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TITLE

A genome-wide association study in divergently selected lines in rabbits reveals novel genomic regions associated with litter size traits

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Summary

Uterine capacity (UC), defined as the total number of kits from unilaterally ovariectomized does at birth, has a high genetic correlation with litter size. The aim of our research was to identify genomic regions associated with litter size traits through a genome-wide association study using rabbits from a divergent selection experiment for UC. A high-density SNP array (200K) was used to genotype 181 does from a control population, high and low UC lines. Traits included total number born (TNB), number born alive (NBA), number born dead, ovulation rate (OR), implanted embryos (IE), and embryo, foetal and prenatal survivals at second parity. We implemented the Bayes B method and the associations were tested by Bayes factors and the percentage of genomic variance (GV) explained by windows. Different genomic regions associated with TNB, NBA, IE, and OR were found. These regions explained 7.36%, 1.27%, 15.87%, and 3.95% of GV, respectively. Two consecutive windows on chromosome 17 were associated with TNB, NBA, and IE. This genomic region accounted for 6.32% of GV of TNB. In this region, we found the *BMP4, PTDGR, PTGER2, STYX* and *CDKN3* candidate genes which presented functional annotations linked to some reproductive processes. Our findings suggest that a genomic region on chromosome 17 has an important effect on litter size traits. However, further analyses are needed to validate this region in other maternal rabbit lines.

Keyword: *divergent selection, GWAS, litter size, QTL, rabbits, uterine capacity.*

Introduction

Litter size has high economic importance in all polytocous livestock species, including rabbits (Cartuche, Pascual, Gómez, & Blasco, 2014) and swine (Quinton, Wilton, Robinson, & Mathur, 2006). However, the selection response for this complex trait, as well for several other reproduction traits, is small. For example, in rabbit selection experiments for litter size the response can be 0.1 kits per generation (see review Khalil & Al-Saef, 2008). This situation encouraged the application of alternative selection strategies based on litter size components such as uterine capacity (UC) (Argente, Santacreu, Climent, Bolet, & Blasco, 1997), ovulation rate (OR) (Laborda, Mocé, Blasco, & Santacreu, 2012), or selection using independent culling levels for OR and litter size (Badawy, Peiró, Blasco, & Santacreu, 2018; Ziadi, Moce, Laborda, Blasco, & Santacreu, 2013).

UC is the prenatal survival when the OR is not a limiting factor of litter size and the uterine horn is crowded with embryos (Argente et al., 1997; Blasco, Argente, Haley, & Santacreu, 1994). This trait can be measured as total number of kits at birth under these conditions (Christenson, Leymaster, & Young, 1987; Mocé, Santacreu, Climent, & Blasco, 2004), since does have a double cervix preventing intrauterine transmigration; and thus, only one uterine horn remains functional and crowed, duplicating its OR when ovariectomies are implemented (Argente et al., 1997; Blasco, Argente, Haley, & Santacreu, 1994). From 1991 to 1998, the Animal Science Department of “Universitat Politècnica de València” carried out an experiment of divergent selection for UC. After ten generations of selection, the divergence between the two divergent lines (high and low UC lines) was 1.50 kits for UC (Blasco, Ortega, Climent, & Santacreu, 2005), with a correlated response in litter size of 2.35 kits

(Santacreu, Mocé, Climent, & Blasco, 2005). Approximately one-half of the response in UC was obtained in the first two generations suggesting the presence of a major locus with large effect segregating in these populations (Argente, Blasco, Ortega, Haley, & Visscher, 2003; Blasco et al., 2005). Thus, a candidate gene strategy was carried out to characterize this locus by comparing polymorphisms and expression levels between the two UC lines of a some promising candidates (Argente et al., 2010; Ballester et al., 2013; Peiró et al., 2008). Some of these genes (progesterone receptor - *PGR,* hydroxysteroid (17-beta) dehydrogenase 4 - *HSD17B4, and* Endoplasmic Reticulum Oxidoreductase 1 - *ERO1*) showed different expression levels in the oviduct of the two UC line, remarkably overexpressed in the low UC line, but these result could not identify any putative causal mutations (Argente et al., 2010; Ballester et al., 2013; Peiró et al., 2008).

The recent availability of an updated rabbit reference genome (Carneiro et al., 2014) and a high-density single nucleotide polymorphisms (SNP) array (Blasco & Pena, 2018) has opened new possibilities for more comprehensive genomic analyses in this species, similar to what is possible in all other major livestock species. Together with these tools, several methods for genome-wide association analyses have been also already developed and applied in many different species (Fan, Du, Gorbach, & Rothschild, 2010). Among them, genome-wide association studies (GWAS) using multi-marker regression approaches can attain better power detection to identify genomic regions associated with a trait than the classical approach of single maker simple regression (López de Maturana et al., 2014; Toosi, Fernando, & Dekkers, 2018).

In this study, we designed a GWAS in rabbit based on the described extreme and divergent lines for UC and applied a Bayesian multi-marker regression approach to identify quantitative trait loci (QTL) affecting litter size traits in this species.

Material and Methods

Ethical statement

Animal manipulations and the experimental procedures were approved by the Ethical Committee of the Polytechnic University of Valencia, according to Council Directives 98/58/EC (European Economic Community, 1998).

Animals and phenotypes

Animals came from an experiment of divergent selection for uterine capacity and a cryopreserved control population (Blasco et al., 2005; Santacreu et al., 2005). After ten generations of selection for uterine capacity, the selection was relaxed. For the current study, we collected blood samples from non-ULO female rabbits. The study involves 90 does of the high UC line, 69 does of the low UC line and 30 does of the control population. All samples of high and low UC lines came from the 11th and 12th generations (Mocé, Santacreu, Climent, & Blasco, 2005; Santacreu et al., 2005). The base population of divergent lines for UC came from the 12th generation of a line selected for number of kits at weaning (named V line). The control population was derived from cryopreserved embryos from the 13th and 15th generations of the V line. The embryos were transferred to receptor does to produce a control population which was contemporary to UC females from 11th generation (Santacreu et al., 2005).

The traits were recorded at the second parity: NBA, as the number of alive kits at parity; NBD, as the number of dead kits; TNB, as the sum of NBA and NBD; OR, calculated as the number of corpora lutea; IE, calculated as the number of implantation sites by laparoscopy at day 12 of the gestation; ES, computed as a ratio IE/OR; FS, as a ratio TNB/IE; and PS, as a ratio TNB/OR (Mocé et al., 2005; Santacreu et al., 2005).

Genotypes and quality control

Genomic DNA was isolated from blood using Favorgen Kit (FABGK 001-2; Favorgen Biotech Corp., Taiwan). We collected 189 samples with a minimum concentration of 20 ηg/μl and minimum volume of 45 μl. The concentrations were estimated with Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and borne out with PicoGreen (Invitrogen Corp. Carlsbad, C.A.). The threshold values for the integrity of DNA were 1.8 OD_{260}/OD_{280} and 1.5 OD_{260}/OD_{320}. The genotyping was performed in The National Genotyping Centre of “Universidad de Santiago de Compostela”.

Does were genotyped using the Affymetrix Axiom OrcunSNP Array (Affymetrix, Inc. Santa Clara, CA, USA) (Blasco & Pena, 2018). The SNP array contains 199,692 molecular markers. Quality control (QC) and genotype calling from raw data in the form of CEL files were implemented with Axiom Analysis Suite v. 4.0 and reanalysed by ZANARDI (Marras et al., 2015). The SNP quality control was performed using the following criteria: call rate ≥ 0.95, P-value > $1.0E^{-7}$ for the χ^2 test for Hardy Weinberg equilibrium, MAF ≥ 0.03 and only SNPs with known chromosome position. Animal samples were excluded from the dataset for values of dish quality control (DQC) < 0.89, missing genotype frequency > 0.03, Plate QC ≤ 0.96 or for failing a Mendelian segregation test. Missing genotypes were imputed by BEAGLE v4.1. SNPs with

imputation quality score $R^2 > 0.75$ were included (Browning & Browning, 2009). After quality control, genotyping data for association analysis consisted of 181 samples and 117,791 SNPs.

Statistical analysis

Preceding to GWAS, we carried out a classical multidimensional scaling plot (Borg & Groenen, 2005) to find putative outliers or the presence of population stratification. The associations between SNPs and phenotypic traits were obtained using Bayes B Method. Briefly, this method computes all SNPs effects jointly and assumed for each marker a different genomic variance (Garrick & Fernando, 2013; Lehermeier et al., 2013). The following statistical model was used for the GWAS analysis:

$$\boldsymbol{y} = \boldsymbol{\mu} + \boldsymbol{X}\,\boldsymbol{b} + \sum_{j=1}^{k} \boldsymbol{z_j}\,\alpha_j\,\delta_j + \boldsymbol{e}$$

in which $\boldsymbol{y}$ is the vector of the phenotypic values; $\boldsymbol{\mu}$ is the trait mean, $\boldsymbol{X}$ is the incidence matrix for systematic effects; $\boldsymbol{b}$ is the vector with the systematic effects of year-season (five levels), line (high UC, low UC or control) and physiological state (lactating or non-lactating does); k is the total number SNP after quality control; $\boldsymbol{z_j}$ is the vector including the genotypic covariate for each SNP or locus j (0, 1 or 2 reference alleles); α_j is the random allele substitution effect for SNPj, which conditional on σ_α^2 is assumed normally distributed $\mathrm{N}(0, \boldsymbol{I} \cdot \sigma_\alpha^2)$; δ_j is the random 0/1 variable that represents the presence (δ_j = 1, with probability 1-π) and the absence (δ_j = 0, with probability π) of the SNP in the model for a given iteration of the Markov chain; and

$\boldsymbol{e}$ is the vector of the residual values with a normal distribution $\mathrm{N}(0, \boldsymbol{I} \cdot \sigma_e^2)$ (Cesar et al., 2014; Onteru et al., 2012). The genomic variance for every SNP was denoted as σ_α^2 and the residual variance as σ_e^2. In Bayesian approaches, variance parameters can be treated as unknown, but having assumed prior distributions (Garrick & Fernando, 2013). In our study, we assigned the prior genomic variance of the SNPs derived from the estimated total genetic variance (Lehermeier et al., 2013). The prior variances for each trait were retrieved from previous experiments (Blasco et al., 2005; García & Baselga, 2002; Ragab, Sánchez, Mínguez, Vicente, & Baselga, 2014) and are displayed in Table 1. A model including line effect can cause a reduction of the statistical power of the divergent selection experiment. The line effect can mistakenly capture markers effects with opposite frequencies between lines. Hence, GWAS analyses were repeated using a model without line effect.

The π value defines the proportion of SNPs having zero effects in each iteration. We performed several analyses before defining this parameter. The π values were evaluated within range of 0.99 to 0.9995. The π values were very high according to the limited number of animals in this study (Ros-Freixedes et al., 2016). In addition, we also performed GWAS at chromosome level with π = 0.95 in order to corroborate the results consistency.

The parameters of the model were estimated with marginal posterior distributions using Markov chain Monte Carlo (MCMC). After some exploratory analyses, a total of 825,000 iterations were performed, with a burn-in period of 225,000 iterations. Only one sample every 60 iterations was saved to avoid the high correlation between

consecutive samples. The GenSel® v. 4.90 software (Garrick & Fernando, 2013) was used for the GWAS analysis.

In our study, 2,171 genomic windows were allocated to the 21 autosomes and the chromosome “X”, containing around 54 SNP markers by each one. Genomic windows were defined for each chromosome according to the rabbit genetic map of OryCun2.0 assembly, and the percentage of the genomic variance explained for non-overlapping genomic windows of one megabase was calculated by marginal posterior density (Cesar et al., 2014; Garrick & Fernando, 2013; Onteru et al., 2012). The genomic windows that explained at least 0.5% of the genomic variance of each trait and with a probability being higher than zero at chromosome level of at least 0.70 were considered to be putative QTL. This threshold of 0.5% was 10 times higher than the expected percentage of genomic variance explained for one window (Cesar et al., 2014; Onteru et al., 2013). In addition, we considered relevant those SNPs markers that overcome at least a Bayes factor of 10, a value commonly considered as evidence of association (Kass & Raftery, 1995; Ros-Freixedes et al., 2016; Stephens & Balding, 2009). The posterior probability of association (PPA) suggested was not used as criterion of association since the low number of records with a high number of SNPs leads always low PPA values, even for real associations (Stephens & Balding, 2009: supplementary information). Hence, additional information such as the results consistency for different models and priors was used to identified the genomic regions associated to the traits.

Linkage disequilibrium, pathways and functional enrichment analysis

The analysis of LD was performed in order to assess its pattern within the consecutive associated windows. The aim of this analysis was to provide support for the association evidence. Hence, those windows with a great span of LD ($r^2 > 0.5$) and with SNPs associated within this LD block were considered as a true association with the trait. We assumed that these SNPs are a tag of the same causal variant. In addition, the LD analysis was performed within line, in order to understand the selection process. The R LDheatmap package was used for this analysis (Shin, Blay, Graham, & McNeney, 2006).

The position of the candidate genes was determined for each QTL using UCSC Rabbit Genome Browser (Rosenbloom et al., 2015). The gene annotations were provided by Ensembl Genes 94 database using Biomart Software (Aken et al., 2016) and "GenerCards" (Stelzer et al., 2016). Moreover, the functional enrichment analyses were performed by Gene Ontology (GO) (Ashburner et al., 2000) and "Database for Annotation, Visualization and Integrated Discovery" (DAVID) v 6.8 (Jiao et al., 2012).

Results and Discussion

Descriptive statistics of phenotypic data

Descriptive statistics for litter size traits of the rabbit lines of UC divergent selection experiment are shown in Table 2. The mean and standard deviation across lines for litter size traits were similar to other rabbit lines (Elmaghraby & Elkholya, 2010; Piles, García, Rafel, Ramon, & Baselga, 2006; Ragab et al., 2014). Apart from OR, there were phenotypic differences between lines in all the traits. The most noticeable differences were for TNB with mean (standard deviation) of 10.11 (2.71), 7.01 (3.08),

and 9.57 (2.82) kits for the high UC line, the low UC line and the control population, respectively; and for IE with 13.08 (2.65), 10.96 (3.04), and 12.07 (2.88) embryos; and for PS with 0.69 (0.17), 0.51 (0.21), and 0.65 (0.21), respectively.

Description of genomic data

A total of 181 rabbits from the two UC lines and for a control line were genotyped with the Affymetrix Axiom OrcunSNP Array, which interrogates 199,692 SNPs. The criteria to exclude SNPs for the GWAS analysis were: minor allele frequency smaller than 0.03 (16.37%), unmapped SNPs (15.82%), mono-high resolution (8.65%), and call rate smaller than 0.95 (8.05%). After filtering, only 59% of SNPs in the array remained. This number was appropriate, taking into account the small phenotypic data size and the selection process performed before the UC experiment (Blasco et al., 1994). Besides, the rabbit lines from “Universitat Politècnica de València” were not considered to design the actual SNP-array. Thus, an important number of SNPs (17,282) was fixed in the experimental UC lines. The average distance between SNPs was 18.90 kb along the genome leading to a LD average around 0.79 for 100 kb, and 0.76 when all genomic data in consecutive pairs SNPs were used. This value seems to be high considering that an average distance of 98 kb showed a LD of 0.5, calculated within rabbit strains (Carneiro et al., 2011).

The multidimensional scaling analysis using genomic data found an evident population stratification (Figure 1). This analysis identified three clusters corresponding to the high UC line, the low UC line, and the control population, respectively. The first two principal components jointly explained 23.6% of the total variance. This would indicate that SNPs captured the population stratification of this experiment. Bayesian multi-marker

regression models are quite robust to population stratification (Toosi et al., 2018). Although the inclusion of line effect reduced the power obtained by the divergent selection, we included the line effect in order to avoid the possible drift effect and check the consistency of the results. We are aware that this type of correction is very stringent. So, we also performed the analysis without line. The variance explained for the main associated region increased considerably (Table 3). However, the conclusions our findings did not change. The regions identified as associated were identical and with the similar order of importance which showed results consistency with and without line effect.

Prior choice

The exploratory analysis of the π value under the model without line effect showed similar results across π values, being not sensitive to them. By contrast, the model with line effect showed a greater increase of shrinkage led to a lower number of windows overcoming the relevant threshold. Additionally, the percentage of the genomic variance explained by these associated windows was greater when the π value was greater. For instance: using a π value of 0.9995 the analysis reported four consecutive genomic windows associated with TNB that explained 16.3% of the genomic variance, whilst using 0.9992, 0.9975, 0.995, and 0.99, these explained 7.4%, 2.8%, 1.4% and 0.6%, respectively. However, the ranking of the relevant genomic windows did not change. Therefore, the π value used in this study was 0.9992 based on the average number of SNPs in the model per iteration (119) and the total number of samples (181).

Genomic windows associated with litter size traits

The GWAS analyses showed associated genomic windows for TNB, NBA, IE, and OR. No associations were evidenced for NBD, ES, FS, and PS.

Total number born and number born alive

The genomic windows associated with TNB are located on chromosome 17 (windows 1903, 1904, 1905 and 1906) (Figure 2). Two of them (1905 and 1906) also showed association with NBA (Figure 3). The genomic variance explained by these two windows was 6.32% for TNB and 1.27% for NBA (Table 3). This result would be in agreement with the high genetic correlation found between NBA and TNB (0.964 +/- 0.008) (García & Baselga, 2002).

The associated genomic region (70.0 - 73.3 Mb) seems to have a major effect on TNB in the UC lines. This could make sense since half of response of selection was obtained in the first two generations of UC divergent selection (Blasco et al., 2005). This region accounted for up to 38.82% and 10.36 % of the genomic variance for TNB and NBA, respectively, under a model excluding the line effect. In addition, the genomic variance explained by all these genomic windows had a probability of being greater than zero at chromosome level of at least 0.95, except the 1906 being greater than 0.75.

Other genomic regions with a smaller effect size than the region associated on chromosome 17 could not have been identified due to the small sample size. In swine, GWAS analyses for TNB and NBA have reported QTLs in several chromosomes. However, the sample size in these studies was greater (>600), and in both studies,

third terminal crossbred lines were used (Onteru et al., 2012; Schneider et al., 2012), generating a much higher LD in their population than in our lines.

Implanted embryos

A large relevant genomic region for IE was found on chromosome 11 (Figure 4). This region involved five associated genomic windows (35.2 – 39.0 Mb), from window 1143 to 1147, accounting for 10% of the genomic variance of IE (Table 3). Besides, the same genomic region on chromosome 17 associated with TNB and NBA explained 5.37% (32.23 % without line) of the genomic variance of IE. Therefore, this region could have a pleiotropic effect on these three litter size traits (TNB, NBA, and IE). These results could be related to the correlated response to selection for IE, shown in the UC divergent selection experiment (Blasco et al., 2005; Santacreu et al., 2005) which is in agreement with the moderate to high genetic correlation between IE and UC (0.66) (Blasco et al., 2005) and IE and TNB (0.46) (Laborda et al., 2012).

Ovulation rate

The results did not show a strong genomic association for this trait due to the low amount of genomic variance explained by each associated window. Moreover, none of the windows were consecutive. Two genomic windows on chromosome 9, window 996 and 993, only explained 1.13% (0.84 % without line) and 1.03% (0.94 % without line) of the genomic variance, respectively (Table 3). Overall, all genomic windows associated with OR accounted for 3.95% (with and without line) of the genomic variance. This result is in contrast to a swine GWAS that found three relevant genomic regions associated with OR explaining 51% of the genomic variance (Schneider, Nonneman, Wiedmann, Vallet, & Rohrer, 2014). The sample size of their study was

considerably greater than in our study, and the swine population had much higher LD and genomic variability. Moreover, in our study animals came from a divergent selection experiment for UC, whose trait had a moderate (0.56) genetic correlation with OR (Blasco et al., 2005). Additionally, the genomic windows associated with OR did not agree with the associated genomic region found for three litter size traits - TNB, NBA, and IE (Figure 5). These results are in concordance with the null correlated response in litter size for OR selection in rabbits and the low genetic correlation estimated between OR and litter size (Laborda, Mocé, Santacreu, & Blasco, 2011).

Associated SNPs in genomic regions

The Bayes factor criteria showed only relevant SNP associations for IE and TNB. These associated SNPs map to chromosome 11 for IE (Figure 6), and chromosome 17 for TNB and IE (Figure 6 and 7). The highest Bayes factor was for a SNP on chromosome 17, associated with TNB. The total number of SNPs between the two traits in chromosome 17 was 14 (five in the window 1905 and nine in the window 1906) (Table 4). This corroborated the remarkable importance of this genomic region on chromosome 17 as a putative QTL. However, the PPAs of SNPs within the putative QTL were low (0.04 to 0.15), which is expected with the small sample size used in our study. Stephens & Balding (2009) pointed out that PPAs have the advantage of being not very sensitive on sample size, power and number of tested SNPs. Despite that, they showed that small sample size can give low PPAs with real associations even under several Bayesian approaches based on different priors (supplementary material). In our study, the putative QTL on chromosome 17 was consistent across the analyses of GWAS, under window and SNP association criteria, allele frequencies and linkage disequilibrium analyses (as shown below). All associated SNPs had an overall

MAF above 0.28. Moreover, the associated SNPs for both TNB and IE showed an even higher value of MAF (from 0.33 to 0.49). The allele frequencies in the control population for these associated SNPs were intermediate (0.43 - 0.45), whilst they were higher for the low UC line (0.64 and 0.75) and very low (0.05) for the high UC line. We assumed that all of these SNPs were associated with the traits (TNB and IE) due to strong LD with their causal variants since selection could have modified the allelic frequency of the SNPs associated with the causal variants. In this case, the joint analysis of the divergent selection would have led to intermediate frequencies, increasing the SNP detection power (Kessner & Novembre, 2015; López de Maturana et al., 2014). Thus, our experiment has been valuable for revealing novel QTLs associated with litter size traits in rabbits.

Linkage disequilibrium analysis

We assessed the LD in the consecutive associated genomic windows on chromosome 11 and 17. The genomic regions associated with IE (chromosome 11) showed a strong LD block amongst the windows 1145, 1146 and 1147. This block was more evident in the low UC line. This suggests that this QTL could have been under higher selection pressure for low UC than for high UC, in agreement with the asymmetric response estimated using the UC lines and the cryopreserved control population (Mocé et al., 2005). This latter study showed the selection response was higher in the low UC line (Mocé et al., 2005; Santacreu et al., 2005). The SNPs that overreached the threshold for IE are indicated with black points in Figure 8. Most of them are mapped in the LD block made up by the three windows (1145, 1146 and 1147). This result is in contrast to the genomic region associated with TNB, NBA, and IE on chromosome 17 displaying several short LD blocks. Most of the associated SNPs within this QTL were in the

window 1906 (Figure 9). This window presents a steady LD block within the control population ($r^2 > 0.8$). This would indicate that the UC selection formed new LD blocks from a large one in the control population.

In our study, both LD and GWAS results support the idea that QTL on chromosome 17 had a major impact on the divergent selection experiment. This hypothesis of an important QTL for litter size in the UC lines is supported by the great response at the second generation, half of the estimated response in this divergent selection experiment, as we said previously (Argente et al., 2003; Blasco et al., 2005).

Gene search and functional annotations

The associated genomic regions disclose 72 coding and noncoding genes (additional file 1: Table S1); nine of them located on the genomic region associated with TNB, NBA and IE (chromosome 17) (Table 3). The top five results of the functional annotation analysis, using the genes in putative QTLs, are shown in Table 5. The human, mice and rabbit functional annotations from DAVID databases gave similar results. Therefore, we described these results using the annotated rabbit genes to subsequently perform a detailed functional seeking for each gene. The most relevant functions were linked to terms such as activity prostanoid receptors, cellular response to prostaglandin, negative regulation of striated muscle tissue development, carbohydrate derivative binding, and cyclin-dependent protein kinase activity. The genes related to reproductive processes and associated with TNB were *PTGDR, PTGER2, BMP4, STYX,* and *CDKN3*. The *PTGDR* and *PTGER2* belong to the prostaglandins receptor family which are essential for the adequate performance of uterus; mainly prostaglandin F receptor that presents underlying functions over the

female reproductive cycle in mammals (Blesson & Sahlin, 2014). Also, a severe deficiency in the *PTGER2* genetic expression decreases fertilization and generates defects in cumulus expansion (Matzuk & Lamb, 2002). Otherwise, *PTGDR* gene presents an important role in the differentiation of germ and Sertoli cells of the embryonic testis in males (Rossitto, Ujjan, Poulat, & Boizet-Bonhoure, 2014). Genes of the transforming growth factor-β superfamily, including *BMP4*, are involved in follicular growth and development in mammals (Al-Samerria, Al-Ali, McFarlane, & Almahbobi, 2015) avoiding the apoptosis of oocytes through regulation of both *Sohlh2 and c-ki* (Ding, Zhang, Mu, Li, & Hao, 2013). Nevertheless, the *BMP4* gene showed no association with OR, but it was associated with TNB and IE in our study. *BMP4* has been also implicated in trophoblast development, implantation, and placentation in humans (Li & Parast, 2014). CDKN3 gene is related to inhibition and reduction of choline, particularly in the neural progenitor cells of the fetal hippocampus, producing cellular apoptosis (Zeisel, 2011). Moreover, the reduction of *STYX* expression disrupts spermatid development (Matzuk & Lamb, 2002). The 1903 window on chromosome 17, associated only with TNB, contains the *ERO1A* gene. This gene did not show a functional annotation directly related to reproductive processes but was identified as overexpressed between the UC lines in a previous study (Ballester et al., 2013). Moreover, it is the precursor of the *ER1L* transcript, which is related to redox homeostasis and oxidative protein folding in the endoplasmic reticulum (Konno et al., 2015).

Regarding genes associated with IE, *BMP4* and *CDKN3* genes (chromosome 17) are annotated to embryo development processes in mice (Goggolidou et al., 2013). In chromosome 11, we found the *CCT5* gene related to sperm quality in bulls (Yathish

et al., 2017). Finally, the genes annotated for OR did not have a direct relationship with this trait or the female reproductive physiology.

Previous candidate gene studies, using the UC divergent lines, showed genes associated with reproductive traits such as progesterone receptor (*PGR*) associated with IE (Peiró et al., 2008), tissue inhibitor of metalloproteinases 1 (*TIMP1*) associated with number of embryos (Argente et al., 2010) and oviduct glycoprotein 1 (*OVGP1*) associated with TNB (Merchán et al., 2009). However, our study did not identify associated genomic regions close to these genes.

In general, the candidate genes found in our study are different from those identified in GWAS for OR, TNB and NBA in swine (Bergfelder-Drüing et al., 2015; Onteru et al., 2012; Schneider et al., 2014). The main associations in these studies did not overlap amongst litter size traits. However, Schneider *et al.* (2012) found overlapping genomic windows for TNB, NBA, NBD and average piglet birth weight in swine; similar to the novel putative QTL found on chromosome 17.

Conclusions

Our study reveals associations between genomic regions and TNB, NBA, IE, OR. Two consecutive genomic windows on chromosome 17 were associated with three traits (TNB, NBA, and IE), and accounted for a meaningful percentage of the genomic variance for TNB, indicating that this genomic region could contain remarkable causal variants for litter size traits in rabbits. In addition, a genomic region on chromosome 11 appears particularly important for IE. The associated genomic regions harboured 72 genes. However, few of these genes were profiled as physiological candidate genes

due to their link to reproductive processes (i.e., *BMP4, PTDGR, PTGER2, STYX, and CDKN3)*. In summary, our results disclosed new putative QTLs for TNB and IE, likely responsible for the large divergent response obtained in the first two generations of selection. However, these results must be validated in independent maternal rabbit lines before being used in breeding programs. This study is the first GWAS for reproductive traits in rabbits and provides a starting point to disentangle the genetic basis of litter size traits in rabbits.

Last Section of Main Text

Abbreviations

ES: Embryo survival; FS: Foetal survival; GO: Gene ontology; GV: genomic variance; GWAS: Genome-wide association study; IE: Implanted embryos; LD: Linkage disequilibrium; MAF: Minor allele frequency; NBA: Number born alive; NBD: Number born dead; OR: Ovulation rate; PPA: the posterior probability of association; PS: Prenatal survival; QC: Quality control; QTL: Quantitative trait loci; SNP: Single nucleotide polymorphism; TNB: Total number born; UC: Uterine capacity, ULO: unilaterally ovariectomized.

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Competing interest

The authors declare that they have no competing interests.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author's contributions

NIE, AB, MAS, and LF conceived and designed the experimental procedures and supervised the study. MAS and AB secured substantial funding. MAS performed the phenotypic data recording and collected blood samples. BSS, and NIE collated, edited and performed the genomic analysis. BSS performed the pathways and enrichment analyses. NIE, MAS, RNP, and AB helped with the interpretation of the results. BSS typed the manuscript. All other co-authors provided manuscript editing and feedback. All authors read and approved the final manuscript.

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Tables

Table 1. Prior variances for Bayes B method.

Table 2. Descriptive statistics of little size traits.

Table 3. Genomic windows associated with total number born (TNB), number born alive (NBA), implanted embryos (IE), and ovulation rate (OR) in rabbits.

Table 4. Relevant polymorphisms (SNPs) for total number born (TNB) and implanted embryos (IE).

Table 5. Top five functional enrichment from the analyses performed through DAVID online web.

Figures

Figure 1. Multidimensional scaling plot of the genomic data. The first component (MDS1) explained 16.73% of the genomic variance and the second component (MDS2) explained 6.90% of the genomic variance. Populations: high uterine capacity line (HUC), low uterine capacity line (LUC) and control population or line selected for number of kits at weaning (V).

Figure 2. Manhattan plot for total number born (TNB) using the percentage of genomic variance explained by each non-overlapping one megabase window.

Figure 3. Manhattan plot for number born alive (NBA) using the percentage of genomic variance explained by each non-overlapping one megabase window.

Figure 4. Manhattan plot for implanted embryos (IE) using the percentage of genomic variance explained by each non-overlapping one megabase window.

Figure 5. Manhattan plot for ovulation rate (OR) using the percentage of genomic variance explained by each non- overlapping one megabase window.

Figure 6. Manhattan plot for implanted embryos (IE) using the Bayes factors by each SNP along the rabbit chromosomes.

Figure 7. Manhattan plot for total number born (TNB) using the Bayes factors by each SNP along the rabbit chromosomes.

Figure 8. Linkage disequilibrium plot of chromosome 11 (35.2 – 40.0 Mb). Physical length is 4756 kb and contains a total of 353 SNPs. The black triangle stands for each one of five associated windows for implanted embryos. The black points are the 38 associated SNP. The colour red is the R-squared from 0.8 to 1.0 (strong LD). The computation was performed using data from (a) all lines, (b) HUC (high UC line), (c) LUC (low UC line) and (d) control population.

Figure 9. Linkage disequilibrium plot of chromosome 17 (72.0 – 73.2 Mb). Physical length is 1278 kb and contains a total of 82 SNPs. The black triangle stands for each one of two associated windows for total number born, number born alive and implanted embryos. The black points are the 14 associated SNP for total number born and implanted embryos. The colour red is the R-squared from 0.8 to 1.0 (strong LD). The computation was performed using data from (a) all lines, (b) HUC (high UC line), (c) LUC (low UC line) and (d) control population.

Supporting Information

View in:

Additional file 1: Table S1. Annotated genes in the genomic regions associated with litter size traits.