R.M., & Robertson, I.S. (1995). Dietary-induced suppression of preovulatory progesterone concentrations in superovulated ewes impairs the subsequent in vivo and in vitro development of their ova. *Anim Reprod Sci*, 39, 89-107.

Moussa, M., Shu, J., Zhang, X.H., & Zeng, F. (2015). Maternal control of oocyte quality in cattle "a review". *Anim Reprod Sci*, 155, 11-27.

Naturil-Alfonso, C., Lavara, R., Millán, P., Rebollar, P.G., Vicente, J.S., & Marco-Jiménez, F. (2016a). Study of failures in a rabbit line selected for growth rate. *World Rabbit Sci*, 24, 47-53.

Naturil-Alfonso, C., Lavara, R., Vicente, J.S., & Marco-Jiménez, F. (2016b). Effects of Female Dietary Restriction in a Rabbit Growth Line During Rearing on Reproductive Performance and Embryo Quality. *Reprod Domestic Anim*, 51, 114-22.

Naturil-Alfonso, C., Marco-Jiménez, F., Jiménez-Trigos, E., Saenz-de-Juano, M.D., Viudes-de-Castro, M.P., Lavara, R., & Vicente, J.S. (2015). Role of embryonic and maternal genotype on prenatal survival and foetal growth in rabbit. *Reprod Domest Anim*, 50, 312-320.

O'Callaghan, D., Yaakub, H., Hyttel, P., Spicer, L.J., & Boland, M.P. (2000). Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. *J Reprod Fertil,* 118, 303-313.

Papadopoulos, S., Lonergan, P., Gath, V., Quinn, K.M., Evans, A.C., O'Callaghan, D., & Boland, M.P. (2001). Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. *Theriogenology,* 55, 1059-1069.

Romanatto, T., Cesquini, M., Amaral, M.E., Roman, E.A., Moraes, J.C., Torsoni, M.A., Cruz-Neto, A.P., Velloso, L.A. (2007). TNF-alpha acts in the hypothalamus inhibiting food intake and increasing the respiratory quotient--effects on leptin and insulin signaling pathways. *Peptides*, 28, 1050-8.

Rommers, J.M., Meijerhof, R., Noordhuizen, J.P.T.M., & Kemp, B. (2004). Effect of feeding program during rearing and age at first insemination on performances during subsequent reproduction in young rabbit does. *Reprod Nutr Dev*, 44, 321-332.

Smith, J.T., Acohido, B.V., Clifton, D.K., & Steiner, R.A. (2006). KiSS-1 neurons are direct targets for leptin in the ob/ob mouse. *J Neuroendocrinol*, 18, 298–303.

Szendrő Z, Szendrő K, Zotte AD. (2012). Management of reproduction on small, medium and large rabbit farms: a review. *Asian-Australas J Anim Sci.,* 25, 738-48.

Tripp, M.W., Ju, J.C., Hoagland, T.A., Riesen, J.W., Yang, X., & Zinn, S.A. (2000). Influence of somatotropin and nutrition on bovine oocyte retrieval and in vitro development. *Theriogenology*, 53, 1581-1590.

Vicente, J.S., Llobat, L., Viudes-de-Castro, M.P., Lavara, R., Baselga, M., & Marco-Jiménez F. (2012). Gestational losses in a rabbit line selected for growth rate. *Theriogenology*, 77, 81-88.

Viudes-de-Castro, M.P., & Vicente, J.S. (1997). Effect of sperm count on the fertility and prolificity traits of meat rabbits. *Anim Reprod Sci,* 46, 313-31.

Wulf, P. & Suter, U. (1999). Embryonic expression of epithelial membrane protein 1 in early neurons. *Brain Res Dev Brain Res*, 116, 169–80.

Yan, J., Zhou, B., Jie, A., Yang, J., Tai, P., Chen, X., Zhang, H., Zhang, M., & Xia, G. (2008). Glucose can reverse the effects of acute fasting on mouse ovulation and oocyte maturation. *Reprod Fertil Dev,* 20, 703-712.

Yu, J., Deng, M., Medvedev, S., Yang, J., Hecht, N,B., & Schultz, R.M. (2004). Transgenic RNAi-mediated reduction of MSY2 in mouse oocytes results in reduced fertility. *Dev Biol,* 268, 195-206.

Yu, J., Hecht, N.B., & Schultz, R.M. (2001). Expression of MSY2 in mouse oocytes and preimplantatory embryos. *Biol Reprod,* 65, 1260-1270.

Table 1. Primers sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analysed and housekeeping genes used (LRP2, as LDL-related protein; EMP1, as Epithelial membrane protein 1; ANXA3, as annexin A3; RBP4, as retinol-binding protein4; CITED1, as Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain; TNFAIP6, as tumor necrosis factor alpha-induced protein 6; EGFLAM, as EGF-like fibronectin type III and laminin G domains; histone (*H2afz*) and *GAPDH*, as housekeeping gene).

Table 2. Primers sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analysed and housekeeping genes used MSY2, as Y-box binding protein 2; MATER as NLR family, pyrin domain containing 5; IPTR1 as inositol 1,4,5-triphosphate receptor 1; IPTR2, as inositol 1,4,5-triphosphate receptor 2; IPTR3, as inositol 1,4,5-triphosphate receptor 3; eIF4E, as eukaryotic translation initiation factor 4E; PARN, as poly(A)-specific ribonuclease; PAPOL-A, as poly(A) polymerase alpha; PAPOL-G, as poly(A) polymerase gamma; GDF9, as growth differentiation factor 9; ZAR1, as zygote arrest 1; YY1, as ubiquitously distributed transcription factor expression; histone (H2afz) and GAPDH, as housekeeping gene).

Figure 1. Relative expression of LDL-related protein (LRP2), Epithelial membrane protein 1 (EMP1), Annexin A3; RBP4: retinol-binding protein4 (ANXA3), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain (CITED1), Tumour necrosis factor alpha-induced protein 6 (TNFAIP6) and EGF-like fibronectin type III and laminin G domains (EGFLAM) for validation of hypothalamus-hypophysis microarray. Relative abundance values are shown in arbitrary units (a.u.), expressed by the mean value ± standard error means.

Figure 2. Relative expression of Y-box binding protein 2 (MSY2), NLR family, pyrin domain containing 5 (MATER), Inositol 1,4,5-triphosphate receptor 1 (IPTR1), Inositol 1,4,5-triphosphate receptor 2 (IPTR2), Inositol 1,4,5-triphosphate receptor 3 (IPTR3), Eukaryotic translation initiation factor 4E (eIF4E), Poly(A)-specific ribonuclease (PARN), Poly(A) polymerase alpha (PAPOL-A), Poly(A) polymerase gamma (PAPOL-G), Growth differentiation factor 9 (GDF9), Zygote arrest 1 (ZAR1), Ubiquitously distributed transcription factor expression (YY1). Relative abundance values are shown in arbitrary units (a.u.), expressed by the mean value ± standard error means.

Figure legends

Figure 1. Relative expression of LDL-related protein (LRP2), Epithelial membrane protein 1 (EMP1), Annexin A3; RBP4: retinol-binding protein4 (ANXA3), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain (CITED1), Tumour necrosis factor alpha-induced protein 6 (TNFAIP6) and EGF-like fibronectin type III and laminin G domains (EGFLAM) for validation of hypothalamus-hypophysis microarray. Relative abundance values are shown in arbitrary units (a.u.), expressed by the mean value ± standard error means.

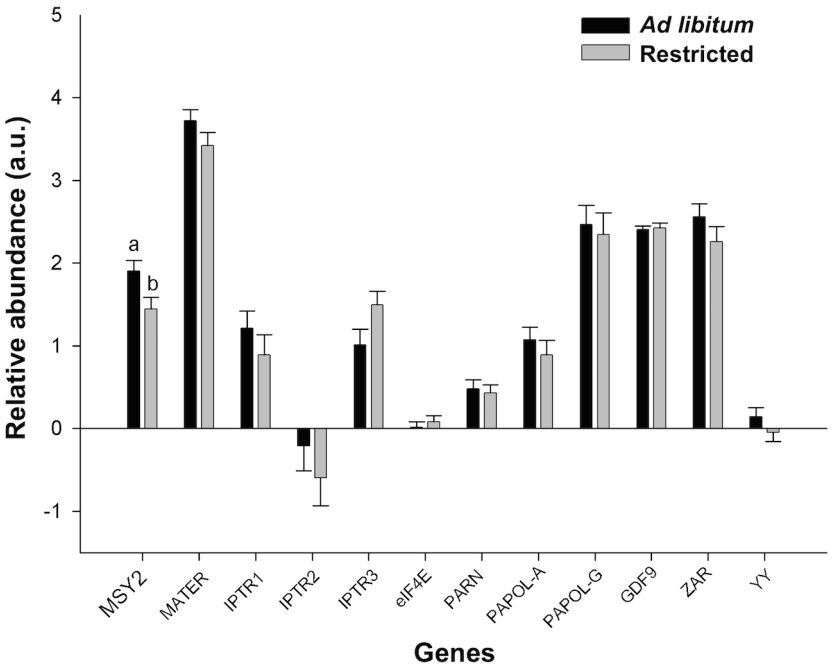
Figure 2. Relative expression of Y-box binding protein 2 (MSY2), MATER: NLR family, pyrin domain containing 5, Inositol 1,4,5-triphosphate receptor 1 (ITPR1), Inositol 1,4,5-triphosphate receptor 2 (ITPR2), Inositol 1,4,5-triphosphate receptor 3 (ITPR3), Eukaryotic translation initiation factor 4E (eIF4E), Poly(A)-specific ribonuclease (PARN), Poly(A) polymerase alpha (PAPOL-A), Poly(A) polymerase gamma (PAPOL-G), Growth differentiation factor 9 (GDF9), Zygote arrest 1 (ZAR1), Ubiquitously distributed transcription factor expression (YY1). Relative abundance values are shown in arbitrary units (a.u.), expressed by the mean value ± standard error means.

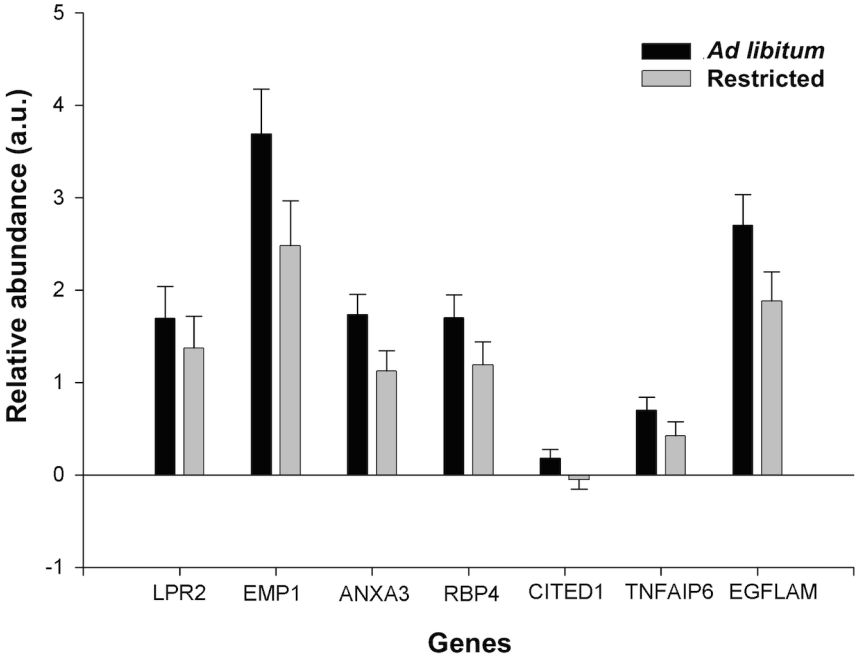
Table 1. Primers sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analysed and housekeeping genes used (LRP2, as LDL-related protein; EMP1, as Epithelial membrane protein 1; ANXA3, as annexin A3; RBP4, as retinol-binding protein4; CITED1, as Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain; TNFAIP6, as tumor necrosis factor alpha-induced protein 6; EGFLAM, as EGF-like fibronectin type III and laminin G domains; histone (*H2afz*) and *GAPDH*, as housekeeping gene).

Gene symbol	Accession number	Forward primer	Reverse primer	Fragment size (bp)	Efficiency (%)	R^2
H2AFZ	AF030235	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85	94.25	0.99
GADPH	L23961	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144	95.68	0.99
LPR2	ENSOCUT00000011039	GCCCAACTTCTTCTCCTGTG	GCATCATTGCTCTTCTCCTCA	153	99.01	0.99
EMP1	ENSOCUT00000021095	AATGTTGGTGTTACTGGCTG	GATGCGTTAATAGAGTCTGAA	110	100.2	0.98
ANXA3	ENSOCUT00000017879	ATCTTAACAACCAGGACAAGCA	TCCACCTTCACACTTTCATCTC	167	100.6	0.99
RBP4	ENSOCUT00000010524	TGGGCACCTTCACAGACAC	TGCCGTCGAAGTTGAGGAG	162	101.86	0.99
CITED1	ENSOCUT00000013306	GGATGAGGAGGTGCTGATGT	CAACCAGACGGAAAGTCTGC	120	94.78	0.99
TNFAIP6	ENSOCUT00000013688	AGTCCCCAATTACGACGATG	ACATGGATGGTTCGGTCATT	99	100.0	0.99
EGFLAM	ENSOCUT00000003325	TGTTTGGCTGACTACGTTGAA	ATTTGGAAACCTCCTGCTGT	164	101.0	0.99

Table 2. Primers sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analysed and housekeeping genes used MSY2, as Y-box binding protein 2; MATER as NLR family, pyrin domain containing 5; IPTR1 as inositol 1,4,5-triphosphate receptor 1; IPTR2, as inositol 1,4,5-triphosphate receptor 2; IPTR3, as inositol 1,4,5-triphosphate receptor 3; eIF4E, as eukaryotic translation initiation factor 4E; PARN, as poly(A)-specific ribonuclease; PAPOL-A, as poly(A) polymerase alpha; PAPOL-G, as poly(A) polymerase gamma; GDF9, as growth differentiation factor 9; ZAR1, as zygote arrest 1; YY1, as ubiquitously distributed transcription factor expression; histone (*H2afz*) and *GAPDH*, as housekeeping gene).

Gene symbol	Accession number	Forward primer	Reverse primer	Fragment (bp)	Efficiency (%)	R^2
H2AFZ	AF030235	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85	94.25	0.99
GADPH	L23961	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144	95.68	0.99
MSY2	ENSOCUT00000015067	CCCACCACCCTTCTTCTACC	CTGGTCTCCCTGTTGTTGGT	262	100.2	0.99
MATER	ENSOCUT00000005758	GCCTGTCCCATGTGATTGTG	GAGGTCCAAGCTCTTCAGGT	60	99.5	0.99
ITPR1	ENSOCUT00000015067	GGGACCTTAACAACCCACCT	GCTGCTTTCCAGAACTGCTT	100	92.27	0.99
ITPR2	ENSOCUT00000010759	CATTTGCCATCGTGTCTGTC	GCACGTCAGCAACAAAGAAG	212	100.7	0.99
ITPR3	ENSOCUT00000005784	AGCTGGTGTGTGACCTCATC	AGCCACTGTGGACTTGGTCT	129	90.11	0.99
eIF4E	ENSOCUT00000024443	CAGGAGGTTGCTAACCCAGA	AGAGATCAGCCGAAGGTTTG	128	97.01	0.99
PARN1	ENSOCUT00000000324	ACCAGCAGATATGCGGAAAG	GCTGTGCTGGTAGCTGTCAA	111	89.57	0.99
PAPOL-A	ENSOCUT00000011534	TGTGGGTGATTGGGTTAGTG	TGGCAGCAATTTTCATATCC	60	85.28	0.99
PAPOL-G	ENSOCUT00000004965	TGACTGCCAGTGCATCAAC	GGGATTTACATGGGCAAGAG	123	100.38	0.99
GDF9	ENSOCUG00000013621	GGGATTCCTAAAGCCAACAG	GTGTTCCACGGCAGTAACG	115	93.95	0.99
ZAR1	XM_008251530	CAGAAGTCTTACAACCCCTACCG	GCAGGAACACCTCGTACGTT	72	88.99	0.99
YY1	ENSOCUT00000025016	GGGCAAGCTATTGTTCTTGG	GGCATTGACCTCTCAGATCC	98	93.37	0.99


Ad libitum
Restricted
Relative abundance (a.u.)
5
4
3
2
1
0
-1
a
b
MSY2
MATER
IPTR1
IPTR2
IPTR3
eIF4E
PARN
PAPOL-A
PAPOL-G
GDF9
ZAR
YY
Genes


Ad libitum
Restricted
Relative abundance (a.u.)
5
4
3
2
1
0
-1
LPR2
EMP1
ANXA3
RBP4
CITED1
TNFAIP6
EGFLAM
Genes