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Follicle-stimulating hormone (*FSHβ*) gene polymorphisms and associations with reproductive traits in Rex rabbits

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ABSTRACT

Follicle-stimulating hormone (FSH) stimulates granulosa cell proliferation and controls the development and maturation of oocytes. In this study with Rex rabbit, there was exploration of the relationships between reproductive traits and SNPs and haplotypes of *FSHβ*, and tested whether FSHβ SNPs would segregate between two lines, using 70 females from the White line and 100 females from the Beaver line. Three SNPs (FSH2, FSH30, FSH31) in exon1 and exon3 of FSHβ were strongly associated with reproductive traits in the combined population. For FSH2, the GG variant was associated with greater ($P < 0.05$) values for total number born (TNB) and number born alive (NBA), compared to those with the TT variant. For FSH30, for the AA variant there was greater ($P < 0.05$) values for TNB and NBA than with the GG and AG variants. For FSH31, the GG variant was associated with greater ($P < 0.05$) values for TNB, litter weight at birth (LWB) and litter size at 21 days of age (LS21), than the AG variant, and with greater ($P < 0.01$) values for NBA than the AG variant. When analyzed separately, FSH2 SNP was associated with LW21 in the Beaver line ($P < 0.05$), whereas FSH30 and FSH31 SNPs were associated with TNB and NBA in the White line ($P < 0.05$). It is concluded that genetic variation in *FSHβ* gene is associated with reproductive traits in the Rex rabbit, therefore, the FSH2, FSH30 and FSH31 SNPs can be used as molecular markers in genetic selection of rabbits.

1. Introduction

The modern European rabbit (*Oryctolagus cuniculus*) was developed from the European wild rabbit (also *O. cuniculus*) on the Iberian Peninsula (Hardy et al., 1995; Fuller et al., 1997) and is one of the most recently domesticated small animal species. The earliest selective breeding has been attributed to French Catholic monks who used rabbits as a food source and there are more than 200 breeds and strains used for a wide variety of purposes (Alves et al., 2015). The Rex genotype originated from a litter of wild grey rabbits in France in 1919, and has since been selected for fur production (Ruben et al., 2014). It was first shown publicly at the Paris International Rabbit Show in 1924 (Ruben et al., 2014) and first imported into China about 30 years ago. Its fur is short, fine, dense, smooth and thus considered attractive.

As with any animal used in production, there is a desire to improve prolificacy of the Rex rabbit in China. A major determinant of prolificacy is follicle-stimulating hormone (FSH), a glycoprotein gonadotropin secreted by basophilic pituitary cells. It is composed of

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an α sub-unit that is highly conserved, even among glycoprotein hormones with different functions, and a β sub-unit that determines the specificity of the action of the hormone (Cooke et al., 1996). The FSHβ sub-unit comprises 111 amino acids, is relatively highly conserved among mammals (Hirai et al., 1990), and is a major factor in regulation of ovarian functions. In the follicle, for example, there is FSH stimulation of granulosa cell proliferation and the secretion of antral fluid from these cells. Furthermore, FSH can stimulate production of receptors for luteinizing hormone (LH) and prolactin (PRL). In addition, FSH stimulates the synthesis and release of estradiol and inhibin, and is involved in the control of development and maturation of oocytes (Cooke et al., 1996). These important functions in ovarian folliculogenesis explain the effects of FSH in the determination of ovulation rate and, therefore, litter size (review: Scaramuzzi et al., 2011).

Even though the FSH gene is highly conserved, there is some variation in the structure of FSHβ. In swine, polymorphisms have been reported to be associated with litter size, suggesting the possible use as molecular markers to improve reproductive performance (Li et al., 1998). In goats, Zhang et al. (2011) also detected polymorphisms in FSHβ and there were indications from the results that polymorphisms in FSHβ affected litter size (LS) and litter weight at birth (LWB).

In rabbits, by contrast, there are very few major genes that are associated with litter size. There appears to be a relationship between the κ-casein gene and reproductive traits (Bolet et al., 2007), and a mutation in the promoter region of the progesterone receptor gene is associated with embryonic traits in lines selected for differences in uterine capacity (Peiró et al., 2008). For FSHβ, the full-length cDNA has been cloned (Sang et al., 2015; Yang et al., 2010) but there has been only one study of the association between FSHβ polymorphisms and litter size (Chen et al., 2009) and no other reproductive traits have yet been studied. The lack of information is problematical because, as with other species, litter size is one of the most important measures of rabbit productivity. There, however, does appear to be opportunities for genetic improvement through selection for litter size in rabbits. For example, Mazouzi-Hadid et al. (2014) reported that colored females give birth to 0.67 more kits per litter than White females, although the opposite has been reported by Zhao et al. (2017) where it was observed that there were 1.37 more kits per litter in a White line than in a colored (Beaver) line. The sample size (30 dams) used by Zhao et al. (2017) was perhaps too small, but the inconsistency in findings emphasizes the need for more research into genetic variation between lines and into the role of variation in the structure of FSHβ.

To investigate the relationships between reproductive traits and FSHβ polymorphisms, the most desirable approach is direct sequencing, a technique considered as the 'gold standard' for genotyping analysis because there is an expectation that there be about 100% sensitivity with use of this technique (Laurie and George, 2009). A desirable refinement of this approach, 'Snapshot' analysis, offers efficiency by improving the throughput for multiple SNPs (Zhang et al., 2014). With the use of 'Snapshot', there can be rapid detection of large numbers of SNPs at the same time with high accuracy at a much lesser cost than with use of more traditional techniques and it is widely used in analysis of human disease (Ye and Huang, 2009).

In the present study, therefore, the 'Snapshot' analysis was used to test whether SNPs and haplotypes in the rabbit FSHβ gene are associated with reproductive traits in the Rex rabbit genotype. There was also assessment of whether the FSHβ SNPs would segregate between the Beaver and White lines because the presence of different FSHβ SNPs are thought to be associated with differences in litter size and other reproductive traits.

2. Materials and methods

2.1. Animal materials and data collection

There was use of two lines of Rex rabbits, the White and the Beaver, because of the similarities between these two lines except for coat color. These two lines were originally imported to China from France in 2009 and have since been continuously selected for three generations. Healthy, mature, purebred, 150-day-old, primiparous and second-parity females (70 White and 100 Beaver) were obtained from the Experimental Rabbit Farm at Shanxi Academy of Agricultural Sciences, Taiyuan, Shanxi, middle China. Litter size and litter weight at both birth and 21 days of age had been used as selection criteria, with a 50% culling rate. The does were placed in cages (each 0.025 m^2) arranged in two rows, with three housing levels in each row, in a semi-open rabbit house. The does were fed pellets (˜16%, crude protein; ˜ 17% crude fiber; 8.3 MJ/Kg digestible energy) by the same technician twice a day, at 08.00 and 17.00. Water was provided *ad libitum*. Litters were raised with their mothers until weaning.

Does were first mated at 20 weeks of age. Non-pregnant does were detected by abdominal palpation and mated for a second time 10 days later. Mating of full-siblings was avoided to reduce inbreeding. Litters were weaned at 35 days of age. Measures of reproductive performance were total number of kits born (TNB), number of kits born alive (NBA), litter weight at birth (LWB), litter size at 21 days of age (LS21), litter weight at 21 days of age (LW21) and litter weight at weaning (LWW).

2.2. Isolation of genomic DNA

At weaning, ear tissue was sampled from all does and preserved at –20 ℃ in 1.5 mL polypropylene tubes containing 70% ethanol. Genomic DNA was isolated using the MiniBEST Unibest DNA extraction kit (Takara Corp. Dalian) and then stored at –20 ℃ until it was used for polymerase chain reaction (PCR) amplification.

2.3. PCR amplification and sequencing

Primers for specific amplification of the complete three exons of rabbit FSHβ were designed using Oligo 6.0 software based on the

published sequence from NCBI (Noguchi et al., 2006, GenBank No.: 5128). The three primer sets were synthesized from the Shanghai Sangon Biological Engineering Technology and Services Co. Ltd., China:

Exon 1: Forward: 5′ -AGACAAGGCAGCCAACCAC-3′,
Reverse: 5′-TCAGAAGCTAGGGAAATTGGTC-3′;
Exon 2: Forward: 5′-GCCAGGATGAAGTCTGTCCA-3′,
Reverse: 5′-GTAGCAGTAGCCGGAACACC-3′;
Exon 3: Forward: 5′-GCTGTCACCATTCTGTCCTG-3′,
Reverse: 5′-TTCAGTGTGTTCCCATCCTCT-3′.

The PCR procedure was performed in a final volume of 20.0 μL containing 50–100 ng DNA templates, 0.3 μM of each primer, 4.0 μmol dNTP, 1.0 U Hs Taq polymerase (Takara, Dalian, China), and 2.0 μL 10 × reaction buffer (adding Mg^{2+}). The PCR cycling conditions were: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 58–60 ℃ for 30 s, and extension at 72 ℃ for 30 s, with final extension at 72 ℃ for 10 min; samples were then stored at 4 ℃ for 10 min. Amplicons were visualized by electrophoresis in 2.0% agarose gels containing 500 ng/mL of ethidium bromide in 1 × TBE buffer with a 100-bp ladder (Takara, Dalian, China) as the molecular weight marker for confirmation of the length of the PCR products. In preparation for sequencing, PCR products were purified with an agarose gel DNA purification kit (Invitrogen, USA). The PCR products of at least ten rabbits of each of the White and Beaver lines were selected and sequenced at the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China. Nucleotide sequence alignments, translations, and comparisons were conducted using DNAMAN (version 5.2.10).

2.4. Genotyping using 'Snapshot'

In all, three target mutations containing SNPs of g. 284 G > T in exon1, g. 2908 G > A and g. 2963 G > A in exon3 were selected for genotyping. There was then genotyping with the 'Snapshot' technique using an ABI 3730 sequencer. The total reaction volume was 10.0 μL, containing 100–200 ng template DNA, 1.0 μmol of each primer, 0.5 U Taq polymerase (Kapa Biosystems Corp., US), and 2.0 μL 2.5 × Buffer IV. The amplification products were digested, prolonged, purified, and then placed in the ABI3730XL sequencer for detecting SNPs. The PCR reaction conditions were: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 30 s, with final extension at 72 ℃ for 10 min; samples were then stored at 4 ℃ for 10 min. The genotyping ratio was 98%, so almost all the SNP sites were successfully genotyped. A BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search was then performed in NCBI to certify the positions of the three SNPs on the rabbit genome (https://www.ncbi.nlm.nih.gov/genome/?term = Rabbit).

2.5. Haplotype analysis

A haplotype was constructed with the three mutations based on a LD (linkage disequilibrium) analysis using Haploview version 4.2 (Barrett et al., 2005).

2.6. Statistical analysis

The allele and genotype frequencies were computed for both lines of rabbits. A χ^2 analysis was used to test for deviation of genotype from Hardy Weinberg Equilibrium. Associations of the SNP genotypes and haplotypes with reproductive traits were analyzed using the GLM procedures in SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The model fitted for genotype association was:

$$y_{ijkl} = \mu + L_i + P_j + G_k + e_{ijkl}$$

where y_{ijkl} is the phenotypes of reproductive traits; μ is the overall mean; L_i is the i^{th} line effect (i = 1, 2, Beaver: 1, White: 2); P_j is the j^{th} parity effect (j = 1, 2, primiparity: 1, multiparity: 2); G_k is the k^{th} genotype effect (k = 1, 2, 3); and e_{ijkl} is the residual. For haplotype association, G_k was replaced by H_k (k = 1, 2…7). Both of the results were presented as $\bar{X} + S_{\bar{X}}$. Before the association analysis, animals with values for TNB smaller than two and greater than ten, and without genotypes, were deleted. There was then assessment of the association between SNP genotypes and reproductive traits in the Beaver and White lines. The GLM procedures in SPSS 19.0 were used and the model fitted was:

$$y_{ijk} = \mu + P_i + G_j + e_{ijk}$$

where y_{ijk} is the phenotype of the reproductive trait; μ is the overall mean; P_i is the i^{th} parity effect (i = 1, 2, primiparity: 1, multiparity: 2); G_k is the k^{th} genotype effect (k = 1, 2, 3); and e_{ijk} is the residual.

Each record for a litter was from a different rabbit. Results from a preliminary correlation analysis indicated litter size was not related with litter weight at 21 days (γ = 0.171, P = 0.075), so a covariate was not included in the models.

The D' and r^2 values among the three SNPs were computed using Haploview 4.2 to represent the extent of linkage disequilibrium (LD) (Barrett et al., 2005). The haplotypes and the frequencies were inferred and computed using PHASE 2.1 (Stephens et al., 2001).

Table 1
Least squares mean ± SEM values for reproductive traits in primiparous and multiparous Rex rabbits in the Beaver (n = 100) and White (n = 70) lines.

Line	Parity	n	TNB	NBA	LWB (g)	LS21	LW21 (g)	LWW (g)
Beaver	Primiparous	65	5.58 ± 0.26	5.48 ± 0.27	328 ± 13	4.96 ± 0.18	1497 ± 53	2933 ± 129
	Multiparous	35	6.00 ± 0.64	6.00 ± 0.64	340 ± 30	5.92 ± 0.42	1578 ± 105	3150 ± 183
White	Primiparous	53	5.77 ± 0.21	5.74 ± 0.22	334 ± 14	5.09 ± 0.20	1398 ± 56	2878 ± 128
	Multiparous	17	5.25 ± 0.61	5.25 ± 0.61	307 ± 33	5.00 ± 0.41	1180 ± 134	3364 ± 426

Within columns, values marked by different letters differ (a, b: $P < 0.05$; A, B: $P < 0.01$).
TNB: total number of kits born; NBA: number of kits born alive; LWB: litter weight at birth; LS21: litter size at 21 days; LW21: litter weight at 21 days; LWW: litter weight at weaning.

3. Results

3.1. Reproductive performance

The data for reproductive performance of the females of the two lines are included in Table 1, with a litter size of no more than seven for new born kits, kits born alive and kits weaned. There were no significant differences between the two lines, or between primi- and multi-parous females in the Beaver line for any trait.

3.2. Allele, genotype and haplotype frequencies of the SNPs

Within the 3770 bp length of FSHβ, comprising three exons and two introns, there was detection of 37 SNPs by direct sequencing and multiple alignments. Nucleotide sequencing data indicated there were eight mutations in exon1 and 16 mutations in exon3, but none in exon2. There were many SNPs in exon1, and two were selected with g.218 G > A and g.284 G > T for genotyping because these two had the largest number of polymorphisms. For the same reason, in exon3, only g.2779 G > C, g.2908 G > A and g.2963 G > A were selected for further genotyping.

Only three SNPs were associated with reproductive traits: g.284 G > T (FSH2) in exon 1; and g.2908 G > A (FSH30) and g.2963 G > A (FSH31) in exon 3. The data for genotypic and allelic frequencies of these three SNPs in the two lines are included in Table 2. The g.284 G > T SNP resulted in the putative amino acid replacement of Ile with Arg, the g.2908 G > A SNP resulted in replacement of Val with Met, and the g.2963 G > A SNP resulted in replacement of Arg with His.

For FSH2, allele T was predominant with frequencies of 0.70 in the Beaver line and 0.54 in the White line. The greatest frequencies were observed for genotype TT (0.47) of the Beaver and genotype GT (0.49) of the White line. For FSH30, the predominant allele was A in the Beaver (frequency 0.51), however, was G in the White (0.57) line. The heterozygote frequencies (AG) were the greatest in both lines (0.48 for Beaver and 0.58 for White). For the FSH31 SNP, allele A was predominant with frequencies of 0.70 in the Beaver and 0.60 in White lines and, again, there was the greatest frequency in the heterozygote (AG) for both lines (0.47 in Beaver and 0.51 in White). Results from use of the Chi-squared analysis indicated the combined population was not in Hardy-Weinberg equilibrium at FSH2 ($\chi^2 = 9.01$, $P < 0.01$) or FSH31 ($\chi^2 = 3.96$, $P < 0.05$), but was in equilibrium at FSH30 ($\chi^2 = 1.59$, $P > 0.05$).

Seven haplotypes were constructed with these three SNPs. Two of these, with frequencies for TGA (0.627) and GAG (0.325) that summed to 0.952, were not associated with any reproductive trait. The other five haplotypes accounted for very low frequencies (less than 0.05).

Table 2
Allelic and genotypic frequencies of the three SNPs in the FSHβ gene in the Beaver and White lines of Rex rabbits; Numbers in parentheses are the sample sizes for the respective genotypes.

Sites	Lines	n	Genotypic frequency			Allelic frequency	
			GG	GT	TT	G	T
FSH2	Beaver	96	0.07 (7)	0.46 (44)	0.47 (45)	0.30 (58)	0.70 (134)
	White	69	0.22 (15)	0.49 (34)	0.29 (20)	0.46 (64)	0.54 (74)
	Total	165	0.13 (22)	0.47 (78)	0.39 (65)	0.37 (122)	0.63 (208)
			AA	AG	GG	A	G
FSH30	Beaver	94	0.05 (5)	0.48 (45)	0.47 (44)	0.51 (95)	0.49 (93)
	White	69	0.14 (10)	0.58 (40)	0.28 (19)	0.43 (60)	0.57 (78)
	Total	163	0.09 (15)	0.52 (85)	0.39 (63)	0.35 (155)	0.65 (171)
			AA	AG	GG	A	G
FSH31	Beaver	96	0.47 (45)	0.47 (45)	0.06 (6)	0.70 (135)	0.30 (57)
	White	67	0.34 (23)	0.51 (34)	0.15 (10)	0.60 (80)	0.40 (54)
	Total	163	0.42 (68)	0.48 (79)	0.02 (16)	0.66 (215)	0.34 (111)

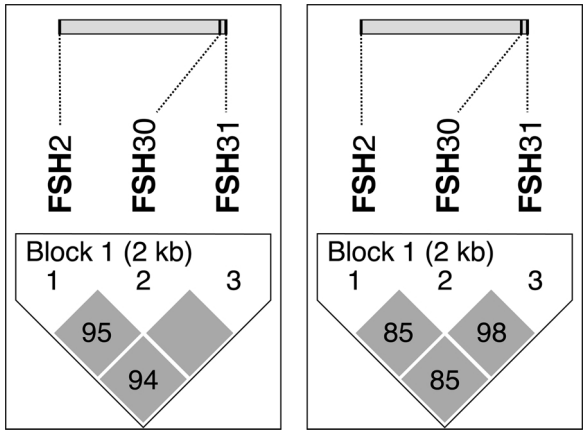

Fig. 1. Linkage disequilibrium among the three SNPs (FSH2, FSH30 and FSH31) identified in the FSHβ gene in Rex rabbits; Shaded diamonds contain the value for D' (A) and r^2 (B) between SNP sites.

The positions of the three significant SNPs in the genome were FSH2-169431333 bp, FSH30-16943958 bp and FSH31-169434013 bp, so these constitute one haplotype block with length of 2680 bp. The results of the LD analysis indicated the D' values were 0.95 between FSH2 and FSH30, and 0.94 between FSH2 and FSH31. Because FSH30 and FSH31 are in close proximity in the genome, these two genes were almost completely linked (Fig. 1). Correspondingly, the r^2 value was 0.85 between FSH2 and FSH30 and also between FSH2 and FSH31, whereas the r^2 value was 0.98 between FSH30 and FSH31 (Fig. 1). These observations indicate that the three SNPs are in strong linkage disequilibrium.

3.3. Associations between FSHβ genotypes and reproductive traits in the combined population

The results of the association analysis indicate all three SNPs had effects on TNB and NBA, but in different ways (Table 3). The g.284 G > T mutation at FSH2 in exon 1 and g.2963 G > A mutation at FSH31 in exon 3 were both associated with reductions in both TNB and NBA; furthermore, rabbits with the AA and TT genotypes of FSH2 and FSH31 had lesser TNB values than rabbits with the GG genotype ($P < 0.05$). By contrast, the g.2908G > A at FSH30 in exon 3 was associated with an increase in litter size, with the AA genotype rabbits having greater values for TNB and NBA than those with the GG genotype ($P < 0.05$). The G > T mutation at FSH2 and G > A at mutation FSH31 were associated with reductions in litter weight, at both birth and 21 days of age, and the G > A mutation at FSH30 was associated with an increase in litter weight. The mutation at FSH31 ($P < 0.01$) was strongly related to a reduction in NBA, and also related to a reduction in LWB ($P < 0.05$). In addition, the SNP at FSH31 was related to litter size at 21 days of age ($P < 0.05$).

In the combined population, does with the GG genotype for FSH2 produced 1.23 kits more than those with the TT genotype. Similarly, in rabbits with the AA genotype for FSH30, the LS value was 1.26 greater than with the GG genotype. For FSH31, does with the GG genotype can be expected to produce 1.18 more kits than those with the AA genotype, and with the GG genotype there was also an increased LWB and LS21.

3.4. Associations between FSHβ haplotypes and reproductive traits

As indicated by data included in Table 3, the GAG haplotype is superior for prolificacy with greater values than the TGA

Table 3
Associations between *FSHβ* genotypes and reproductive traits in the combined population of Rex rabbits (Values are least squares means ± SEM).

Sites	n	Genotype	TNB	NBA	LWB (g)	LW21 (g)	LS21 (g)	LWW (g)
FSH2	19	GG	6.56 ± 0.40^{b}	6.56 ± 0.39^{b}	365 ± 14	1494 ± 148	5.40 ± 0.48	2970 ± 290
	63	GT	5.84 ± 0.20^{ab}	5.70 ± 0.20^{ab}	339 ± 13	1435 ± 60	5.24 ± 0.20	2943 ± 137
	51	TT	5.33 ± 0.26^{a}	5.33 ± 0.26^{a}	320 ± 15	1392 ± 68	4.95 ± 0.22	3025 ± 155
FSH30	53	GG	5.51 ± 0.24^{a}	5.42 ± 0.25^{a}	325 ± 13	1434 ± 68	5.16 ± 0.23^{a}	2935 ± 151
	68	AG	5.68 ± 0.21^{a}	5.51 ± 0.21^{a}	327 ± 12	1437 ± 52	4.90 ± 0.19^{a}	2946 ± 131
	13	AA	6.77 ± 0.39^{b}	6.77 ± 0.39^{b}	371 ± 28	1516 ± 187	5.90 ± 0.46^{a}	3311 ± 356
FSH31	15	GG	6.80 ± 0.35^{b}	6.80 ± 0.35^{Bb}	382 ± 24^{b}	1464 ± 158	6.08 ± 0.49^{b}	3251 ± 300
	65	AG	5.63 ± 0.21^{a}	5.46 ± 0.22^{Aa}	319 ± 12^{a}	1461 ± 59	4.90 ± 0.18^{a}	2904 ± 138
	52	AA	5.62 ± 0.24^{a}	5.52 ± 0.25^{ABa}	334 ± 13^{ab}	1451 ± 62	5.13 ± 0.24^{a}	2999 ± 139

Within columns, values marked by different letters differ (a, b: P < 0.05; A, B: $P < 0.01$); TNB: total number of kits born; NBA: number of kits born alive; LWB: litter weight at birth; LS21: litter size at 21 days; LW21: litter weight at 21 days; LWW: litter weight at weaning; Total does used for association analysis: 133 for FSH2; 134 for FSH30; 132 for FSH31.

Table 4
Associations between *FSHβ* haplotypes and reproductive traits in Rex rabbits; (Values are least squares means ± SEM).

Haplotype	*n*	Traits					
		TNB	NBA	LWB(g)	LS21(g)	LW21(g)	LWW(g)
TGA	53	5.79 ± 0.28	5.65 ± 0.28	339 ± 15	5.08 ± 0.26	1500 ± 76	3036 ± 166
GAG	82	5.90 ± 0.29	5.73 ± 0.28	335 ± 15	4.86 ± 0.26	1409 ± 77	2887 ± 152
P		0.555	0.555	0.659	0.908	0.481	0.767

TNB: total number of kits born; NBA: number of kits born alive; LWB: litter weight at birth; LS21: litter size at 21 days; LW21: litter weight at 21 days; LWW: litter weight at weaning.
Total number of does used for haplotype association analysis was 135.

haplotype. In Table 4, there are lists of the data for reproductive performance of females with TGA or GAG haplotype. None of the associations between haplotype and reproductive trait were significant, but there were indications that rabbits with the GAG haplotype had the greatest combination of prolificacy with the least litter weight at birth (Table 4). Because of a greater post-natal mortality, the litter size and weight of the GAG rabbits at 21 days of age were also less than those of the rabbits with the TGA haplotype, leading to a lesser litter weight at weaning in the rabbits with the GAG haplotype.

3.5. Associations between FSHβ genotypes and reproductive traits in the Beaver and White lines

In the Beaver line, only FSH2 was associated with LW21, with rabbits of the GG genotype having values greater than those with GT and TT genotypes (Table 5; $P < 0.01$). By contrast, in rabbits with the FSH30 and FSH31 genotypes there were no associations with any of the reproductive traits ($P > 0.05$; Table 5). In contrast, in the White line, for the FSH2 genotype there was no association with any reproductive trait whereas with the FSH30 and FSH31 genotypes there were associations with TNB and NBA (Table 6). White line rabbits with the AA genotype for FSH30 had greater values for TNB and NBA than those with the GG and AG genotypes ($P < 0.01$). For FSH31, White line rabbits with the GG genotype had greater values for TNB and NBA than those with AA and AG genotypes ($P < 0.01$; Table 6).

4. Discussion

In the combined population of Rex rabbits, there were three significant associations between the FSHβ SNPs and NBA, TNB, LWB, and LS21. All three SNPs were located in exon1 and exon3, which is inconsistent with the findings of Chen et al. (2009) where there was detection of a c.176 A > G SNP in exon2 of FSHβ that was associated with NBA in the NZW rabbit. Nevertheless, it is clear that the various FSHβ polymorphisms of rabbits with various genotypes explain variations in reproductive traits. The results for the association analysis in the present study when conducted separately for the two lines also indicate that the different SNPs had effects that were specific for the Beaver and White lines, but there needs to be further exploration of this result with larger populations.

For pigs, Zhao et al. (1999) reported a 229bp Alu insertion in intron 1 of the FSHβ gene, between bases 809 and 810, and the litter size was 1.5 greater in the homozygotic rabbits with this insertion than in the homozygote without the insertion. The results of the present study indicate that all three putative mutations led to amino acid replacement that could affect the secondary protein structure of FSHβ and thus increase/decrease number of ovulations in Rex rabbits.

Because the D' values among the three SNPs were close to 1, it can be inferred that these SNPs were all in strong linkage disequilibrium (LD). Obviously, the GAG haplotype is the most advantageous considering the TNB and NBA for animals with this haplotype. It is noteworthy that the difference between haplotypes in TNB and NBA were not significant as SNPs, probably because the haplotypes were inferred from PHASE 2.1, which can't reflect the real distribution of haplotypes. The values for LS21, LW21 and

Table 5
Associations between *FSHβ* genotypes and reproductive traits in the Beaver line of Rex rabbits (Values are least squares means ± SEM).

Sites	*n*	Genotype	TNB	NBA	LWB (g)	LW21 (g)	LS21 (g)	LWW (g)
FSH2	13	GG	5.91 ± 0.67	5.86 ± 0.67	332 ± 36	2208 ± 172^{Aa}	5.33 ± 0.55	3648 ± 370
	27	GT	6.02 ± 0.53	5.96 ± 0.53	354 ± 29	1491 ± 136^{Bb}	5.42 ± 0.44	3088 ± 307
	37	TT	5.56 ± 0.37	5.49 ± 0.36	332 ± 20	1593 ± 106^{ABb}	5.34 ± 0.34	3163 ± 245
FSH30	38	GG	5.86 ± 0.37	5.78 ± 0.38	342 ± 19	1585 ± 97	5.60 ± 0.33	3139 ± 220
	33	AG	5.91 ± 0.45	5.83 ± 0.46	350 ± 23	1555 ± 105	5.37 ± 0.36	3116 ± 239
	4	AA	6.25 ± 0.93	6.25 ± 0.95	341 ± 48	1697 ± 213	5.75 ± 0.73	3369 ± 472
FSH31	36	GG	5.44 ± 0.55	5.19 ± 0.39	314 ± 25	1347 ± 111	4.93 ± 0.35	2895 ± 285
	33	AG	5.87 ± 0.49	5.50 ± 0.27	311 ± 17	1375 ± 86	4.88 ± 0.27	2882 ± 206
	7	AA	6.60 ± 0.82	6.90 ± 0.49	393 ± 31	1331 ± 162	6.14 ± 0.52	3148 ± 364

Within columns, values marked by different letters differ (a, b: $P < 0.05$; A, B: $P < 0.01$); TNB: total number born; NBA: number born alive; LWB: litter weight at birth; LS21: litter size at 21 days; LW21: litter weight at 21 days; LWW: litter weight at weaning.
Total does used for association analysis in the Beaver line: 77 for FSH2; 75 for FSH30; 76 for FSH3.

Table 6
Associations between *FSHβ* genotypes and reproductive traits in the White line of Rex rabbits (Values are least squares means ± SEM).

Sites	*n*	Genotype	TNB	NBA	LWB (g)	LW21 (g)	LS21 (g)	LWW (g)
FSH2	9	GG	6.33 ± 0.58	6.33 ± 0.56	368 ± 37	1224 ± 154	4.29 ± 0.59	2565 ± 346
	36	GT	5.89 ± 0.29	5.72 ± 0.28	334 ± 18	1392 ± 77	5.43 ± 0.29	3009 ± 182
	14	TT	5.00 ± 0.46	5.29 ± 0.45	302 ± 30	1240 ± 113	4.77 ± 0.43	2932 ± 283
FSH30	15	GG	5.00 ± 0.41^{Aa}	5.00 ± 0.40^{Aa}	307 ± 26	1315 ± 115	4.71 ± 0.38	2717 ± 278
	35	AG	5.77 ± 0.27^{ABa}	5.60 ± 0.26^{ABa}	327 ± 17	1352 ± 81	4.96 ± 0.27	2893 ± 197
	9	AA	7.00 ± 0.53^{Bb}	7.00 ± 0.51^{Bb}	384 ± 34	1395 ± 176	6.00 ± 0.58	3278 ± 379
FSH31	16	AA	5.19 ± 0.40^{Aa}	5.19 ± 0.38^{Aa}	314 ± 25	1347 ± 111	4.93 ± 0.35	2895 ± 285
	32	AG	5.69 ± 0.28^{ABa}	5.50 ± 0.27^{ABa}	311 ± 17	1375 ± 86	4.88 ± 0.27	2882 ± 206
	10	GG	6.90 ± 0.51^{Bb}	6.90 ± 0.49^{Bb}	393 ± 31	1331 ± 162	6.14 ± 0.52	3148 ± 364

Within columns, values marked by different letters differ (a, b: $P < 0.05$; A, B: $P < 0.01$). TNB: total number of kits born; NBA: number of kits born alive; LWB: litter weight at birth; LS21: litter size at 21 days; LW21: litter weight at 21 days; LWW: litter weight at weaning.
Total does used for association analysis in White line: 59 for FSH2; 59 for FSH30; 58 for FSH31.

LWW for the TGA haplotype, however, were greater than the values for the GAG haplotype, in contrast to the values for TNB and NBA. By age 21 days and weaning, the effects of environment become more important than the genetic effects, so a larger sample size is needed to detect effects, and the gene × environment interaction should be considered when analyzing the reproductive performance of rabbits.

The results from using the Hardy-Weinberg analysis indicated that the FSH2 and FSH31 genotypes deviated from the equilibrium, probably because the experimental population had a history of artificial selection for reproductive traits, and might also contain some specific family genomic structure. These factors could explain why the frequency of the T allele in the FSH2 genotype and the frequency of the A allele in the FSH31 genotype were greater than the frequencies of the G alleles in both genotypes.

The values for TNB, NBA, LWB, LS21 in the White primiparous does were all greater than the values in Beaver counterparts, an observation consistent with those of Zhao et al. (2017) in China, but inconsistent with those of Mazouzi-Hadid (2014) when there was study of a colored Rex population in Algeria. This inconsistency is probably due to innate differences in prolificity between the breeds, but could also reflect effects of the environment. For the primiparous White Rabbit strain R and the Sichuan White Rex Rabbit, the average values are 7.20–7.60 for TNB, 6.98–7.27 for NBA and 349.0–396.1 g for LWB (Yu et al., 2003, 2004; Wen et al., 2017), all of which are greater than the values in the population of animals used in the present study.

In conclusion, this study led to the discovery of three novel SNPs in exon1 and exon3 of the rabbit FSHβ gene that affect TNB, NBA, LWB and LS21, with the G allele in FSH2, A allele in FSH30 and G allele in FSH31 genotypes all being related to reproductive traits. Interestingly, the G allele in the FSH2 genotype was associated with a greater LW21 in the Beaver line, whereas the A allele in FSH30 and G allele in FSH31 genotypes were both associated with TNB and NBA in the White line. These SNPs could perhaps be used for selection to improve the reproductive performance of the Rex rabbit. The next goal should be to conduct genome-wide studies to verify these three associations, in association with studies on the function of FSHβ in domestic rabbits.

Conflict of interest

The authors have no conflicts of interest.

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