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Additional Information

# Role of embryonic and maternal genotype on prenatal survival and fetal growth in rabbit

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## ABSTRACT

The aim of this work was to evaluate the influence of maternal and embryonic genotype on prenatal survival and fetal growth during pregnancy. Embryos were recovered at 48 h of gestation from two different donor lines (R=46 and A=40) and transferred to nulliparous recipient does (26 R and 24 A). Each recipient doe received six embryos into one oviduct from line R and six embryos form line A into the other. Laparoscopy was performed at day 14 to determine implantation rate. Recipient females were slaughter at day 14, 24 and 30 (12, 24, 14, respectively) to determine the number of live foetuses and the weight of live foetuses, fetal placenta and maternal placenta. A transcriptome analysis was performed to search for differences between fetal placentas at day 14 and 24 of development. Prenatal survival at Days 14 and 24 was affected by embryonic genotype and determined by maternal genotype at Day 30. Fetal weight at Day 14 was influenced by both genotypes, being the weight higher for group A/A (0.29±0.01 g vs. 0.19±0.01 g, for group R/R). However, both genotypes were determinant for fetal placenta weight at Day 24, while those genotypes affected maternal placenta weight at Day 30. Nevertheless, no differences in fetal placenta at transcriptome level and progesterone and IGF-I plasma levels in recipient does were found. In conclusion, results indicate that the influence of embryo and maternal genotype on the prenatal survival and growth seem to be changing over gestation.

Embryo development and survival, as well as a successful pregnancy, are dependent on a well-established and functional placenta. Yet the influence of embryonic and maternal genotypes on placental weight is controversial. While both genotypes had an influence on fetal and placenta weight in mouse and pig (Al-Murrani and Roberts, 1978; Barkley and Fitzgerald, 1990; Biensen et al., 1998; Wilson et al., 1998), Mocé et al. (2004a) stated that fetal weight in the last term of gestation depends on the maternal genotype, and fetal-placental weight depends on the embryonic genotype in rabbit. However, recently Vicente et al. (2013) showed that embryonic genotype affects fetal weight, but both embryonic and maternal genotype affect fetal-placental weight in the last term of gestation. In fact, fetal growth in late gestation is dependent upon the correct growth and development of the placenta (Chaddha et al., 2004).

The establishment of a healthy and functional placenta is a crucial element in the embryonic and fetal development. The development and interrelationships between maternal and fetal vascular networks in the placenta is critical for the successful development of the offspring (Yllera et al., 2003).

Therefore, due to the relevant role of the placenta and the fetal-placental interface, several works have focused in the study of placenta transcriptome (Buffat et al., 2007; Zhou et al., 2009; Salilew-Wondim et al., 2013; Whitehead et al., 2013; Gu et al., 2014). The advantage of microarray analysis is the simultaneous measurement of the expression patterns of large numbers of genes (Lockhart et al., 1996). These studies showed differences at transcriptomic level between porcine placentas with different placental efficiency (Zhou et al., 2009; Gu et al., 2014), placentas with intrauterine growth

restriction and fetal growth restriction (Buffat et al., 2007; Whitehead et al., 2013), and between bovine placentas derived from artificial insemination, *in vitro* fertilization and somatic cell nuclear transfer (Salilew-Wondim et al., 2013).

In this work, we set out to evaluate the effect of maternal and embryonic genotype on prenatal survival and placenta and fetal weights over the course of pregnancy. In addition, fetal placenta transcriptome at Days 14 and 24 of pregnancy was addressed.

## MATERIAL AND METHODS

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

### 1. Ethical Statement

The Ethics and Animal Welfare Committee of the Polytechnic University of Valencia approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

### 2. Animals

Animals used as donors and recipients came from two commercial lines generated at the Universidad Politécnica de Valencia. One (named line R) is a synthetic line selected since 1990 by individual selection on daily weight gain from weaning to slaughter age (28 and 63 days, Estany et al., 1992) and the other one (named line A) came form a New Zealand White selected since 1980 by a family index for litter size at weaning (Estany et al., 1989). Animals were kept under controlled (16 h light : 8 h dark) photoperiod and fed with a commercial rabbit diet (on dry matter basis: 17.5% crude protein, 3.5% ether

extract, 16.7% crude fiber, 2938 kcal/kg).

**3. Embryo transfer**

The scheme for the embryo transfer procedure is presented in Figure 1.1. At the age of five months, a total of 86 nulliparous does were used as donor females; 46 does from the line R and 40 does from the line A. Does were injected 25 IU of eCG intramuscular (Intervet International B.V., Bowmeer-Holland) to induce receptivity. After 48 hours, females were artificially inseminated with a heterospermic pool of fertile males from the same selected line to randomise male effect. At the time of artificial insemination, females were injected with 1 µg of buserelin acetate (Hoechst, Marion Roussel, Madrid, Spain) to induce ovulation. Then, does were slaughtered 48 hours after insemination. Embryos were collected at room temperature by flushing the oviducts and the first one-third of the uterine horns with 5 mL of embryo recovery media consisting of Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 0.2% (w/v) bovine serum albumin (BSA) and antibiotics (penicillin G sodium 300,000 IU, penicillin G procaine 700,000 IU, and dihydrostreptomycin sulphate 1250 mg; Penivet 1; Divasa Farmavic, Barcelona, Spain). After recovery, morphologically normal embryos (classified as normal when they presented correct developmental stage, homogeneous cell size and cytoplasm aspect, and spherical zona pellucida and mucin coat) were kept at room temperature (20-25ºC) in dark light until transfer to recipient females.

A total of 600 embryos were transferred. Receptive females (according to the turgidity and colour of the vulva) were induced to ovulate by injection of 1 µg of buserelin acetate (Hoechst, Marion Roussel, Madrid, Spain) 48 hours before transfer. Females were anaesthetised by intramuscular injection of 5 mg/kg of

xylazine (Rompún, Bayer AG, Leverkusen, Germany) following intravenous injection of 15 mg/kg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). Embryo transfer was performed using the laparoscopic technique described by Besenfelder and Brem (1993). At the age of 5 months, a total of 26 nulliparous females from line R and 24 from line A were used. The number of embryos transferred per oviduct was standardised to 6, so that all recipients received 12 embryos (six embryos from line R into one oviduct and six embryos from line A into the other). Transfers to right or left uterine horns were randomised. According to the transfers, four groups were obtained: $R^{[embryo]}/R^{[mother]}$ (R/R), $R^{[embryo]}/A^{[mother]}$ (R/A), $A^{[embryo]}/A^{[mother]}$ (A/A), and $A^{[embryo]}/R^{[mother]}$ (A/R).

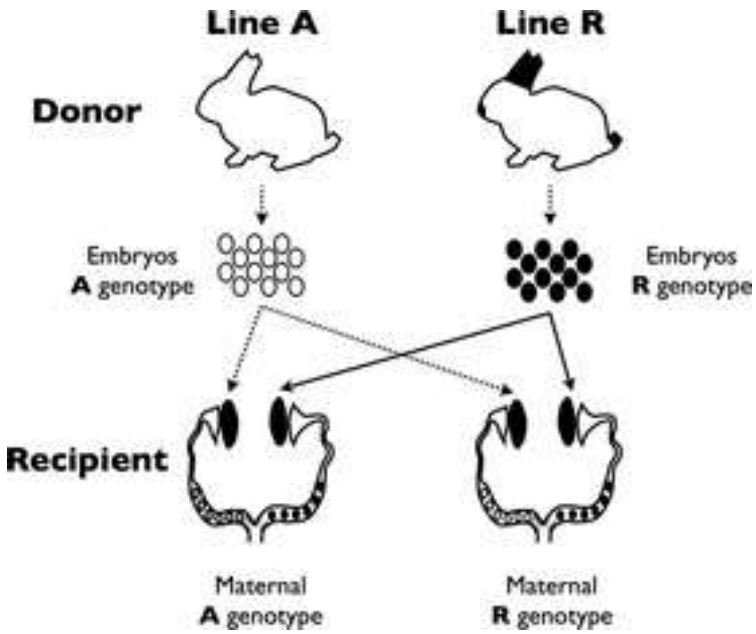


**Figure 1.1.** Schematic illustration of the embryo transfer model used in this study to determine maternal genotype and fetal genotype effect on prenatal survival and fetal growth. A/ R (A) embryo transferred into R foster mother; R/R (R) embryo transferred into R foster mother; R/A (R) embryo transferred into A foster mother; A/A (A) embryo transferred into A foster mother. Transferred embryos were gestated in foster mothers for 14, 24 and 30 days and then collected to record prenatal survival, fetal and placental weights.

**4. Prenatal survival rate and samples at Day 14, 24 and 30**

Implantation rate in each horn (number of implanted embryos at Day 14 from total embryos transferred) was assessed by laparoscopy, according to the procedure previously described (Llobat et al., 2012; Vicente et al., 2012). Recipient females were sequentially euthanized at Day 14 (n=12), Day 24 (n=24) and Day 30 (n=14). Then, prenatal survival was assessed, and live foetuses were weighted after placental membranes and fluids were removed. Fetal placenta and adjacent maternal placenta from each foetus were dissected separately and individually weighted. Samples from fetal placental tissue were stored for RNA expression analysis at -80ºC.

**5. RNA Extraction**

PolyA RNA was extracted from fetal placental tissue at Day 14 and Day 24 of group RR and group AA.

In the case of Day 14 fetal placentas, total RNA was isolated from 10 samples per experimental group. In the case of Day 24 fetal placentas, seven samples per experimental group were used. A traditional phenol/chloroform extraction by sonication in the Trizol reagent was performed. Then, RNA was purified by RNA Clean-up columns (Nucleospin, Madrid, Spain), and concentration, quality and integrity of RNA were evaluated by Nanodrop 1000 and Bioanalyzer 2100 (Agilent Technologies, Madrid, Spain).

**6. Microarray analysis**

For the two-colour microarray analysis, four biological replicates were used Day 14 fetal placentas, including two dye swaps to compensate dye-bias. For Day 24, four biological replicates were used including one dye-swap.

Total RNA (100 ng) was amplified using QuickAmp Labelling Kit (Agilent Technologies, Madrid, Spain), following 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 (Qiagen 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, and then 55 µL of the mixture were hybridised into the Rabbit 44X oligonucleotide array G2519F (Agilent Technologies, Madrid, Spain). After 17 hours at 65ºC, hybridised 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 the Benjamini and Hochberg false discovery rate (FDR), and differences of $P<0.05$ were considered significant. All data sets related to this study were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE62491.

**7. RT-PCR**

To validate the microarray results RT-PCR for six genes (*VEGF*, *ERBB3*, *TGFB2*, *IGF1*, *ITGA1*, *INFG*) were carried out in 20 independent samples for Day 14 fetal placentas and 14 samples Day 24 fetal placenta. 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 Reverse Transcriptase Quantitect kit (Qiagen Iberia S.L, Madrid, Spain) according to the manufacturer's instructions. Real-time PCR reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every PCR was performed from 5 µL diluted 1:20 cDNA template, 250 nM of forward and reverse primers (Table 1.1) and 10 µL 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 (15s) and 60ºC (60s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with a continuous registration of changes in fluorescent emission intensity. The products of RT-PCR were confirmed by bromure ethide-stained 2% agarose gel electrophoresis in 1x Bionic buffer (Sigma Aldrich Química S.A, Alcobendas, Madrid, Spain). Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. 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 housekeeping normalization factor (Weltzien et al., 2005). 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.

**8. Progesterone and IGF I serum levels**

Whole blood was collected from 14 females at Day 14, 21 and 28 of gestation with the aid of a Vacutainer-heparin tube (LH/Li Heparin Tube TAPVAL®, MonLab, SL. Barcelona, Spain). Blood was centrifuged (1500 x g, 10 min at 4ºC) and plasma was stored at -80ºC until assaying. Plasma levels of progesterone (steroid C21, preg-4-ene-3,20-dione) and Insulin-like Growth Factor-I (IGF-I) were determined by direct enzyme immunoassay technique following the manufacturer's instructions (Rabbit Progesterone Elisa Test, Endocrine Technologies, Inc. Newark, USA; IGF-I Elisa Kit, Diagnostic Systems Laboratories, Inc. Texas, USA) . Sensitivities of the tests used were 0.1 ηg/mL mL for progesterone, and 1.1 ηg/mL for IGF-I.

**Table 1.1.** Primers sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analyzed and housekeeping genes used (*VEGF*, as Vascular Endothelial Growth Factor; *ERBB3*, as Epidermal Growth Factor Receptor 3; *TGFB2*, as Trasnforming Growth Factor β2; *IGF1*, as Insulin-like Growth Factor-I; ITGA1: Integrin alfa-1; INFG: as Interferon Gamma; Histone (*H2afz*) and *GAPDH*, as housekeeping gene.

| Gene | Accession number | Sequence | Fragment size (pb) | Efficiency (%) | Correlation ($R^2$) | Reference |
|---|---|---|---|---|---|---|
| ***VEGF*** | AY196796 | For - 5' CTACCTCCACCATGCCAAGT<br>Rev - 5' CACACTCCAGGCTTTCATCA | 236 | 95.5 | 0.99 | Saenz-de-Juano et al. 2011b |
| ***ERBB3*** | AF333179 | For - 5' GTCACATGGACACGATCGAC<br>Rev - 5' AAGCAGTGGCCGTTACACT | 191 | 96 | 0.98 | Saenz-de-Juano et al. 2011a |
| ***TGFB2*** | NM_001082660 | For - 5' GACCCCACATCTCCTGCTAA<br>Rev - 5' CACCCAAGATCCCTCTTGAA | 165 | 98 | 0.95 | Saenz-de-Juano et al. 2011a |
| ***IGF1*** | ENSOCUT00000014681 | For - 5' GTGGATGCTCTTCAGTTCGT<br>Rev - 5' CAGCCTCCTCAGATCACAG | 81 | 100.5 | 0.99 | Naturil-Alfonso et al. 2011 |
| ***ITGA1*** | ENSOCUT00000011375 | For-5' GCCTGTTCTTGATGATTCTCTACC<br>Rev-5'GCATCTTTCCTTGTTTCCACAG | 81 | 100.0 | 0.99 | Saenz-de-Juano et al.2012 |
| ***INFG*** | NM_001081991 | For-5' GTCTGCATTCTAGCCACTG<br>Rev-5' ATTCAGGGGCAGTCACAGTT | 151 | 100.5 | 0.99 | Llobat et al. 2012 |
| ***H2afz*** | AF030235 | For - 5' AGAGCCGGCTGCCAGTTCC<br>Rev - 5' CAGTCGCGCCCACACGTCC | 85 | 99.5 | 0.99 | Mamo et al. 2008 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***GAPDH*** | L23961 | For- 5' GCCGCTTCTTCTCGTGCAG<br>Rev-5' ATGGATCATTGATGGCGACAACAT | 144 | 96.5 | 0.99 | Navarrete-Santos et al. 2004 |

**9. Statistical analysis**

All traits were analysed by a generalised linear model (GLM), using the SPSS software package, version 16.0 (SPSS Inc, Chicago Illinois, USA 2002). Results are reported as least square means ± SEM. Means were considered statistically different at $P \leq 0.05$.

A probit link function was used to determine the effect of maternal and embryonic genotypes on implantation rate and fetal survival at Day 14 and 24 and 30 of gestation, respectively. The GLM fitted to analyse these traits included as fixed effects the embryonic genotype (R or A), maternal genotype (R or A) and their interactions (groups RR, RA, AR, AA). Number of implanted embryos at Day 14 per recipient was included as a covariate in the analysis of fetal survival at Day 24 and 30.

To analyse the fetal and placental (foetus and maternal) weights were analyzed with a GLM including as fixed factors maternal genotype (R or A), gestation day (14, 24, 30) and their interaction was used. Progesterone and IGF-I plasma levels, were also analyzed with a GLM including as fixed factors maternal genotype (R or A), gestation day (14, 21, 28) and their interaction. Moreover, placenta and fetal weights were analysed including the current number of live foetuses at Day 14, 24 and 30 of gestation as covariate.

Data of relative mRNA abundance were normalised by a Nepierian logarithm transformation and evaluated using a GLM too.

## 1. Prenatal survival rate

Prenatal survival rate was affected by embryonic genotype at Day 14 and 24 but not at Day 30. The total implantation rate at Day 14 was 0.75 ± 0.04 of total transferred embryos (447/600). The implantation rate was lower for embryonic genotype R (0.57± 0.04 and 0.69 ± 0.04, for genotype R and A, respectively, Figure 1.2). These embryonic effects were also observed at Day 24 (Figure 1.2 A).

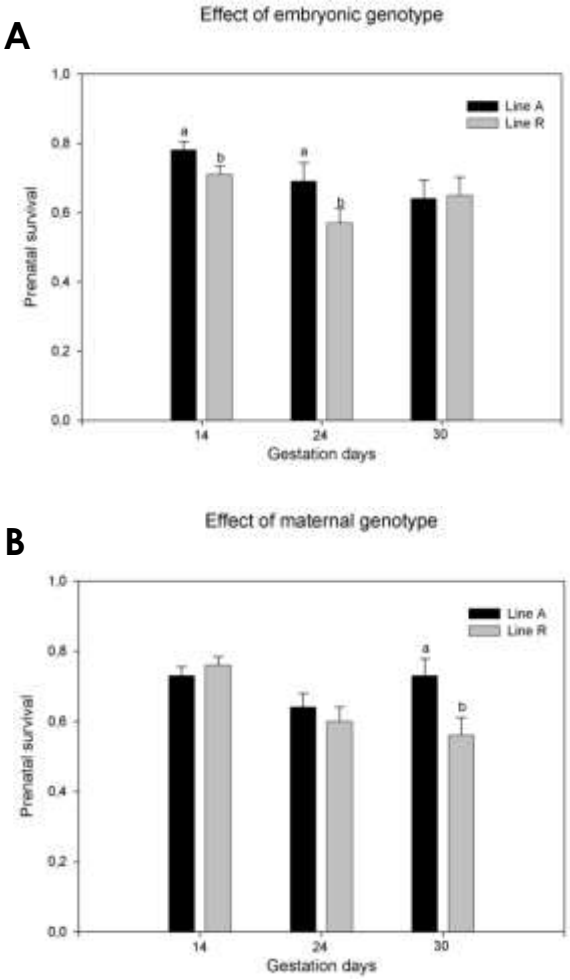


**Figure 1.2.** Prenatal survival at Day 14, 24 and 30 for the different lines. A: Fetal survival for the embryonic genotype effect; B: Fetal survival for the maternal genotype effect.

Nevertheless, when the number of implanted embryos at Day 24 was included as a covariate, a significant interaction between both genotypes was observed. Concretely, group R/R presented lower live fetuses rate (0.48 ± 0.05 vs. 0.68 ± 0.04, 0.60 ± 0.05, 0.70 ± 0.05, for genotypes interaction A/A, A/R and R/A, respectively, Figure 1.3). At Day 30, maternal genotype influenced the prenatal survival; maternal genotype A presented a higher prenatal survival rate (0.73 ± 0.05 vs. 0.56 ± 0.05, genotype A vs. genotype R, respectively, Figure 1.2 B).

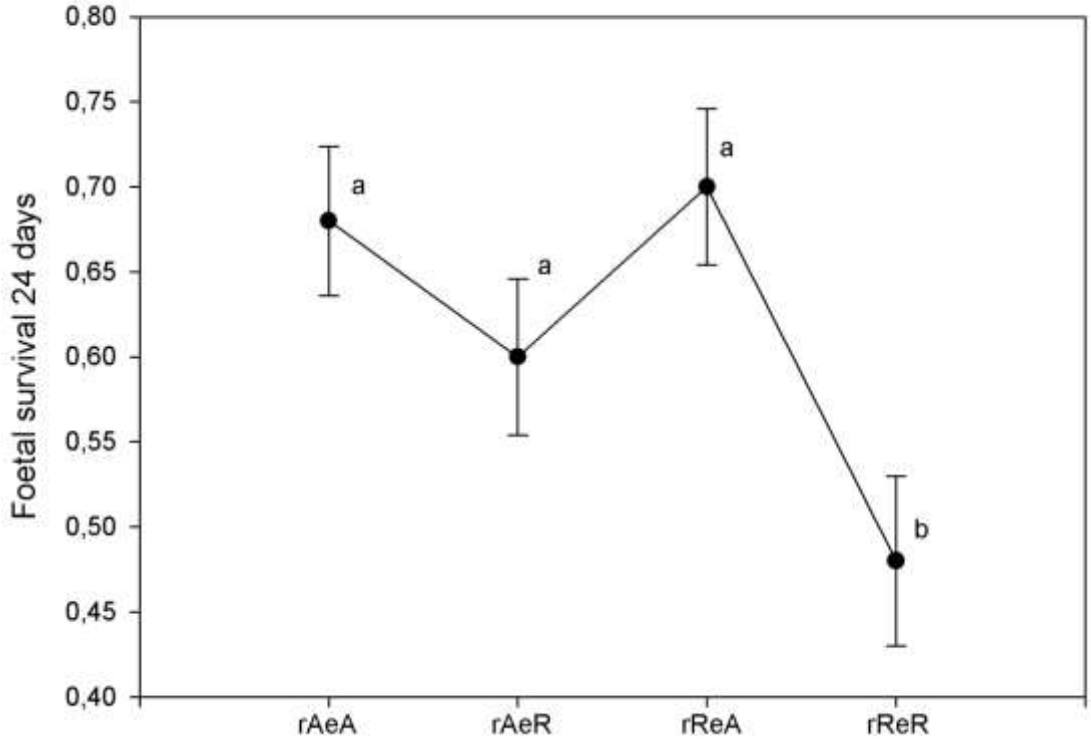


**Figure 1.3.** Interaction of fetal survival at Day 24 when the covariate implantation rate is included, for: group A/A ($A^{[embryo]}/A^{[mother]}$), group A/R ($A^{[embryo]}/R^{[mother]}$), group R/A ($R^{[embryo]}/A^{[mother]}$) and group R/R ($R^{[embryo]}/R^{[mother]}$). a,b values are statistically different (p-value < 0.05).

## 2. Fetal and placental weight

Both fetuses and placentas (fetal and maternal) were weighted at Day 14, 24 and 30 (Figure 1.4).

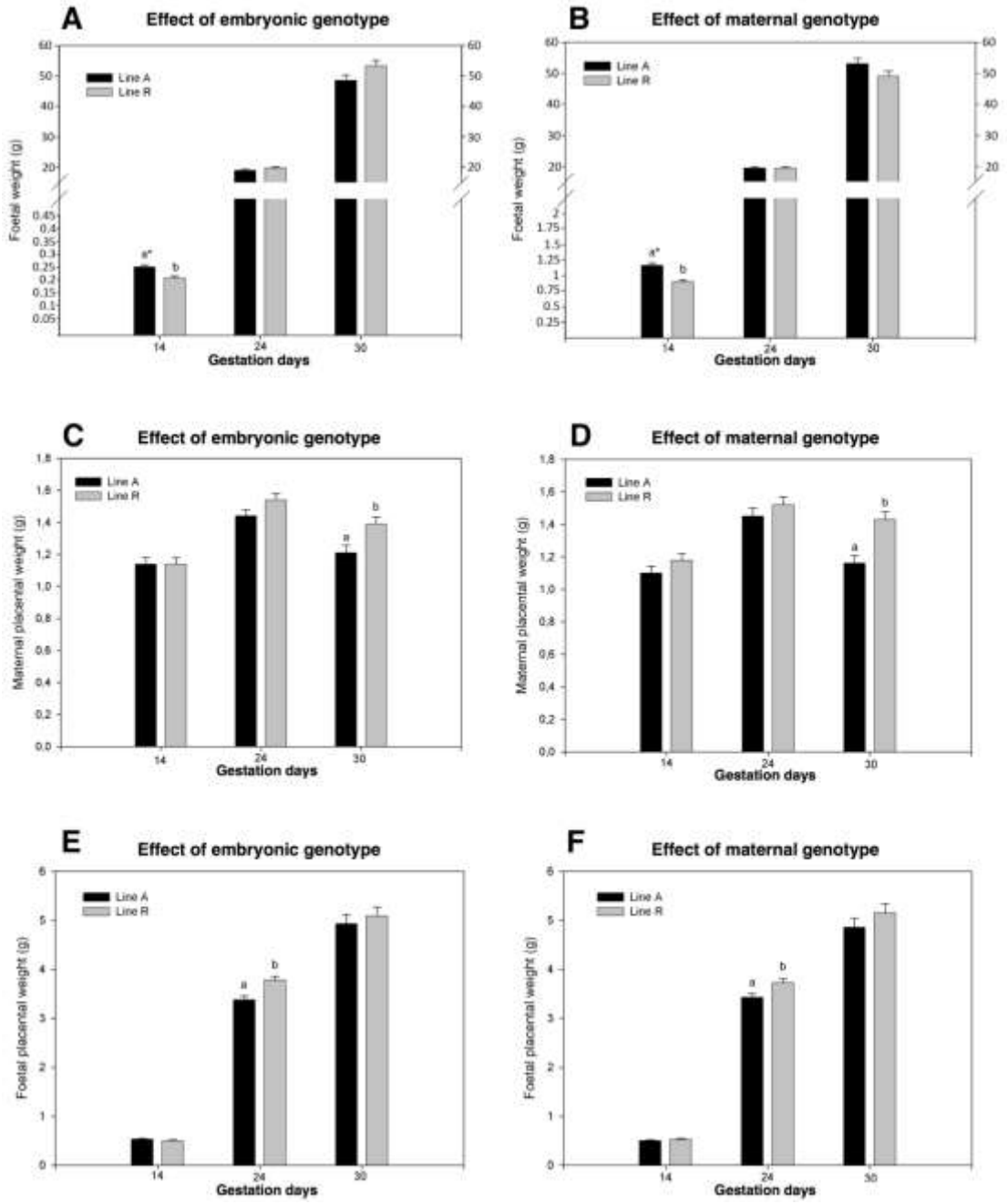


**Figure 1.4.** Fetal and placental (fetal and maternal) weights at Day 14, 24 and 30 of gestation. A: Fetal weight for embryonic genotype effect; B: Fetal weight for maternal genotype effect; C: Fetal placenta weight for embryonic genotype effect; D: Fetal placenta weight for maternal genotype effect; E: Maternal placenta weight for embryonic genotype effect; F: Maternal placenta weight for maternal genotype effect. a,b values are statistically different (p-value < 0.05).

Fetal weight at Day 14 was affected by both embryonic and maternal genotypes (Figure 1.4 A and B, respectively). Specifically, the interaction showed that group A/A has higher weight, while group R/R showed lower weight (0.29 ± 0.01 g vs. 0.19 ± 0.01 g, respectively, Figure 1.5). However, fetal weight at Day 24 did not vary between the embryonic and maternal genotype (Figure 1.4 A and B). At Day 30 foetus weight was almost significantly affected by the embryonic genotype (p-value=0.054), being higher for embryonic genotype R (53.40 ± 1.74 g and 48.50 ± 1.81 g, for embryonic genotype R and A, respectively, Figure 1.4 A).

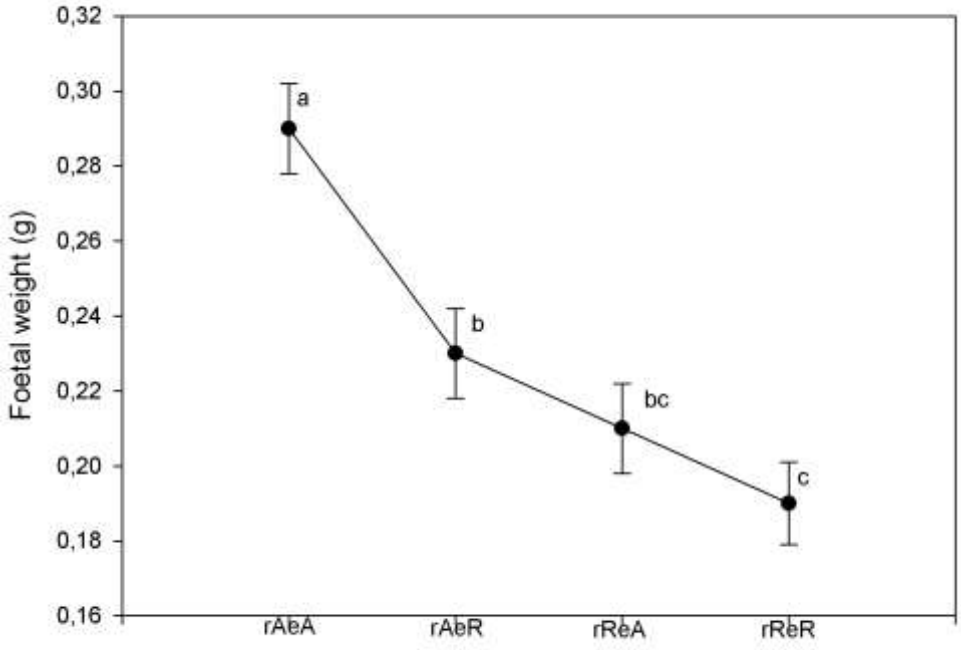


**Figure 1.5.** Interaction of fetal weight at Day 14 for: group A/A ($A^{[embryo]}/A^{[mother]}$), group A/R ($A^{[embryo]}/R^{[mother]}$), group R/A ($R^{[embryo]}/A^{[mother]}$) and group R/R ($R^{[embryo]}/R^{[mother]}$). a,b,c values are statistically different (p-value < 0.05).

When maternal placental weight was compared, we did not observe differences at Day 14 (Figure 1.4 C, D), but when the number of implanted embryos was included as covariate, maternal genotype became significant,

being higher for maternal genotype R (1.24 ± 0.05 g vs. 1.04 ± 0.05 g, genotype R and line A, respectively, Figure 6). However, at Day 24 maternal placental weight was similar for the embryonic and maternal genotype (Figure 1.4 C and D). Conversely, maternal placental weight presented differences for the embryonic and maternal genotype at Day 30 (Figure 1.4 C and D), being heavier for embryonic and maternal genotype R (1.39 ± 0.05 g and 1.52 ± 0.05 g, respectively) than for genotypes A (1.21 ± 0.05 g and 1.45 ± 0.05 g, for embryonic and maternal genotypes, respectively). Nevertheless, the interaction between embryonic and maternal genotype was not significant. Respect to the fetal placental weight at Day 14 was similar for embryonic and maternal genotypes. However, at Day 24, the weight was affected by both embryo and maternal genotype, being higher for the group R/R (3.92 ± 0.12 g vs. 3.23 ± 0.11 g, for group R/R and A/A, respectively, Figure 1.4 E and F). On the contrary, fetal placental weight at Day 30 did not present differences for the embryonic and maternal genotype (Figure 1.4 E and F).

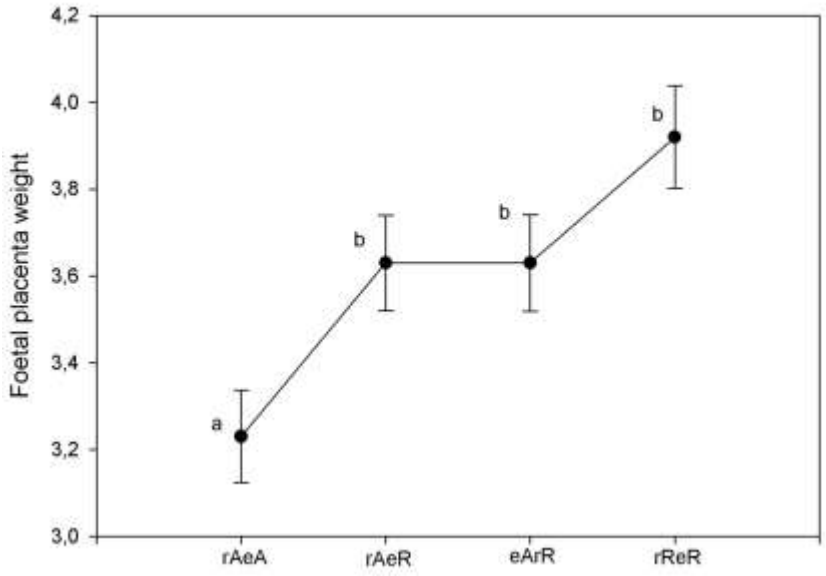


**Figure 1.6.** Interaction of fetal placenta weight at Day 24 for group A/A ($A^{[embryo]}/A^{[mother]}$), group A/R ($A^{[embryo]}/R^{[mother]}$), group R/A ($R^{[embryo]}/A^{[mother]}$) and group R/R ($R^{[embryo]}/R^{[mother]}$). a,b values are statistically different (p-value < 0.05).

### 3. Effect of group (R/R and A/A) on fetal placental gene expression at Day 14 and Day 24

Limma analysis after normalization did not reveal any significant changes in gene expression, neither at Day 14 not at Day 24. A total of six genes represented on the microarray (*VEGF*, *ERBB3*, *TGFB2*, *IGF1*, *ITGA1*, *INFG*) were selected and tested using RT-PCR. These genes were selected because they represent likely important moments as embryo development, implantation events and placenta formation. According to microarray results, no significant differences were observed between groups neither at Day 14 not at Day 24 (Figure 1.7 and 1.8, respectively).

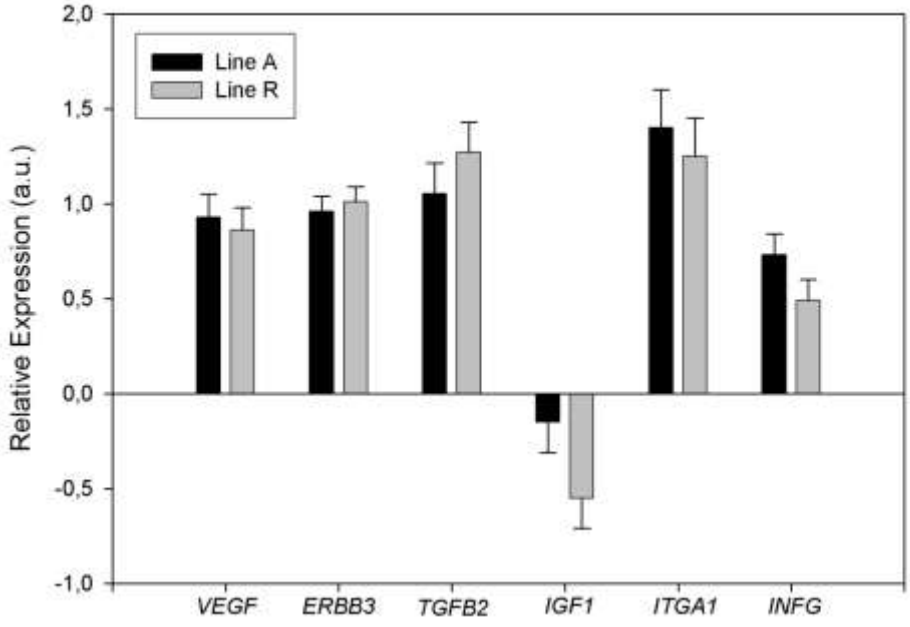


**Figure 1.7.** Relative expression of vascular endothelial growth factor (*VEGF*), Epidermal Growth Factor receptor 3 (*eRBB3*), Transforming Growth Factor-B2 (*TGFB2*), Insulin-like Growth Factor I (*IGF1*), Integrin alpha-I (*ITGA1*) and Interferon-gamma (*IFNG*) for validation of Day 14 fetal placentas microarray. Relative abundance values are shown in arbitrary units (a.u), expressed by the mean value ± standard error means.

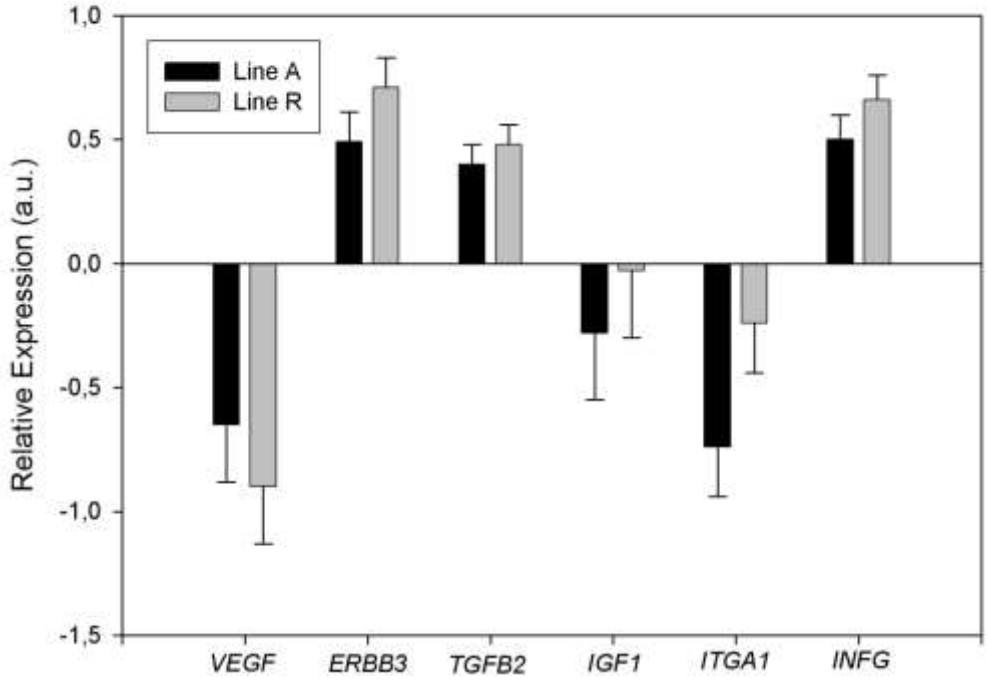


**Figure 1.8** Relative expression of vascular endothelial growth factor (*VEGF*), Epidermal Growth Factor receptor 3 (*eRBB3*), Transforming Growth Factor-B2 (*TGFB2*), Insulin-like Growth Factor I (*IGF1*), Integrin alpha-I (*ITGA1*) and Interferon-gamma (*IFNG*) for validation of Day 24 fetal placentas microarray. Relative abundance values are shown in arbitrary units (a.u), expressed by the mean value ± standard error means.

**4. Effect of maternal genotype on progesterone and IGF1 levels**

Progesterone plasma levels at Day 14, 21 and 28 of gestation were similar between maternal genotypes R and A, with higher levels at Day 14 and decreasing levels at days 21 and 28 of gestation (Figure 1.9 A). As well, plasma levels of IGF1 were not different between recipients of the different lines at Day 14, 21 and 28, with lower levels at Day 14 and reaching higher levels at Day 21 and 28 (Figure 1.9 B).

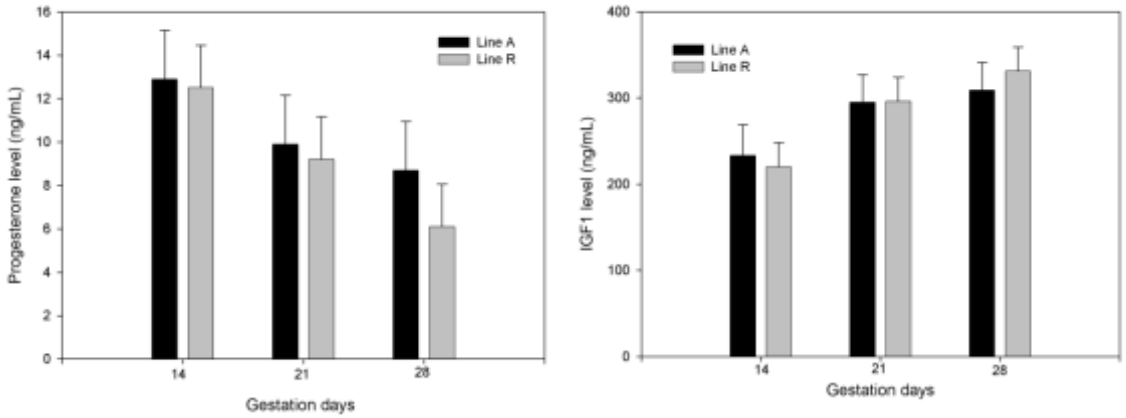


**Figure 1.9.** Effect of maternal genotype on progesterone and IGF1 serum levels at Day 14, 21 and 28 of gestation. A: Progesterone levels; B: IGF1 levels.

## DISCUSSION

Survival and fitness of offspring depend on complex systems of provisioning resources between parents and offspring, resulting in intricate coadaptations to variations in supply and demand (SenthamaraiKannan et al., 2011). In eutherian mammals, fetal growth and epigenetic preadaptive responses for birth depend on the proper function of the placenta, which acts as an interface between the mother and foetus. Many studies describing genetic differences in prenatal survival in polytocous species have been performed (Brien, 1986; Blasco et al., 1993; Argente et al., 2003; Holt et al., 2004; Mocé et al., 2004b; Foxcroft et al., 2006; Freking et al., 2007; Laborda et al., 2012; Vicente et al., 2012; 2013). However, few studies have been done to elucidate how maternal genotype, embryonic genotype and their dialogue can modify survival rate over the course of pregnancy. To examine this, we studied the effect of embryonic and maternal genotype at different stages of pregnancy (Day 14, 24 and 30) by reciprocal transfers. The embryo transfer model that we used has the following advantages: (*i*) two fetal genotypes are transferred into

two different maternal genotypes, which allows us to study the fetal and maternal genotype effects, *(ii)* the female rabbit has a uterus formed by two independent horns (each horn possess its own cervix) and it have advantages in identification of the offspring and (*iii*) laparoscopic embryo transfer is a non-invasive and feasible technique.

Our results show that at Day 14 the fetal survival was significantly regulated by the embryonic genotype but not the maternal genotype. This finding correlates with previous studies (Youngs et al., 1994; Ernst et al., 2000, in mouse, Ashworth et al,. 1990; Kaminski et al., 1996, in swine and Vicente et al., 2013 in rabbits). However, at Day 24, when the covariate implantation rate was included fetal and maternal genotype interaction was determined, while at Day 30 (last term of gestation) occurs a change and fetal survival was significantly regulated by the maternal genotype. Mocé et al. (2004b), working with rabbits divergently selected by high and low uterine capacity found an interaction between both genotypes at Day 28. These authors suggest that the embryonic genotype had an effect on fetal survival only in a favorable maternal genotype. These results are in agreement with those reported by Moler et al. (1981), who detected a recipient and recipient x donor interaction effect on survival in mice to term. In our case, we did not observe this effect at post-implantation stages. Maybe, the relevant differences in the selection criteria of lines used in this study would explain this discrepancy.

In terms of fetal and placental weights, our results show that at Day 14 the fetal weights were significantly regulated by both maternal and embryonic genotypes. This findings correlates with previous studies that showed that maternal genotype has been described as the key factor in determining fetal

weight (Pomp et al., 1989; SenthamaraiKannan et al., 2011). Yet our results suggest that embryonic genotype may also influence fetal weight after implantation, and the maternal placenta weight. When the pregnancy progresses (at Day 24), these effects of the influence of embryonic and maternal genotypes on fetal weights and the maternal placental weights were not observed. Nevertheless, at last term of gestation (Day 30), maternal genotype seems to take a role, increasing embryo mortality. Thus, at Day 30 also appears an effect of maternal genotype, which indicates that the recipient endometrium also plays a relevant role at the last term of gestation. However, when we studied two endocrine factors highly related to the development and maintenance of the endometrium and to the mobilization of maternal resources for gestation, we did not observe differences between both maternal genotypes. Inspite of this, specifically, R/R presented higher maternal placental weight than the other groups, being group A/A the one with lowest maternal placental weight. This could be a sign of placentomegaly to ensure a sufficient fetal growth and survival in this environment as a consequence of either a potential restriction before a fast growth from foetuses genotype R or a lower functionality of placenta.

As the placenta is an interface receiving signals from both mother and foetus and a platform for maternal-fetal interaction, we analyzed fetal-placental to evaluate the fetal genotype effects on prenatal survival analysis compared gene expression with an embryo transfer system using the two inbred lines (A/A and R/R). The fact that no differences were observed in gene expression in fetal placentas at both Day 14 and Day 24 of gestation was surprising, considering that embryonic genotype had influence on the prenatal survival. Although, in

terms of prenatal survival, the vast majority of the studies indicated a strong maternal uterine genotype effect (inbred strains (Fekete, 1947; Baunack et al., 1986), genetically selected lines (Brumby, 1960; Moore et al., 1970a,b; Aitken et al., 1977; Al-Murrani and Roberts, 1978; Moler et al., 1981), or cross-nursing and sib analysis studies (Cox et al., 1959; El-Oksh et al., 1967)). Our findings indicate that the influence of embryo and maternal genotypes on rabbit prenatal survival and growth seem to change over gestation.

In conclusion, embryonic genotype seems to influence prenatal survival, but additionally, at last term of gestation maternal genotype can affect embryonic mortality. Moreover, at early gestation (Day 14), embryonic genotype has an effect on fetal weight, while both embryonic and maternal genotype affected placental weights at Day 24 and 30, respectively. These findings highlight the need to consider both maternal and embryonic genetic effects in the neonatal survival over the course of pregnancy.

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