Effects of *FecB* Mutation on Estrus, Ovulation, and Endocrine Characteristics in Small Tail Han Sheep

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The Booroola fecundity gene (*FecB*) has a mutation that was found to increase the ovulation rate and litter size in Booroola Merino sheep. This mutation is also associated with the fecundity of small-tail han (STH) sheep, an important maternal breed used to produce hybrid offspring for mutton production in China. Previous research showed that the *FecB* gene affects reproduction in STH sheep, based on litter size records. However, the effects of this gene on estrus, ovulation, and endocrine characteristics in these sheep remain unclear. Here, we analyzed the traits mentioned earlier and compared them among the three *FecB* genotypes of STH ewes using estrus synchronization. Overall, 53 pluriparous ewes were selected from among 890 STH ewes and subjected to *FecB* genotyping for experiments to characterize estrous and ovulation rates. *FecB* heterozygous (+B) ewes presented an earlier onset of estrus (42.9 $\pm$ 2.2 h) and a shorter estrous cycle (17.2 $\pm$ 0.2 days) ($P \leq 0.05$). The ovulation rates increased with the increasing copy number of the B allele ($P \leq 0.01$). Ovulation time showed no significant differences among the three *FecB* genotypes. The serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone, estrogen (E_2), and progesterone (P_4) were measured in 19 of the ewes. Serum concentrations of E_2 and FSH dramatically varied around the time of behavioral estrus. In *FecB* mutant homozygous (BB) ewes, E_2 concentration had two peaks, which were higher ($P \leq 0.05$) than those of ++ genotypes. FSH concentration of BB ewes was higher ($P \leq 0.05$) than that of the ++ ewes just after estrus. The expression of the estrogen receptor 1 (*ESR1*) gene in the +B genotype was higher than in the other genotypes. Based on the data for the reproductive performance of STH ewes with the three *FecB* genotypes, our study suggests that the development of follicles in ewes with the B allele is dependent on the response to FSH regulated by E_2 in the early stage. +B ewes, exhibiting moderate ovulation and litter size and a shorter estrous cycle, can be highly recommended in sheep crossbreeding systems for commercial mutton production. Moreover, this study provides useful information to conserve better and use the genetic resources of STH sheep in China.

Keywords: sheep, *FecB* gene, estrus, ovulation, reproduction hormones, hormone receptors

INTRODUCTION

The *FecB* gene, also called *BMPR1B* (bone morphogenetic protein receptor type 1B), was initially identified in prolific Booroola Merino sheep. These sheep have a mutation (A746G) in the coding region of the *FecB* gene, leading to the substitution of glutamine for arginine in the protein (1–5). The *FecB* gene was shown to have an additive effect on ovulation rate and an effect on litter size that varies from additive to dominant depending on the genotype (6). Moreover, the *FecB* gene might be associated with a higher concentration of follicle-stimulating hormone (FSH) in peripheral blood during the estrous cycle (7–9). It has been reported that progesterone concentrations in Booroola ewes were 25% higher than in control Merino ewes during days 9–11 of the estrous cycle, although the difference did not reach significance (7, 10). *In vitro*, in the absence or presence of FSH, granulosa cells from BB ewes were found to be less responsive to the activity of *GDF5* and *BMP4*, resulting in higher progesterone secretion (3). In addition, it was suggested that granulosa cells carrying this *FecB* mutation might result in reduced proliferation but earlier responsiveness to LH in small follicles (11, 12). The estrous cycle is an external phenotype that is influenced by reproductive hormones (13). Small-tail han (STH) sheep are well known as a high-fecundity breed. The *FecB* mutation in this breed originates from Central Asian sheep, unlike the mutation in Booroola sheep (14). The average litter size of STH primiparous ewes is 2.00, and that of pluriparous ewes is 2.61. Many reports about frequency distributions of the *FecB* gene described that segregations of this gene are found in STH sheep. The +B and BB genotypes are the dominant genotypes in this breed, and the frequency of the B allele is greater than 0.5 (15). The *FecB* gene was shown to significantly affect litter size in STH sheep and other Chinese sheep breeds (16). Because of their excellent reproductive performance, STH sheep are widely used as the female parent in crossbreeding to improve the production efficiency of commercial mutton sheep. In the modern sheep industry, laparoscopic artificial insemination (AI) is being used to maximize reproductive potential. To achieve successful laparoscopic AI, the sheep breed and the signs of behavioral estrus are key factors to be taken into account (17). Based on research on Booroola Merino sheep, it was assumed that the *FecB* gene affects reproductive performance (4, 18). Unlike Booroola Merino sheep, STH sheep are a breed exhibiting year-round estrus. However, there has been little research on the effects of the *FecB* gene on the phenotypes of estrous time, estrous onset, estrous duration, estrous cycle, and subsequent ovulation time in this sheep breed. Therefore, there is a need to evaluate these factors.

Here, estrus and ovulation time traits were measured in three groups of estrus-synchronized STH ewes with different *FecB* genotypes. Combining estrus and ovulation data, we further analyzed the differences in hormonal patterns of the three genotypes in estrous cycles. By analyzing the expression profiles of hormone receptors in granulosa cells of preovulatory follicles in the different genotypes, we also analyzed how hormones coordinate with their receptors to generate signaling cascades that regulate follicle selection and maturation. This research should provide additional insight into the effect of the *FecB* gene on estrus and ovulation events and benefit future sheep reproductive production in China.

MATERIALS AND METHODS

Animals and Genotyping of *FecB* Gene by TaqMan Assay

Jugular vein blood and litter size data of 890 healthy non-pregnant ewes aged approximately 3 years were collected randomly from a herd in southwest Shandong Province, China. These ewes were genotyped for the *FecB* gene by the TaqMan method. Primer sequences of *FecB*-TaqMan and its probe sequences are listed in **Table 1**. The 6-μl reaction system contained 1 μl of DNA, 3 μl of 2× TaqMan Universal Master Mix II (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.3 μl of each *FecB*-TaqMan primer, 0.15 μl of two probe primers, and 1.9 μl of H_2O. Polymerase chain reaction (PCR) amplification was performed on a Roche LightCycler 480 II System (Roche, Basel, Switzerland) under the following PCR conditions: incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. The allelic discrimination data were analyzed using endpoint genotyping with the hydrolysis probe protocol.

Pluriparous ewes ($n = 53$) with similar body conditions were selected and divided into three groups based on the *FecB* genotype (23 wild-type ++ ewes, 16 heterozygote +B ewes, and 14 homozygous mutant BB ewes) for subsequent experiments.

Experimental Treatment and Design
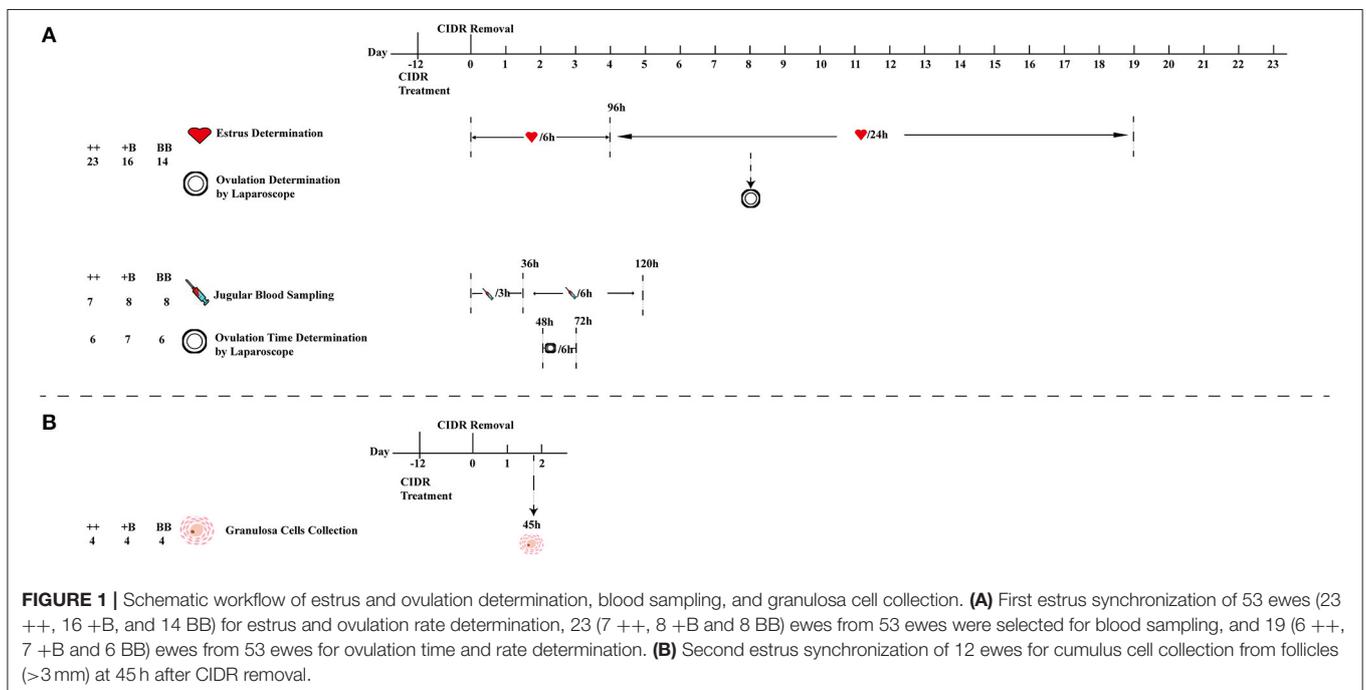
During the spring, estrus synchronization in these three groups was performed using a controlled internal drug release device (CIDR) (EAZI-BREED® CIDR® Sheep and Goat Device, Pfizer Animal Health, Auckland, New Zealand) with 300 mg of progesterone in all ewes for 12 days without any use of exogenous hormones for superovulation. Only 250,000-IU vitamin A and 25,000-IU vitamin D were injected to protect the vaginal epithelium when the CIDR was inserted. Estrus synchronization was performed on ewes twice with an interval of 14 days. In the first synchronization of estrus in 53 sheep of the three genotypes, estrus response, estrus onset, estrus duration, ovulation time, ovulation rate, and hormonal patterns in these estrous cycles were recorded. In the second experiment, cumulus cells were collected from 12 ewes. All ewes were maintained in a barn with food and water *ad libitum*.

Estrus Determination

After removal of the CIDR, eight rams with proven high serving capacity were selected as teasers wearing an apron around their hypogastrium to detect estrus in the ewes. The 53 experimental ewes (23 ++, 16 +B, 14 BB) and rams were raised separately. CIDRs were removed at 6:00 a.m. Teasing was initially performed every 6 h (at 6:00, 12:00, 18:00, and 24:00) for 96 h (19–22). Teasing was subsequently performed

TABLE 1 | TaqMan primer and probe sequences for genotyping and Quantitative PCR primer information.

Gene	GenBank ID	Usage	Primer sequence (5′–3′)	
BMPR1B	NC_019463.2	Genotyping	Forward primer	CCAGCTGGTTCCGAGAGACA
			Reverse primer	CTTATACTCACCCAAGATGTTTTCATG
			Probe-A	AAATATATCAGACGGTGTTG-MGB
			Probe-G	AAATATATCGGACGGTGTTG-MGB
FSHR	NM_001009289.1	Quantitative PCR	Forward primer	CAAAGATCCTCCTGGTCCTGTTC
			Reverse primer	GTTCCTGGTGAAGATGGCGTAG
LHCGR	NM_001278566.2		Forward primer	GCTGAGAGTGAACTGAGTGACTG
			Reverse primer	CTGGTTCGGGAGCACATTGG
INHA	NM_001308579.1		Forward primer	GACTGGACAGACAGGAGACC
			Reverse primer	AGGACAACGCAGCAGGAG
ESR1	XM_027972563.1		Forward primer	TGCCATGTTTCAAACCCATCTTC
			Reverse primer	TCTATGACCAATGACCTCTCTGTG
CYP19A1	NM_001123000.1		Forward primer	TCGTCCTGGTCACCCTTCTG
			Reverse primer	CGGTCTCTGGTCTCGTCTGG
CYP17A1	NM_001009483.1		Forward primer	CTTACCATTGACAAAGGCACAGAC
			Reverse primer	GCTTAATGATGGCGAGATGAGTTG
RPL19	XM_004012836.2		Forward primer	F:AATGCCAATGCCAACTC
			Reverse primer	R:CCCTTTCGCTACCTATACC

FIGURE 1 | Schematic workflow of estrus and ovulation determination, blood sampling, and granulosa cell collection. **(A)** First estrus synchronization of 53 ewes (23 ++, 16 +B, and 14 BB) for estrus and ovulation rate determination, 23 (7 ++, 8 +B and 8 BB) ewes from 53 ewes were selected for blood sampling, and 19 (6 ++, 7 +B and 6 BB) ewes from 53 ewes for ovulation time and rate determination. **(B)** Second estrus synchronization of 12 ewes for cumulus cell collection from follicles (>3 mm) at 45 h after CIDR removal.

only once a day at 6:00 a.m. until the initiation of the next estrus. Following established definition and procedures, traits of estrus response, estrus onset, and estrus duration of ewes of the three groups in the first estrous cycle were recorded and calculated (22). The first estrus was defined from the ewes' first accepted mount, and the estrous cycle was defined as the interval from the last refused mount to the next one. Details of the experimental process of estrus determination are presented in **Figure 1**.

Ovulation Determination

We randomly selected 6 ++ ewes, 7 +B ewes, and 6 BB ewes from among the 53 ewes of the three experimental groups to determine the ovulation time and rate by a modified version of the method reported by Romano et al. (22, 23). Laparoscopy was performed to determine ovulation at 48, 54, 60, 66, and 72 h after CIDR removal. The intervals from CIDR removal and estrus onset to first ovulation were respectively defined as the times elapsed from CIDR removal and estrus

onset to the time halfway between the first appearance of preovulatory follicles and their disappearance. The intervals from CIDR removal and estrus onset to the last ovulation were respectively defined as the times elapsed from CIDR removal and estrus onset to the time halfway between the last appearance of preovulatory follicles and their disappearance. The ovulation rate (the number of corpora lutea on both ovaries) was also recorded for all 53 sheep in the three groups using laparoscopy on day 8 after CIDR removal (**Figure 1**).

Blood Sampling and Hormonal Assays

Blood samples for testing hormone levels were collected from 7 ++, 8 +B, and 8 BB ewes from among the 53 ewes at the same time to determine the time of ovulation in the same batch. After removal of the CIDR at 06:00 a.m., 10 ml of jugular vein blood was collected in BD SST tubes (BD, San Diego, CA, USA) every 3 h for a 36-h period (24). From 36 to 120 h, blood was collected every 6 h. Jugular vein blood was kept at room temperature for 30 min, followed by centrifugation at $1,500 \times g$ for 10 min (25). The concentration of follicle-stimulating hormone (FSH) was determined using a commercial FSH [^{125}I]-labeled radioimmunoassay (RIA) kit (B03-FSH; Beijing North Institute of Biological Technology, BNIBT, Beijing, P. R. China). The sensitivity of the kit was <1.0 mIU/ml. The coefficient of variation (CV) in each batch was <10%, and the CV across the batch was <15%. Luteinizing hormone (LH) was quantified using a commercial LH [^{125}I]-labeled RIA kit (B04-LH; BNIBT). The kit sensitivity was 1.0 mIU/ml, and intra- and inter-batch CVs were <10 and <15%, respectively. The estradiol (E$_2$) concentration was tested using an E$_2$ RIA kit (B05-E2; BNIBT), the sensitivity of which was <2 pg/ml. The CVs for each batch and between the batches were <10 and 15%, respectively. Progesterone (P$_4$) was quantified using RIA kits (B08-P; BNIBT) with a sensitivity of <0.1 ng/ml, intra-batch CV of <10%, and inter-batch CV of <15%. To ensure the accuracy of hormone detection, triplicate assays were performed for each sample. The details are presented in **Figure 1**.

Granulosa Cell Collection and Real-Time Quantitative Polymerase Chain Reaction

Ewes (4 ++, 4 +B, and 4 BB) were used for estrus synchronization. At 45 h after CIDR removal, cumulus–oocyte complexes were aspirated from follicles > 3 mm in diameter with a syringe containing Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific Inc.). Then, the cumulus–oocyte complexes were washed with Dulbecco's phosphate-buffered saline and digested with hyaluronidase. After 5 min of centrifugation at 2,000 rpm, the granulosa cells were gathered and lysed in 1 ml of Trizol (Thermo Fisher Scientific Inc.). The lysate was immediately frozen in liquid nitrogen and stored at −80°C for further RNA extraction.

Total RNA from cumulus cells was isolated following the Trizol protocol. One microgram of RNA was reverse-transcribed with PrimeScript RT Reagent Kit (TaKaRa, Shiga,

Japan). Real-time quantitative PCR was performed on the LightCycler 480 Real-Time PCR system (Roche, Switzerland) with the SYBR Premix Ex Taq kit (TaKaRa), as described (26). Primer sequences for quantitative PCR are listed in **Table 1**. Ribosomal protein L19 (RPL19) was used as an internal reference gene, and the $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels in messenger RNA analysis (27).

Data Analysis

Statistical analysis was performed using the generalized linear model Statistical Analysis System release 8.12 (SAS Institute Inc., NC, USA). All data are presented as the mean ± standard error of the mean, and P ≤ 0.05 was considered significant. P ≤ 0.01 was considered extremely significant. The statistical analyses of genotype frequency, allele frequency, and polymorphism information content were performed with reference to the formulas reported by Guo et al. (28). Traits in estrus determination, ovulation time, hormone concentrations in serum, and gene expression were continuous data. The normality of these continuous data was analyzed using the Shapiro–Wilk normality test. One-way analysis of variance was used to analyze the effect of *FecB* mutations on these normally distributed data. The data of the first estrus and estrus cycle were not normally distributed, so the Kruskal–Wallis nonparametric test was applied. Repeated-measures analysis of variance was adopted to analyze how hormonal (FSH, LH, E$_2$, and P$_4$) concentrations changed in different *FecB* genotypes over time. The estrus response was a binary variable, so Pearson's Chi-squared test was adopted to analyze the estrus response. Litter size and ovulation, traits measured as counts, are usually modeled by the Poisson distribution, so we analyzed the genotypic effects on litter size and ovulation rate through a generalized Poisson regression with log–link function using the generalized linear model GENMOD procedure of SAS. For litter size data, we set $Y_j = 0, 1, \ldots, \infty$ to be the litter size of individual sheep. The expectation (E) and the variance of litter size were calculated as $E\left(Y_j\right) = var\left(Y_j\right) = \mu_j$, where $\mu_j = \exp(\alpha_0 + X_j\beta + Z_j\gamma)$, α_0 is the intercept, β is a 3×1 vector for parity, and γ is a 3×1 vector for genotype. The ovulation data from 53 and 19 sheep were analyzed separately, letting $Y_j = 0, 1, \ldots, \infty$ be the ovulation rate of an individual. The expectation and the variance of ovulation rate were equivalent, $E\left(Y_j\right) = var\left(Y_j\right) = \mu_j$, where $\mu_j = \exp(\alpha_0 + X_j\beta)$, α_0 is the intercept, and β is a 3×1 vector for genotyping.

RESULTS

Characteristics of the *FecB* Gene in Small-Tail Han Ewes and Its Association With Litter Size

Using a TaqMan probe-based method, the allele and genotype frequencies of the *FecB* gene in experimental STH ewes were determined, as presented in **Table 2**. The frequencies of ++, +B, and BB genotypes were 0.16, 0.46, and 0.38, respectively; accordingly, the frequencies of the + and B alleles were 0.4

TABLE 2 | Genetic characteristics of *FecB* gene in STH ewes.

Polymorphic site	Genotype	Number of samples	Genotype frequency	Allele	Allele frequency	$\chi^2(P)$	Polymorphism information content
FecB	++	142	0.16	+	0.40	0.61 (0.44)	0.47
	+B	413	0.46	B	0.60		
	BB	335	0.38				

TABLE 3 | Litter size of STH ewes in different parities and *FecB* genotypes.

Genotype	Litter size (Mean ± SEM)			
	First	**Second**	**Third**	**Overall mean**
++	1.77 ± 0.08 (115)[A]	1.73 ± 0.09 (73)[A]	2.05 ± 0.22 (20) [A]	1.78 ± 0.06 (208)[A]
+B	2.04 ± 0.04 (330)[B]	2.25 ± 0.06 (224)[b]	2.76 ± 0.11 (85) [B]	2.21 ± 0.03 (639)[B]
BB	2.32 ± 0 0.05 (251)[C]	2.51 ± 0.07 (162)[c]	3.00 ± 0.17 (54) [B]	2.46 ± 0.04 (467)[C]
	P (Genotype) < 0.01**			
	P (Parity) < 0.01**			

Numbers of litter size analyzed for each genotype are indicated in brackets. Values with same capital letter for same column have no significant difference ($P > 0.05$). Values with different lowercases or capital for same line differ significantly ($P \leq 0.05$). Double asterisk (**) indicates extreme significance ($P \leq 0.01$).

and 0.6, respectively. Chi-squared fitness testing showed that *FecB* gene mutations were under Hardy–Weinberg equilibrium in the randomly selected ewes ($P > 0.05$). The polymorphism information content of the *FecB* gene exceeded 0.3, reflecting moderate polymorphism, implying that the selection potential of the sites was considerable. For the polymorphic sites of *FecB*, the results of variance analysis as shown in **Table 3** indicated that the litter size increased with increasing copy numbers of the B allele. Genotype and parity were shown to have significant effects on litter size. In each parity group, the mean litter size of the ++ genotype ewes was significantly smaller than in the other two genotypes. With increasing parity, the differences in litter size between BB and +B genotypes decreased. There was no significant difference in litter size between the BB ewes and +B ewes in terms of parity above two.

Estrus Performance After Estrus Synchronization

A total of 53 STH ewes of similar ages (2.9 ± 0.1 years) and weights (72.5 ± 1.9 kg) were selected for an estrus determination experiment based on the estrus synchronization technique. After CIDR removal, apart from the estrus duration (32.6 ± 1.4 h), all other estrous cycle indexes were significantly affected by *FecB* (**Table 4**). The first estrus for +B STH ewes was 23.6 ± 1.9 h from CIDR removal, which was earlier than in the ++ and BB genotypes at 32.6 ± 2.4 and 30.4 ± 1.6 h, respectively ($P < 0.05$). The cumulative estrus response is shown in **Figure 2**; all ewes presented estrus. The STH ewes with the +B genotype had an earlier response to estrus synchronization treatment, whereas the response of BB STH ewes had a narrower distribution.

The onset of estrus in +B STH ewes was 42.9 ± 2.2 h from CIDR removal, which was also earlier than in ++ and BB

TABLE 4 | Estrus and ovulation comparison in STH ewes of three *FecB* genotypes.

Genotype	++	+B	BB	Total
Number of samples	23	16	14	53
Age (y)	2.8 ± 0.1	2.8 ± 0.2	3.0 ± 1.1	2.8 ± 0.1
Weight (kg)	73.8 ± 2.7	73.0 ± 4.6	70.1 ± 3.1	72.5 ± 1.9
Estrus response	23	16	14	53
First estrus (h)	32.6 ± 2.4[a]	23.6 ± 1.9[b]	30.4 ± 1.6[a]	29.3 ± 1.3
Estrus onset (h)	51.5 ± 2.0[a]	42.9 ± 2.2[b]	50.0 ± 1.7[a]	48.5 ± 1.3
Last estrus (h)	65.0 ± 2.4[a]	56.3 ± 2.9[b]	63.4 ± 2.9[ab]	61.9 ± 1.6
Estrus duration (h)	32.4 ± 2.3	32.6 ± 2.3	33.0 ± 3.1	32.6 ± 1.4
Estrus cycle (d)	17.9 ± 0.1[a]	17.2 ± 0.2[b]	17.9 ± 0.2[a]	17.7 ± 0.1
Ovulation Rate	1.09 ± 0.02[A]	2.38 ± 0.13[b]	3.07 ± 0.35[c]	2.02 ± 0.17

Values with same lowercase for same line have no significant difference ($P > 0.05$). Values with different lowercases or capital for same line differ significantly ($P \leq 0.05$) or extreme significantly ($P \leq 0.01$).

genotypes with 51.5 ± 2.0 and 50.0 ± 1.7 h ($P < 0.05$). Compared with 17.9 ± 0.1 and 17.9 ± 0.2 days in the STH ewes with ++ and BB genotypes, a shorter estrous cycle of 17.2 ± 0.2 days was observed in the ewes with the +B genotype. The findings suggested that STH ewes with *FecB* heterozygosity (+B) may exhibit an earlier first estrus, earlier estrus onset, and a shorter estrous cycle.

Ovulation Times and Rates in Small-Tail Han Ewes

Ovulation times and rates in STH ewes among the three genotype groups were observed laparoscopically. The mean interval from CIDR removal to first ovulation in STH ewes was 58.1 ± 2.7 h (**Table 5**). We noted that the interval from CIDR removal to
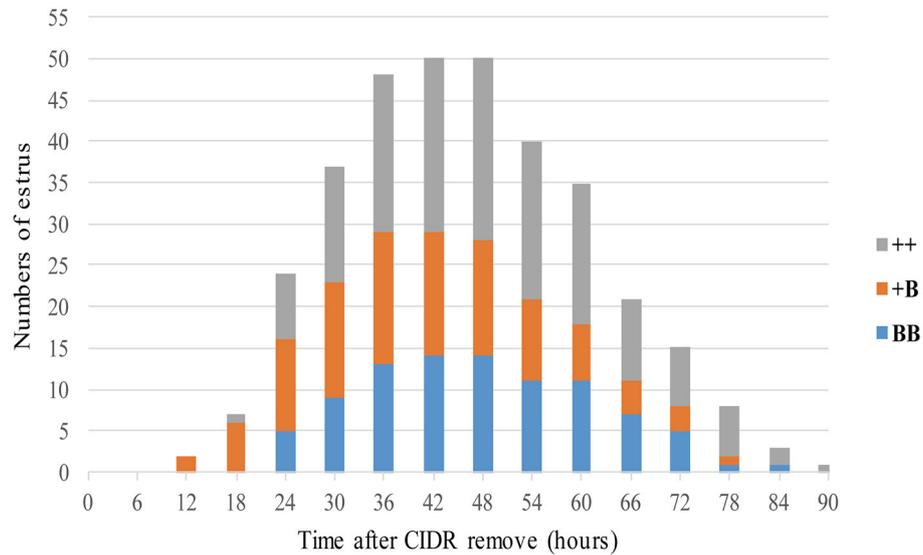
FIGURE 2 | Numbers of ewes in estrus among three *FecB* genotype groups after CIDR removal.

TABLE 5 | Ovulation traits of STH ewes in three *FecB* genotypes.

Items	Genotype		
	++	+B	BB
Number of samples	6	7	6
Interval from CIDR removal to first ovulation(h)	63.0 ± 5.8	52.2 ± 4.4	58.0 ± 3.3
Interval from estrus onset to first ovulation(h)	9.5 ± 2.9	10.2 ± 0.7	9.3 ± 2.0
Interval from CIDR removal to last ovulation(h)	63.3 ± 5.8	55.8 ± 4.4	62.0 ± 3.6
Interval from estrus onset to last ovulation(h)	9.8 ± 3.1	13.8 ± 3.4	13.3 ± 2.3
Ovulation Rate	1.17 ± 0.41^a	2.43 ± 0.98^B	2.00 ± 0.63^b

Values with same lowercase for same line have no significant difference (P > 0.05). Values with different lowercases or capital for same line differ significantly (P ≤ 0.05) or extreme significantly (P ≤ 0.01).

first ovulation for +B STH ewes appeared to be shorter (52.2 ± 4.4 h), albeit not significantly ($P = 0.30$). The mean intervals from first estrus and estrus onset to first ovulation in STH ewes were 26.3 ± 2.0 and 9.7 ± 1.2 h, respectively. The three *FecB* genotype groups did not differ significantly in any of the variables related to ovulation time. For the ewes with *FecB* mutation, the ovaries did not expel eggs simultaneously. Endoscopy performed every 6 h to estimate the time of ovulation indicated that all ovulations occurred in a period no longer than 6 h. The ovulation rate was measured successfully in the 53 STH ewes (**Table 4**). The ovulation rate of BB ewes (3.07 ± 0.35) was significantly higher than that of +B ewes (2.38 ± 0.13; $P \leq 0.01$). Meanwhile, the ovulation rate of +B ewes was also highly significantly higher than that of ++ ewes (1.09 ± 0.02; $P \leq 0.01$). The *FecB* mutation significantly influenced the ovulation rate of STH ewes.

Serum Concentrations of P_4, Follicle-Stimulating Hormone, Luteinizing Hormone, and E_2 During a Synchronized Estrous Cycle in Small-Tail Han Ewes

The serum concentrations of FSH, LH, E_2, and P_4 were measured during an intact synchronized estrous cycle in 7 ++, 8 +B, and 8 BB ewes for 5 days after CIDR removal. Data presented in **Figure 3** show the mean endocrine profiles of these ewes. Upon CIDR withdrawal, the P_4 concentration rapidly dropped to a low level. E_2 and FSH concentrations started to increase and peaked at approximately 27 h, which was aligned with the first estrus time. At 60 h after CIDR removal, the peaks of E_2 and P_4 dropped before the LH peak. FSH concentration peaked at 72 h after the LH peak. These four hormone concentrations peaked over 60–72 h.

The time of the first estrus of +B STH ewes was earlier than those of the other two genotypes. To investigate the changes of endocrine characteristics in serum before and after estrus, mean FSH, LH, E_2, and P_4 concentrations were calculated in relation to their levels of each individual's time of the first estrus (hour 0; see **Figure 4**). In the three genotypes, P_4 dropped at around hour 0, whereas P_4 concentrations in BB ewes were higher than those in +B and ++ ewes. E_2 increased from hour 0 to 3 in all three genotypes. In BB ewes, E_2 exhibited two peaks (-6 and 3 h), at which the E_2 concentrations were higher ($P \leq 0.05$) than in the ++ genotypes. In ++ and +B ewes, the FSH concentrations dropped before hour 0. The FSH concentrations of ++ and BB ewes increased after hour 0 and peaked at hour 3. BB ewes had significantly higher ($P \leq 0.05$) FSH concentrations than ++ ewes. LH concentrations increased at -3 h and decreased at 3 h, particularly in BB ewes. LH concentrations at 3 h were lower ($P \leq 0.05$) than in the ++ ewes, whereas those in +B ewes were intermediate.
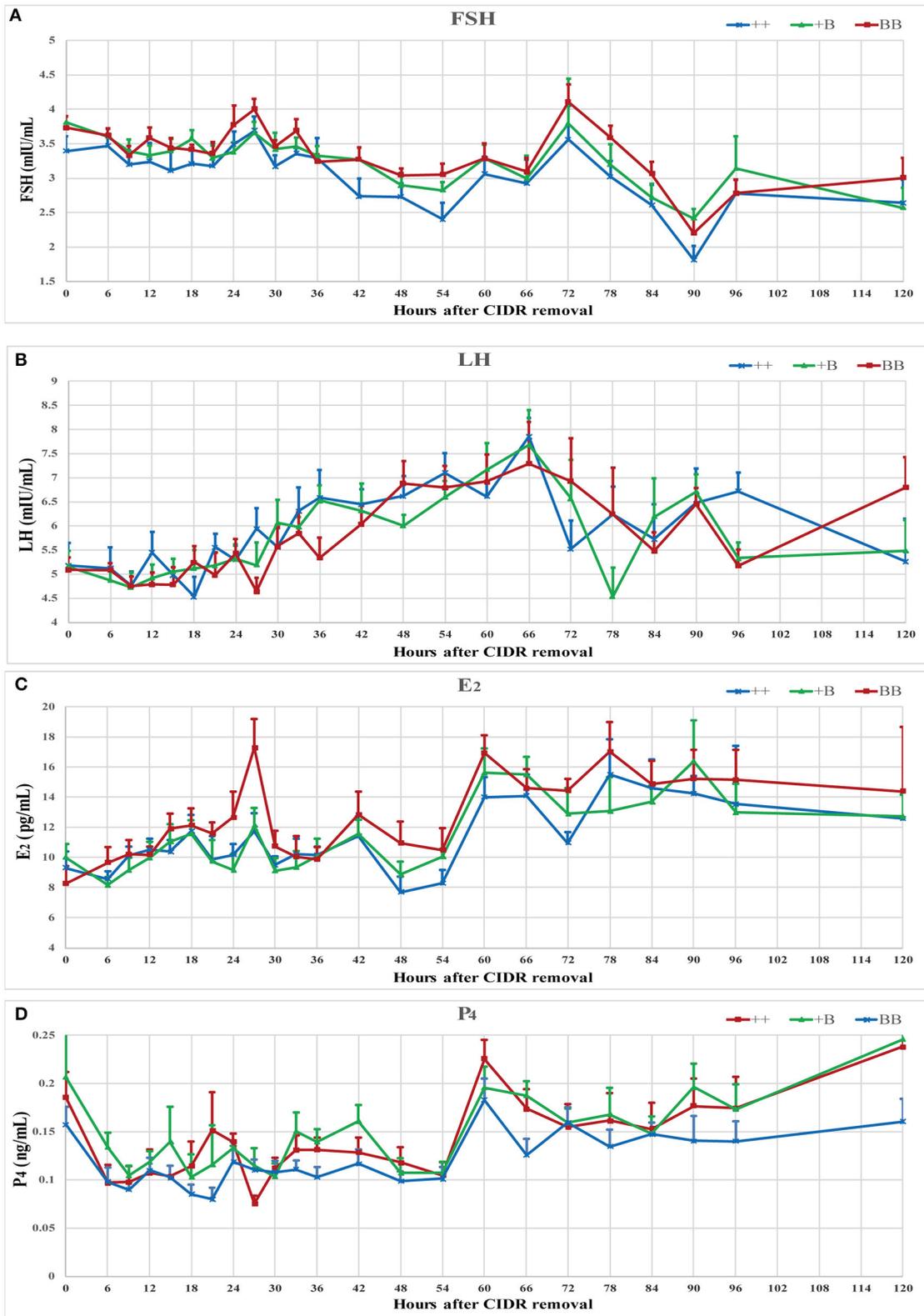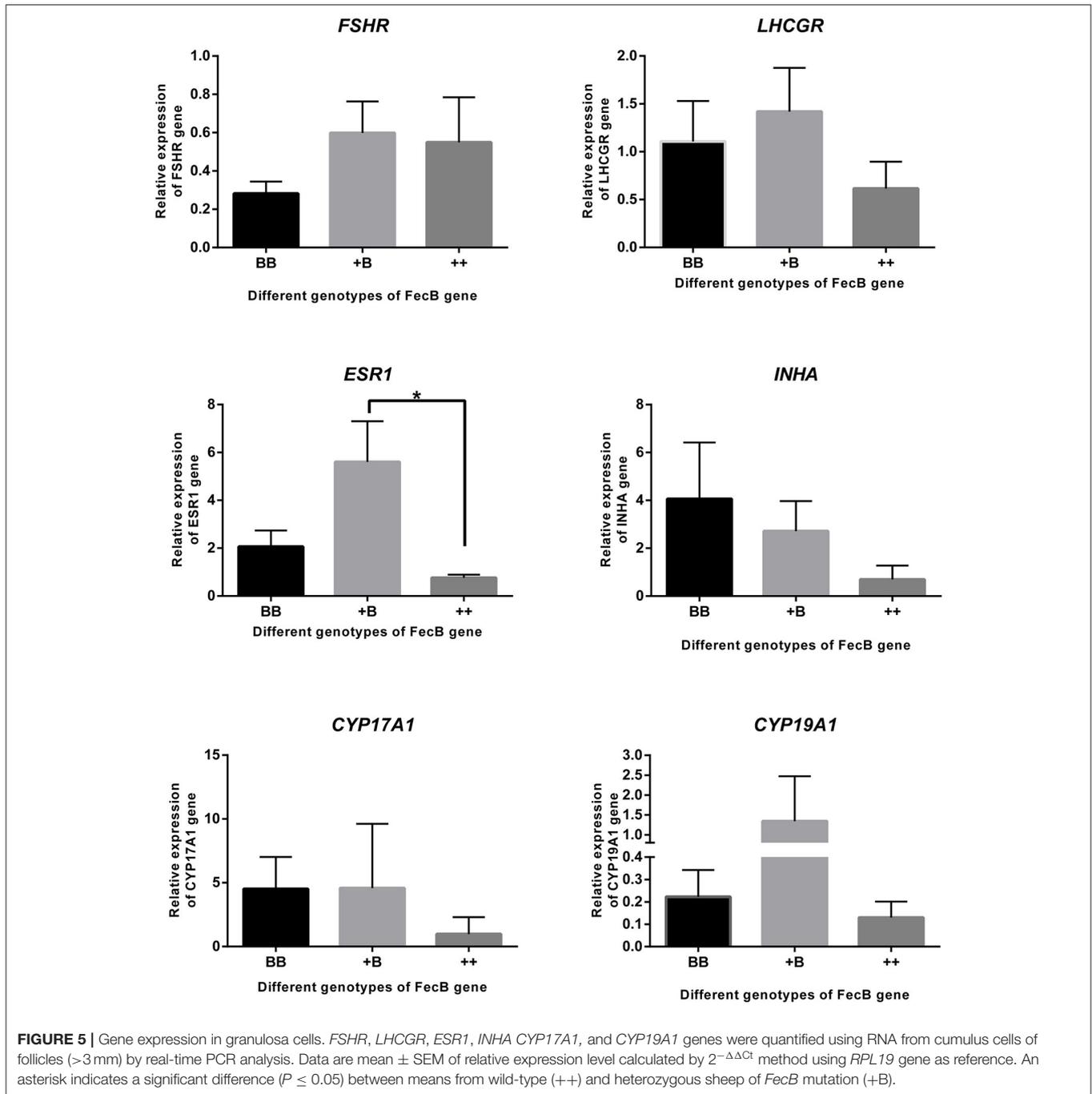
FIGURE 3 | Endocrine profiles of reproductive hormone (FSH, LH, E$_2$, and P$_4$) in ewes of three *FecB* genotypes during a synchronized estrous cycle. **(A)** Mean ± SEM of serum FSH (mU/ml), **(B)** Mean ± SEM of serum LH (mU/ml), **(C)** Mean ± SEM of serum E$_2$ (pg/ml), and **(D)** Mean ± SEM of serum P$_4$ (pg/ml).

FIGURE 4 | Mean ± SEM of serum FSH, LH, E$_2$, and P$_4$ concentration before and after initiation of first estrus (hour 0 is initiation of first estrus) in different FecB genotype ewes. Values with same capital letter for same column have no significant difference ($P > 0.05$). Values with different lowercases or capital for same line differ significantly ($P \leq 0.05$). **(A)** Mean ± SEM of serum FSH (mU/ml), **(B)** Mean ± SEM of serum LH (mU/ml), **(C)** Mean ± SEM of serum E$_2$ (pg/ml), and **(D)** Mean ± SEM of serum P$_4$ (pg/ml).
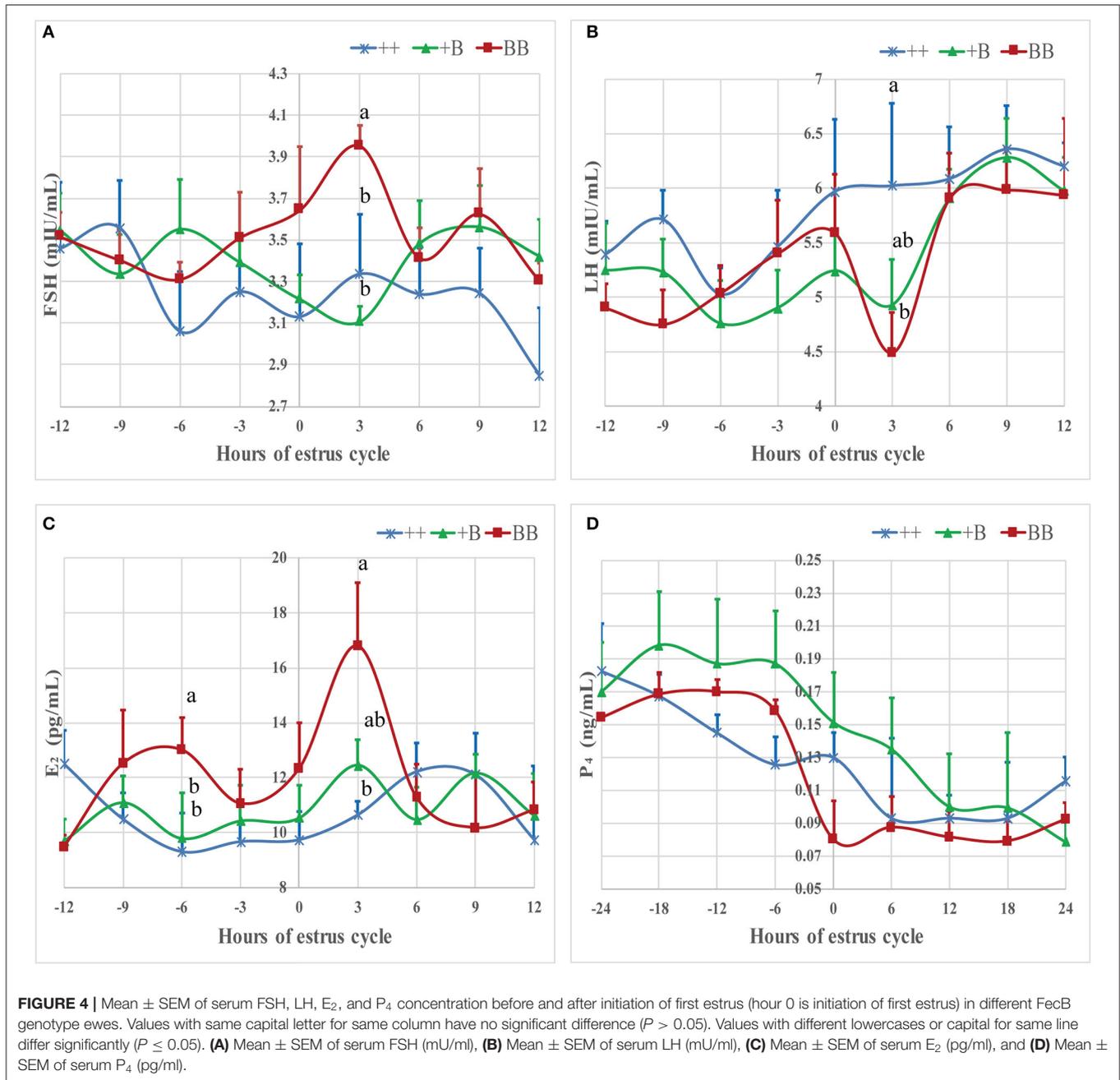
Gene Expression in Granulosa Cells

Taking together the results of the temporal trends in reproductive hormone levels and estrus and ovulation times, granulosa cells were derived from preovulatory dominant follicles approximately 45 h after CIDR removal. Total RNA was extracted and subjected to quantitative real-time PCR to analyze the expression of steroidogenic genes, cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*) and cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*); gonadotropin receptors, follicle-stimulating hormone receptor (*FSHR*) and luteinizing hormone/choriogonadotropin receptor (*LHCGR*); estrogen receptor 1 (*ESR1*); and the inhibin subunit alpha (*INHA*) (**Figure 5**). Expressions of the genes for the FSH and LH receptors were detected during the preovulatory stage, whereas no significant differences in the expression of these two genes were observed across the three genotypes. The expression of *ESR1* in the +B genotype was higher ($p \leq 0.05$) than in the wild-type (++) genotype. *ESR1* expression in the BB genotype was higher than that in the wild type, but this did not reach significance. There was a trend for an increase of *INHA* expression with increasing *FecB* mutant copy number ($P = 0.08$). The expression of *CYP17A1* and *CYP19A1*, markers of preovulatory follicles, also appeared at 45 h after CIDR removal when we collected the sample.

FIGURE 5 | Gene expression in granulosa cells. *FSHR, LHCGR, ESR1, INHA CYP17A1,* and *CYP19A1* genes were quantified using RNA from cumulus cells of follicles (>3 mm) by real-time PCR analysis. Data are mean ± SEM of relative expression level calculated by $2^{-\Delta\Delta Ct}$ method using *RPL19* gene as reference. An asterisk indicates a significant difference ($P \leq 0.05$) between means from wild-type ($++$) and heterozygous sheep of *FecB* mutation ($+B$).

DISCUSSION

STH sheep are derived from ancient northern Mongolian sheep, which are widely raised throughout northern China. The effect of *FecB* mutation in STH matches that identified in Booroola Merino sheep: it can significantly increase the litter size of STH sheep (16, 29, 30). Genetic analysis of the *FecB* gene in this study showed that the three *FecB* genotypes are still not fixed in this STH sheep population collected from a population set aside for conservation. The *FecB* gene showed moderate polymorphisms,

meaning that no significant selection response has occurred in the last decade (15, 31). This is good news regarding the protection of genetic diversity and of benefit for efforts to exploit the genetic resources of STH sheep in China.

The *BMPR1B* gene is a member of the transforming growth factor-β superfamily, which is critical for skeletal development, organ formation, and embryo development. Many studies have shown that *FecB* mutations influence ovulation rate, litter size, reproductive endocrinology, adrenal gland size, body mass, follicular development, fetal growth, milk production, and

sperm concentration, among others (32–36). With regard to reproductive endocrinology, such mutations can control external estrous characteristics and affect oocyte development. However, little research has been reported on the correlation between *FecB* mutation and estrous characteristics in STH sheep. Here, the estrous indexes of *FecB* heterozygous (+B) STH sheep differed from those in the other genotype groups, presenting an earlier first estrus, earlier estrus onset, and a shorter estrous cycle. However, no differences were observed in ovulation time among the three *FecB* STH genotypes. An investigation of ++ and +B ewes from a three-quarters Romney Marsh × one-quarter Booroola Merino flock after spontaneous estrus induced with progesterone treatment during the reproductive season also found that the *FecB* gene cannot change the duration of ovulation (37). The duration from the first estrus to ovulation was longer in +B STH sheep than in the ++ and BB groups. These results indicate that the *FecB* mutation accelerated the onset of estrus in heterozygote +B STH sheep but had no effect on the onset of ovulation. The +B genotype constituted almost half (0.46) of the total in the STH sheep group; the fecund +B genotype sheep are usually used as maternal parents in crossbreeding programs in commercial mutton production (15). *FecB* gene mutation can result in a change in the estrous phenotype, which is usually the main indicator for determining whether to perform AI (38). When using estrus synchronization with AI in mutton production, we should establish marker-assisted mating and breeding programs. Considering the production cost, prohibition on the use of castrated rams for estrus detection, and the full use of high-breeding-value rams, timed insemination is the optimal mating strategy (39). Combining previous research results and the data of ovulation time from CIDR measured in the three STH *FecB* genotype groups, it was recommended that fixed-time AI be performed between 48 and 60 h after CIDR removal (40, 41).

These results are consistent with previous findings showing that *FecB* mutations appear to have no effect on endocrine profiles (42). In our research, ewes with an *FecB* mutation showed premature estrus. We calculated the individual endocrine data after the time of the first estrus. Before estrus, the ewes with the BB genotype exhibited a peak in E_2 level, which was the highest among the three genotypes. After estrus, E_2 exhibited a second peak. During the growing follicle stage, E_2 downregulates the synthesis and secretion of LH and FSH *via* negative feedback to the hypothalamus and pituitary (43). Our results showed that FSH was not markedly inhibited in the BB genotype; on the contrary, FSH showed significant elevation with a peak at 3 h after estrus. This is in accordance with research on the peripheral FSH concentrations in an F_2 population from a cross between Booroola Merino and Scottish Blackface sheep during the breeding season (44). As the follicles mature, E_2 levels peak, and then positive feedback begins, triggering the release of preovulatory LH and an FSH surge (45). The interval from estrus onset to first ovulation suggested that the ewes started estrus approximately 10 h after estrus was detected. LH secretion in ewes peaked at 9 h after estrus. The concentration of FSH was lower than the peak at 3 h after estrus. Increasing FSH of BB ewes accelerated follicle development, meaning that the follicles reached maturity while still having a small diameter.

Before ovulation, the FSH concentration decreased during follicle selection, and LH increased to enable the follicles to survive and proceed to ovulation (46). During antral follicle development, the expression of estrogen receptors and *CYP19A1* and E_2 concentrations significantly increase in granulosa cells (47). The expression pattern of estrogen receptors was similar to that of *CYP19A1* among the three genotypes; in our results, only the expression of *ESR1* was affected by *FecB* mutation. *CYP17A1* and *CYP19A1* play important roles in the steroidogenic pathway. *CYP17A1* expression was higher in the large follicles. LH *via* *LHCGR* induced the messenger RNA expression of *CYP17A1* in ovaries (48, 49). In the large follicles that we collected, we detected the expression of *CYP17A1* and *LHCGR*. The expression in the ewes with the *FecB* mutation was slightly higher than in the wild type. We also detected the expression of *FSHR* in mature follicles, in agreement with previous findings (50). These two receptors, *FSHR* and *LHCGR*, have been reported to adjust the FSH and LH signaling cascades in granulosa cells and to regulate follicular development and ovulation (51).

Here, STH ewes of ++, +B, and BB genotypes showed significant increases in ovulation rate, and *FecB* mutation carriers had significantly larger litters than the wild-type sheep. These findings are in accordance with previous research reported by Davis et al. (52, 53). Combining the data on litter size, we conclude that the effect of the *FecB* gene on litter size is based on the regulation of the ovulation rate. However, litter size is influenced not only by ovulation rate but also by fertilization, embryonic survival, implantation, placentation, and parity (54). Moreover, *FecB* homozygotes were reported to have a smaller litter size than the heterozygotes in Australian Booroola Merino sheep (55). Therefore, an excessive ovulation rate should be avoided in ewes to prevent lower reproductive efficiency, and sheep with *FecB* heterozygosity exhibiting a higher ovulation rate and larger litter size are recommended for commercial hybrid sheep production in China.

CONCLUSION

With the exception of ovulation rate, our results show that the estrus performance of *FecB* heterozygote ewes differed from that of *FecB* homozygote and wild-type ewes, as revealed using estrus synchronization. The development of follicles in ewes with the B allele depended on the response to FSH in the early stage of estrus. *FecB* heterozygote ewes, exhibiting moderate ovulation and litter size and a shorter estrous cycle, can be highly recommended in sheep crossbreeding systems for commercial mutton production. Moreover, the findings obtained in this study should be useful for improving conservation and to better exploit the genetic resources of STH sheep.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All experimental procedures conducted in this study were performed in accordance with the guidelines set by the Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences, and approved by the committee (no. IASCAAS-AE-03, 12 December 2016).

AUTHOR CONTRIBUTIONS

XW, XG, and MC designed this study. XW and XG performed the experimental, processed and analyzed the data, drafted the manuscript, and prepared all the figures and tables. XH and XC collected some serum samples. XW, XG, XZ, and JZ detected the ovulation time and rate. QL, RD, WH, and MC contributed to revisions of the manuscript. All authors read and approved the final manuscript.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.709737/full#supplementary-material

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