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CORRELATED RESPONSE IN EARLY EMBRYONIC DEVELOPMENT IN RABBITS SELECTED FOR LITTER SIZE VARIABILITY

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Abstract: A divergent selection experiment for litter size variability was carried out in rabbits. The litter size variability was estimated as the phenotypic variance of litter size within female. The aim of this study was to assess the effect of selecting for litter size variability on early embryonic development and survival after 7 generations of divergent selection (high and low variability lines). A total of 30 non-lactating multiparous does per line were used. The ovulation rate and early embryonic development were analysed using Bayesian methodology. Ovulation rate was not affected by the selection process. At 28 h of gestation, embryonic development and survival were similar in both lines. At 48 h of gestation, the majority of embryos in the high line were in the early morulae stage. The high line had a higher proportion of early morulae (79.54 *vs*. 53.43%; *P*=0.94) and a lower proportion of compacted morulae (20.46 *vs*. 46.57%; *P*=0.93%) than the low line. At 72 h of gestation, the high line had 1.59 fewer embryos than the more homogeneous line (*P*=0.85), due to reduced embryonic survival (0.60 *vs*. 0.74; *P*=0.93). The high line continued to show a higher proportion of early morulae (21.01 *vs*. 3.69%; *P*=0.93) and a lower proportion of compacted morulae and blastocysts (78.99 *vs*. 96.31%; *P*=0.94) than the low line at 72 h of gestation, indicative of reduced embryonic development. In conclusion, selection for homogeneity in litter size had a positive impact on embryonic traits.

Key Words: rabbit, blastocyst, embryonic survival, morula, ovulation rate, residual variance.

INTRODUCTION

The environmental sensitivity of animals has a considerable impact on their productivity (Rauw and Gomez-Raya, 2015). Selection to reduce environmental variance improves the performance of animals in adverse environments (Mulder *et al.,* 2013). A divergent selection experiment for litter size variability was successfully carried out in rabbits, where after seven generations of selection, the line selected to increase litter size variability showed greater variability (+1.37 kit²) and a more decreased litter size (–0.71 kits) than the low line (Blasco *et al.,* 2017) due to the lower (–1.38 embryos) number of implanted embryos (Argente *et al.,* 2014a). This line also showed less resilience, i.e., greater sensitivity to illness and stressful conditions (García *et al.,* 2012; Argente *et al.,* 2014b). Maternal stress increases the failure rate of blastocyst implantation (Liu *et al.,* 2015; Burkuš *et al.,* 2015) by changing the expression patterns of genes involved in embryo development (Marco-Jiménez *et al.,* 2013; Silva *et al.,* 2013). Asynchrony between embryonic development and oviduct function plays an important role in early embryonic loss (Geisert and Schmitt, 2002). Our hypothesis is that a lower implantation rate in the line selected for increased litter size variability is associated with retarded embryonic development related to oviduct function.

The aim of this study was to assess the effect of selection for litter size variability on early embryonic development and survival in rabbits.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the Research Ethics Committee of Miguel Hernández University, Elche, on 21 June 2011 (reference 98 number DTA-MJA-001-11), in accordance with Council Directives 98/58/EC and 2010/63/EU.

Animals

The animals were the 7th generation of a divergent selection experiment for litter size variability, measured as the phenotypic variance of litter size within does after correcting for the effects of year-season and parity-lactation status (first parity or lactating/non-lactating at mating for other parities). The high line was seleted to increase litter size variability or to decrease homogeniety in litter size, and the low line was selected to decrease litter size variability or to increase the homogeniety in litter size. Details of the experimental protocol were previously reported in Argente *et al.* (2014a). All animals were bred at the farm of the Miguel Hernández University in Elche, Spain. They were kept under a constant photoperiod consisting of 16 h continuous lighting and 8 h continuous darkness, with controlled ventilation.

Traits

A total of 30 non-lactating multiparous does per line were used in this experiment. Does were euthanised at 28, 48 or 72 h post-mating by intravenous administration of sodium thiopental at a dose of 50 mg/kg of body weight (Thiobarbital; B. Braun Medical S.A., Barcelona, Spain). The entire reproductive tract was removed immediately. The ovulation rate (OR) was estimated as the number of corpora haemorrhagica. The numbers of normal embryos (NE), abnormal embryos and oocytes were counted after collection by perfusion of each oviduct and uterine horn with 10 mL of Dulbecco's phosphate buffered saline containing 0.2% bovine serum albumin. Embryos were classified as normal if they presented homogeneous cellular mass and intact zona pellucida and mucin coating (Maurer, 1978), determined using a binocular stereoscopy microscope (Mz 9.5-600x; Leica, Wetzlar, Germany). At 28 h of gestation, normal embryos were classified as either 2-cell or 4-cell embryos. At 48 h of gestation, normal embryos were classified as early morulae (EM) or compacted morulae (CM). At 72 h of gestation, normal embryos were classified as EM, CM or blastocysts (B). In all cases, the number of 2-cell, 4-cell, EM, CM and B, were expressed as a percentage of normal embryos. The early embryonic survival (EES) was estimated as the number of normal embryos divided by the ovulation rate.

Statistical analyses

All traits were analysed with a model including the fixed effects of line and season. The model for OR also included the pregnancy stage (28, 48 or 72 h post-mating) as a fixed effect. The traits were analysed using Bayesian methodology. Bounded flat priors were used for all unknowns. Residuals were independently normally distributed with mean 0 and variance Iσ_e^2. The priors for the variances were also bounded uniform. Features of the marginal posterior distribution for all unknowns were estimated using Gibbs sampling. The Rabbit program developed by the Institute for Animal Science and Technology (*http://www.dcam.upv.es/dcia/ablasco/Programas/THE PROGRAM Rabbit.pdf*; Valencia, Spain) was used to analyse the differences between lines. After some exploratory analyses, we used a chain of 60 000 samples with a burn-in period of 10 000 samples, with only 1 of every 10 samples saved for inferences. Convergence was tested using the Z criterion of Geweke (Sorensen and Gianola, 2002), and Monte Carlo sampling errors were computed using the time-series procedures described by Geyer (1992). The Monte Carlo standard errors were small in all Bayesian analyses, and a lack of convergence was not detected by the Geweke test. An advantage of the Bayesian approach using Markov chain Monte Carlo (MCMC) procedures is the ease of computing confidence intervals and probabilities (see reviews by Blasco, 2001 and 2005). Bayesian statistics provides a new approach for describing the uncertainty against classical statistics. For example, we can calculate the median for each line and the precision of our estimation, allowing us to determine the smallest interval with a 95% probability of containing the true value (the highest posterior density interval at 95%). This interval is not dependent on the estimate provided, and can be asymmetric about the median. Aside from this, we were also interested in estimating differences between the high and low lines selected for litter size variability (D_{H-L}), as we could thus also calculate the probability of this difference being greater than zero [$P(D_{H-L}>0)$].

RESULTS AND DISCUSSION

Table 1 shows the features of the estimated marginal posterior distribution of the differences between the high and low lines (D_{H-L}) for ovulation rate and early embryonic development. When $|D_{H-L}|>0$, we consider there to be enough evidence of a difference between the high and low lines if the probability (P) of $|D_{H-L}|$ is greater than 0.80 (Table 1). The probability (*P*) should not be confused with the *P*-value (Blasco 2001, 2005). Johnson (2013) showed that the evidence provided by *P*-values is lower than indicated, for example, a *P*-value of 0.05 only provides 67-75% of evidence, therefore approximately 25% of false positives appear with a *P*-value of 0.05. The *P* is the actual probability, so we chose 80% to represent sufficient evidence. According to the value of *P*, both lines were found to have a similar ovulation rate (*P*=0.70).

At 28 h of gestation, the high and low lines exhibited a similar number of recovered embryos (*P*=0.55) and embryonic survival rate (*P*=0.63). There was no difference in embryonic development between lines (*P*=0.75). We observed a similar proportion of embryos in the 2-cell and 4-cell stages (approximately 50% in each stage) in both lines. Previous studies in the literature have indicated that the majority of embryos are in the 2-cell stage at 25 h post-mating (Peiró *et al.,* 2007) and in the 4-cell stage at 30 h post-mating (Peiró *et al.,* 2015). Our results are in agreement with these studies, confirming that embryonic development at 28 h post-mating represents an intermediate stage between 25 and 30 h post-mating.

At 48 h of gestation, no differences were found for the number of recovered embryos (*P*=0.58) and embryonic survival (*P*=0.72) between the high and low lines. The majority of embryos in the high line were classified as early morulae (79.54%). The high line had 26.81% (2.8 embryos) more early morulae (*P*=0.94) and 26.16% less compacted morulae than the low line (20.46 *vs.* 46.57%; *P*=0.93). Hence, increasing litter size variability had a negative effect on early embryonic development.

At 72 h of gestation, the difference between the high and low lines for the number of normal embryos had increased, where the high line had 1.59 fewer embryos than the more homogeneous line (*P*=0.85). Embryonic survival was also

Table 1: Features of the estimated marginal posterior distribution of the differences between the high and low lines selected for litter size variability.

	High line[a]	Low line[a]	D_{H-L}	HPD$_{95\%}$		*P*
OR[b]	12.43 (2.30)	12.08 (2.35)	0.35	−1.01	1.57	0.70
28 h post-mating[c]						
NE	9.95 (2.86)	10.24 (2.37)	−0.29	−3.44	3.06	0.55
2C (%)	42.64 (29.35)	52.86 (32.21)	−10.18	−41.85	23.10	0.75
4C (%)	57.36 (33.54)	47.14 (31.34)	10.81	−21.43	40.45	0.75
EES	0.81 (0.10)	0.84 (0.12)	−0.05	−0.25	0.18	0.63
48 h post-mating[c]						
NE	9.92 (2.92)	10.20 (2.35)	−0.27	−3.65	2.85	0.58
EM (%)	79.54 (38.68)	53.43 (37.52)	26.81	−6.06	62.12	0.94
CM (%)	20.46 (37.40)	46.57 (38.67)	−26.16	−60.50	8.28	0.93
EES	0.79 (0.11)	0.85 (0.12)	−0.06	−0.28	0.14	0.72
72 h post-mating[c]						
NE	7.56 (2.12)	9.18 (3.01)	−1.59	−4.76	−1.42	0.85
EM (%)	21.01 (25.71)	3.69 (20.19)	17.36	−6.42	39.86	0.93
CM (%)	27.38 (34.65)	33.63 (37.19)	−7.17	−42.28	26.07	0.67
B (%)	51.61 (44.43)	62.68 (44.67)	−11.27	−50.74	31.70	0.71
EES	0.60 (0.10)	0.74 (0.11)	−0.14	−0.34	0.05	0.93

[a]mean (standard deviation). [b]30 does per line. [c]10 does per line. D_{H-L}: median of difference between the high and low lines. HPD$_{95\%}$: highest posterior density region at 95% *P*: probability of the difference being >0 when $D_{H-L}>0$ and probability of the difference being <0 when $D_{H-L}<0$. OR: ovulation rate. NE: Number of normal embryos. 2C: 2-cell embryos. 4C: 4-cell embryos. EM: early morulae. CM: compacted morulae. B: blastocysts. 2C, 4C, EM, CM and B were expressed as a percentage of normal embryos (NE). EES: early embryonic survival (NE/OR).

lower in the high line compared to the low line (0.60 *vs.* 0.74; *P*=0.93). The line selected to increase litter size variability had a higher proportion of early morulae (21.01% in the high line *vs.* 3.69% in the low line; *P*=0.93) and a lower proportion of compacted morulae and blastocysts (78.99% in the high line *vs.* 96.31% in the low line; *P*=0.94). Therefore, the high line continued to show reduced embryonic development compared to the homogeneous line at 72 h of gestation. The embryonic development in this line was lower than that reported in other maternal lines (Mocé *et al.,* 2004; Peiró *et al.,* 2007; Argente *et al.,* 2010), in which a lower percentage of early morulae (10-14%) and a higher percentage of compacted morulae and blastocysts (85-95%) were reported. Several studies have reported that embryos with a slower developmental rate have higher mortality than more developmentally advanced embryos during gestation (Torres *et al.,* 1987) and at birth (Murakami and Imai, 1996). The high line had fewer implanted embryos than the low line after 7 generations of selection (10.18 embryos in the high line *vs.* 11.49 embryos in the low line; Argente *et al.,* 2014a). This difference was maintained for litter size at birth (7.46 kits in the high line *vs.* 8.17 kits in the low line; Argente *et al.,* 2014a). These results are in agreement with lower embryonic development observed in the high line at 48 and 72 h of gestation, representing a negative effect on embryonic survival.

In previous studies, the line selected to increase litter size variability has been reported to have a higher subclinical immune response, related to higher sensitivity to microorganisms usually found on the farm and a higher cortisol level (García *et al.,* 2012; Argente *et al.,* 2014b). These results are consistent with the high line being more sensitive to stress, in addition to a greater risk of illness. We hypothesise that embryonic development is delayed in the high line due to stress and their higher susceptibility to illness. Embryonic development can be delayed under stress conditions by the disruption of proteins involved in embryonic growth (see review by Puscheck *et al.,* 2015). For example, a lack of DICER1, MATER, ZAR1, PADI6 and SEBOX does not allow the embryo to develop beyond the 2-cell stage, and the embryo is unable to reach the 8-cell or morulae stage in the absence of either SMARCA4, DNMT1, DNMT3A, TET, KLF4, OCT4, NANOG or SOX2 (see review by Argente, 2016). Moreover, the survival of an embryo that reaches the oviduct environment in a less developed state than the oviduct will be compromised in the early stages of pregnancy due to asynchrony (see review by Geisert and Schmitt, 2002). It has been reported that, although less developed embryos can survive beyond implantation, they probably die soon afterwards due to foetal competition for uterine space and a poor blood supply (Mocé *et al.,* 2004; Argente *et al.,* 2008). Selection for litter size variability modifies early embryo development starting from 48 h of gestation, leading to decreased embryo development and fewer normal embryos in the line selected to increase litter size variability. These results demonstrate the negative relationships of litter size variability with embryonic development and survival in the early stages of gestation.

CONCLUSIONS

Selection for litter size variability did not seem to affect ovulation rate. Nevertheless, there was a negative correlated response in early embryonic development and survival.

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