FREQUENCY OF BMP15 AND GDF9 MUTATIONS INCREASING LITTER SIZE AND THEIR PHENOTYPIC EFFECTS IN OLKUSKA SHEEP POPULATION*

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Abstract

Two mutations affecting the ovulation rate and litter size are segregating in Olkuska sheep population, *FecX⁰* in the *BMP15* gene, and the G7 site mutation in *GDF9* gene. Homozygous carriers of both mutations are hyperprolific, contrary to the sterility observed in homozygous carriers of most other *BMP15* and *GDF9* mutations. The objective of this study was to assess frequency and phenotypic effects of both mutations. Blood samples were obtained from 740 individuals, 111 rams and 629 ewes, out of which 91 rams and 561 ewes were successfully genotyped for the *BMP15* and *GDF9* loci. The reproductive performance included a number of lambs born/born alive and a number of lambs reared until 60 days of age, and for a subset of ewe ovulation rates. The study proved a high frequency of the *FecX⁰* mutation in two flocks that have been selected for many years for increased litter size (0.7–1.0 in breeding ewes and rams respectively), and a moderate frequency in another 19 private flocks (0.4–0.5). The frequency of the *GDF9* mutation was low, with only 50 sheep out of 312 genotyped being carriers of the *GDF9*/G7 mutation, including three homozygous carriers. The *FecX⁰* mutation in the *BMP15* had a significant effect on both litter size and the ovulation rate. The single copy in heterozygous carriers increased litter size by 0.255 (0.063), while the effect of two copies in homozygous genotypes was +0.874 (0.081) lambs born. Due to the low frequency of the *GDF9* mutation, it can only be preliminarily concluded that litter size has been increased in double carriers of both the *BM15* and *GDF9* mutation, which may suggest their additive interaction. The positive effect of both mutations supports their direct use in selection programmes.

Key words: litter size, sheep, major genes, ovulation rate

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Litter size, as an important factor influencing profitability of lamb production has been widely studied for decades. This ongoing research has resulted in the identification of a number of sheep populations worldwide, where the ovulation rate is affected by segregation of a single major gene or genes.

All four major fecundity genes known so far, are affecting the growth of follicles and influence ovulation rates, resulting in higher litter size. Three loci belonging to the TGF- β family, Transforming Growth Factor B, include Booroola gene BM-PR1B (Bone Morphogenetic Protein Receptor 1B); BMP15 (Bone Morphogenetic Proteins) and GDF9 (Growth Differentiation Factor-9), while B4GALNT2 gene, encodes a glycosylation enzyme that is not related to the BMP family. These major genes regulate expression and secretion of the hormones that affect follicular growth and ovulation rates.

The best known Booroola mutation (FecB^B) responsible for high prolificacy in Australian Merino population, was discovered after 20 years of extensive research (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001). The same mutation was later found in Garole and Javanese sheep (Davis *et al.*, 2002), and in a number of Chinese breeds, such as Hu, Small Tail Han, Cele, Duolang and Chinese Merino prolific strains (Hua and Yang, 2009). In homozygous Booroola carriers, an additive effect on ovulation rate (OR), and a partially dominant effect on litter size (LS) are observed.

Most of the BMP15 mutations described so far: Inverdale (FecX^I), Hanna (FecX^H), Belclare (FecX^B), Galway (FecX^G), Lacaune (FecX^L); and mutations in Rasa Aragonesa (FecX^R) and Tunisian Barbarine Sheep (FecX^{Bar}) (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004; Bodin *et al.*, 2007; Martinez-Royo, 2008; Monteagudo, 2009; Lassoued *et al.*, 2017, respectively), result in the same phenotypic pattern. Ewes that are heterozygous carriers of these mutations are highly prolific, whereas homozygous carriers are infertile, with typical morphological changes of streak ovaries. There is also evidence of novel mutations in Iranian sheep, Lori and Mehraban: one in Exon 1 (c.379G>A) (Nadri *et al.*, 2016); and a second in Exon 2, G→A, 57 position of the amplified 312b fragment (Zamani *et al.*, 2015). Another mutation, T755C, was found in four Iranian breeds: Afshari, Ghezel, Lori-Bakhtiari and Shal (Amini *et al.*, 2018). The latest study by Dolebo *et al.* (2019) suggests that BMP15 was the main candidate gene responsible for enhanced reproduction in prolific Ethiopian Bonga ewes, however the exact causative mutation has not been confirmed. The authors also identified a number of genes associated with other reproduction traits that have epistatic effects on litter size.

At present, five unique mutations in the GDF9 gene (chromosome 5) have been identified: FecG^H in Belclare and Cambridge sheep (Hanrahan *et al.*, 2004), FecG^T in Icelandic sheep (Nicol *et al.*, 2009), FecG^E in Santa Inês sheep (Silva *et al.*, 2011), FecG^A in Araucana creole sheep (Bravo *et al.*, 2016) and FecG^V in Brazilian Ile de France population (Souza *et al.*, 2014).

The FecG^I mutation found by Hanrahan *et al.* (2004) proved to have a phenotypic effect in two Chilean breeds, Chilota and Aracuana (Paz *et al.*, 2014); another one from a Hanrahan study FecG⁷ was present in Norwegian White sheep (Våge *et al.*, 2013). There is also an indication of an association of the GDF9 polymorphism

(GDF9/G1 and GDF9/G4 sites) and litter size in Slask and Volgograd sheep breeds in Russia (Gorlov et al., 2017). However, there is also evidence that neither the G1 nor the G4 mutation were associated with litter size in a number of Iranian sheep breeds: Afshari, Ghezel, Lori-Bakhtyari and Shal breeds (Eghbalsaied et al., 2017).

Out of these five named mutations, the Fecundity Embrapa (FecG^{E}) and Fecundity Araucana (FecG^{A}) mutation, do not result in sterility in homozygous ewes. Contrarily, a significant increase in OR (2.22 ± 0.12 vs. 1.22 ± 0.11) and LS (1.78 vs. 1.13) were observed in homozygous carriers compared to non-carrier of the FecG^{E} gene (Silva et al., 2011), as well as in homozygous Araucana creole sheep (0.66 additional lambs) (Bravo et al., 2016).

It may be noted that the expression of BMP15 and GDF9 seems to be related to the size of antral follicles, as suggested by Foroughinia et al. (2017), after analyzing ovaries of Iranian Afshari sheep with the highest and lowest number of antral follicle counts, although the material was limited (5 ewes and 10 ewes respectively). This study also proved the importance of multiple genes expression of the ovulation rate.

A new fecundity major gene B4GALNT2, located at chromosome 11 provides a new mechanism of ovulation rate regulation in sheep (Drouilhet et al., 2013). The gene has been discovered in Lacaune sheep, and is named FecL . Its mutated prolific allele (FecL^{L}) results in high ovulation rates and large variation in litter size.

Recently, an extensive screening study carried out on the 25 most numerically important French meat sheep breeds identified the FecL^{L} mutation in the Mouton Vendéen breed, and the FecX^{Gr} mutation in the Noire du Velay breed (Chantepie et al., 2018).

The Olkuska sheep, a native Polish longwool breed, belongs to this wide group of super prolific sheep, with litter sizes up to seven lambs with good maternal abilities. The Olkuska sheep went through a bottleneck in the 1980s when only about 100 breeding ewes were used for over a decade (Drobik and Martyniuk, 2014). Successful in-situ conservation efforts over many years increased the population size of this endangered breed, such that in 2018, the population had increased to 1291 ewes, in 54 flocks (<http://owce.bioroznorodnosc.izoo.krakow.pl/liczebnosc#owca-olkuska>).

The initial observations, such as a high variability of mean OR and LS among related females, and low within-individual variation suggested the existence of an autosomal major gene segregating the same way as the Booroola mutation (Davis, 2005). High heritability and repeatability of the OR, and segregation analysis confirmed segregation of the single gene with a major effect on OR.

However, it was proved that neither the FecB^{B} nor FecX^{I} are present in the Olkuska population (Davis et al., 2002). In 2013, within research project 3SR (Sustainable Solutions for Small Ruminants), two novel, non-conservative mutations in the BMP15 gene associated with increased LS and OR were identified in the Grivette and Olkuska sheep breeds (FecX^{Gr} and FecX^{O} respectively) (Demars et al., 2013). Both homozygous Grivette $\text{FecX}^{\text{Gr}}/\text{FecX}^{\text{Gr}}$ and $\text{FecX}^{\text{O}}/\text{FecX}^{\text{O}}$ Olkuska ewes were hyperprolific, instead of exhibiting sterility as in the case of other known homozygous BMP15 mutations.

The mutation in GDF9 gene (V371M) was also identified in Olkuska sheep. It corresponds to GDF9/G7 mutation, identified by Hanrahan *et al.* (2004), which had no phenotypic effect in Cambridge and Belclare sheep. Contrarily, Olkuska ewes with one allele with V371M substitution showed an increase in litter size of +0.55 lambs (Kaczor, 2017).

While it seems that the genetic background of high prolificacy of Olkuska sheep has been better understood, the next important questions to be addressed are the frequency of both mutations in the population, and their effect on litter size and ovulation rates. The current study aimed at evaluation of reproductive performance of carriers and non-carriers of both mutations, estimation of gene effects and potential interactions between both mutations.

Material and methods

Material

The study was carried out on Olkuska sheep biological material coming from various sources. Blood was collected in the autumn of 2015 from 321 Olkuska sheep held in 18 private flocks, that were located in five voivodeships (provinces), including the region of the origin of the breed. Blood sampling was conducted by a veterinarian using a vacuum system and vials with sodium edetate. The biological material obtained in 2015 supplemented the material collected over time in the Olkuska sheep population and was stored in the Department of Genetics and Animal Breeding of Warsaw University of Life Sciences.

The available material included: samples from 254 animals from the Żelazna experimental flock at the Warsaw University of Life Sciences (WULS); 53 animals from the experimental flock at the Wrocław University of Environmental and Life Sciences; 24 animals from the University of Agriculture in Krakow; and 88 ewes used in the 3SR (Sustainable Solutions for Small Ruminants) project.

In total, material was obtained from 740 individuals, 111 rams and 629 ewes, out of which 91 rams and 561 ewes were genotyped for BMP15 and GDF9 loci (Table I). Additionally, the genotypes for 25 rams were established on the basis of their parents and progeny, in each case, at least compatible information on three relatives were required to establish a genotype of the ram (e.g. three daughters of CC genotype for BMP15).

The reproductive performance data included a number of lambs born/born alive and number of lambs reared until 60 days of age. Data on the ovulation rate, estimated as the number of corpora lutea (CL) observed by laparoscopy were collected by the Department of Genetics and Animal Breeding, WULS, from 1992, when the flock was first established, until the autumn of 2012. After the discovery of *FecX⁰* mutation, only genotyping was carried out to identify carriers. In total, 122 genotyped ewes included in the current study had information on their ovulation rate, recorded by laparoscopy from the 1999 mating season onward.

Table I. Distribution and origin of the experimental material

Flock	Years of blood collection	Voivodeship	Total N	N of Rams		N of Ewes		
				All	Gen	All	Gen	Liter size (OR)
Experimental flocks								
RZD Żelazna	2006 till 2015	Łódzkie	254	61	49	193	180	120 (122)
UP Wrocław	2015	Dolnośląskie	53	5	2	48	33	23
UP Kraków Bielany	2012	Małopolskie	24	1	1	23	23	23
Private flocks								
5 flocks including Imbramowice	2006–2010 2015	Małopolskie	172	19	15	153	137	116
4 flocks	2015	Podkarpackie	95	9	9	86	80	71
7 flocks	2015	Śląskie	97	11	11	86	73	70
2 flocks	2010–2015	Świętokrzyskie	45	5	4	40	35	29
Additional rams		–	0	0	25	0	0	0
Total		6	740	111	116	629	561	452

N – number of blood samples.

Gen – number of samples genotyped for FecX^o within BMP15 gene.

Liter size (Ovulation rate) – number of samples with phenotypic information on ewes; Ovulation rate records available only for Żelazna flock.

Additional breeding rams – genotype was determined based on progeny and/or parents. Confirmed with minimum 3 information sources.

Methods

The DNA was isolated using the QIAamp DNA Blood kit (Qiagen). After extraction the concentration of genomic DNA was measured in the NanoDrop N2000, which allowed preparation of samples for detection analysis of SNP mutations. The quantified DNA was stored at –20°C until further processing.

All animals with available samples were genotyped for polymorphism of BMP15 N337H (g.1009A>C Gene Bank accession number AC28089) using a method described by Demars et al. (2013). Reactions were carried out in an ABI 7500 Real-Time thermocycler, and the results were obtained using the standard software for allelic discrimination (Applied Biosystems).

The G7 (g.1111G>A, Gene Bank accession number AC28089) mutation in the GDF9 gene was identified by the TaqMan allele discrimination method (ABI 7300 HT, Sequence Detection System, Applied Biosystems Foster City, CA). Using the Primer Express program (Applied Biosystems), specific probes and primers labeled fluorescently with 5' (FAM and VIC) reporter dyes (TaqMan SNP Genotyping Assays) were designed for each of the alleles.

The sequence of primers and probes for the GDF9 gene were as follows: Primers_F 5'-GCTCCATGACTTTAGACTTAGCTTTAGT 3' _R 5' GGACAGTCCCCTTTA-CAGTATCGA-3'

Probes VIC CTGGATTGTGGCCCC, FAM ACTGGATTATGGCCCC. The reaction was carried out in a volume of 25 µl, the reaction mixture contained: 20ng DNA, 1x20 TaqMan SNP Genotyping Assay, 2x TaqMan Genotyping Master Mix (Applied Biosystems). Thermal reaction conditions were: 10 min 95°C; 40 cycles (92°C – 15s, 60°C – 60s). The data obtained were analyzed using SDS 2.0 software (Applied Biosystems).

The frequency of the FecX⁰ mutation in the BMP15 gene was estimated in animals born between 2004 and 2015, in two groups separately: in the experimental Żelazna flock, and in the remaining population that included 18 private flocks and 2 flocks belonging to Wrocław and Kraków universities. The approach took into account the genetic distinction of the Żelazna flock due to the long-term limited exchange of the breeding stock with other flocks, as well as the substantial amount of available biological material and performance records collected over years in the Żelazna flock. The frequency of FecX⁰ mutations was also analyzed in the breeding populations in 2010, 2012 and 2015. The breeding population being defined as ewes and rams present in a flock and taking part in the mating season in a given year.

The analysis regarding the frequency of the mutation in the GDF9 gene was conducted on 26 lambs (including 7 ram-lambs) born in 2015 in the Żelazna flock, as well as 39 rams and 314 dams from private flocks, and from the Wrocław and Kraków experimental farms born between 2004 and 2015.

The analysis of reproductive performance was carried out on 452 ewes genotyped for BMP15, and 242 ewes genotyped for GDF9, present in the breeding populations in years 1999-2015. The following traits were taken into account:

- Fertility: percent of ewes lambing to all ewes participating in the mating season;
- Prolificacy: the number of lambs born to the number of ewes lambing. The results were analyzed and are presented as the average litter size of individual ewes;
- Percentage of lambs reared: number of lambs reared until 60 days of age to all lambs born;
- Fecundity: percent of lambs reared to all ewes participating in the mating season.

The impact of the mutation in the BMP15 gene on the ovulation rate was analyzed on 122 ewes only in the Żelazna flock.

Statistical analysis

The effect of the FecX⁰ mutation in the GDF9 gene on litter size was estimated by comparing groups of carriers to wild non-carriers. The effect of the number of mutant alleles (1 or 2) on the size of the litter and the level of ovulation was analyzed using a mixed model, with the components of variance estimated using the maximum likelihood (REML) method. The analysis was carried out using the MS Excel 2007 program, the R environment (version 3.2.3) and the lme4 package (Bates

et al., 2015). In the litter size model, the genotype and the age of dams (from 1 to 8) were considered as permanent effects, while the ID of the ewe and the flock as random effects. Analysis was performed for individuals with genotypes AA, AC and CC (FecX⁰ mutation), and individuals with genotypes GG (wild) and GA (heterozygotes) for the GDF9 mutation, as only 3 homozygous carriers were identified. The year of lambing was not statistically significant, and therefore, was excluded from the model. Due to the small number of variable levels, the analysis excluded ewes older than 8 years.

The effect of the FecX⁰ mutation on the ovulation rate was analyzed on the basis of the average of all available OR records for ewes of the CC, AC and AA genotype. In the model, estimating the impact of genotype on the ovulation rate, the age of the dam was considered as a permanent effect, while dam ID and the record number (laparoscopy in a single ewe was performed from 1 to 4 times in a given season), as a random effect. The year of recording was not statistically significant, and therefore, was excluded from the model. As in the case of litter size, due to the small number of variable levels, records for dams older than 8 years were excluded from the analysis.

Results

Frequency of FecX⁰ mutation in BMP15 gene

The analysis of the frequency of the mutant allele C was carried out separately for two parts of the Olkuska population: the Żelazna flock and then the remaining 20 flocks.

The frequency of C allele in ewes born between 2004 and 2015 was much higher in the Żelazna flock, reaching 0.9 in 2013 and 2015, while in the remaining 20 flocks the frequency of the C allele changed over time, with a range of 0.2 to 0.4 (Figure 1 A).

The lower frequency of the C allele in ewes born between 2012 and 2014 in the Żelazna flock, was due to the wild genotype of rams used in the previous mating season. For example, in the 2013 mating season, an unrelated breeding ram from a private flock had been purchased, which after genotyping proved to be bereft of the beneficial C mutation in the BMP15 gene.

The frequency of the C allele in rams was biased due to the small number of males that were genotyped in consecutive years. Nevertheless, in rams, the C allele has in general a higher frequency than in ewes, up to 0.55 in private flocks. All rams born and genotyped in the Żelazna flock after 2009 were carriers of the mutation (Figure 1 B).

The number of ewes, homozygous and heterozygous carriers and non-carriers, lambled between 2004 and 2015 in the Żelazna flock, and in the private flocks, are shown in Figure 2 A, B. Most performance data were collected for individuals born in 2008, 2010 and 2015, which is related to the periods when biological material was collected (autumn 2010 and 2015).

Figure 1 A, B. Frequency of C allele and the number of carriers in ewes (A) and rams (B) born 2004–2015

In ewes born since 2009 in the Żelazna flock, mainly AC or CC genotypes were observed, with two exceptions. Three ewes born in 2010 were non-carriers (AA), and in 2012, two such ewes were born in the flock (Figure 2 A). In the private flocks and both experimental flocks (belonging to Wrocław and Kraków universities), a decrease in the number of ewes born carrying the AA genotype between 2013 and 2015 was observed, in comparison to 2007–2012 (Figure 2 B).

Figure 2 A, B. Distribution of three genotypes in BMP15 gene in ewes born 2004–2015 in the Żelazna flock (A) and in private and other research flocks (B)

The vast majority of dams in Olkusa population are carriers of the $FecX^o$ mutation, but frequency is very different among flocks. For example, in the experimental flock of the Wrocław university experimental farm, only 33% of the genotyped ewes were carriers of the mutation. This contrasts with the private flock from Imbramowice, where the successful selection for high litter size has been carried out since the establishment of the flock in 1994 (Korczynski, 2007) in 2015, as many as 78% of the animals were carriers of the mutation. In other private flocks with at least 10 successfully genotyped individuals, the frequency of carriers was in the range from 38% to 100%.

The frequency of the $FecX^0$ mutation has been changing in subsequent mating seasons in breeding ewes and rams (Figure 3 A, B). In the Żelazna flock, the number of rams used for mating varied from six (2012, 2015) to eight (2010), and the average number of genotyped ewes was 44 per year. The frequency of the C allele in the breeding stock in the Żelazna flock was the highest in 2015, when it reached 78% in ewes, and 100% in rams. The frequency of the $FecX^0$ mutation in the remaining flocks increased slightly in ewes, reaching 40.4%. In rams, it was highest in the 2012 breeding season, and dropped to 31.6% in 2015 season (Figure 3 A B).

Figure 3 A, B. Frequency of C allele in ewes (A) and rams (B) participating in the 2010, 2012 and 2015 mating seasons in the Żelazna flock and in other flocks

Frequency of mutations in locus GDF9

None of 26 lambs genotyped at the GDF9 locus in the Żelazna flock were a carrier of the V371M (g.1111G>A) mutation. In total, 6 rams and 46 dams from the private flocks and Wrocław and Kraków experimental farms born after 2004 were carriers (Figure 4). The share of heterozygous ewes in the population remained at a very low level (Figure 5). The frequency of the mutated V371M allele was the highest in 2007, reaching 23.1%.

Figure 4. Number of ewes (F) and rams (M) with genotypes GG, GA and AA in GDF9 locus recorded in private flocks and in experimental flocks of Wrocław and Kraków universities

Figure 5. Distribution of genotypes GG, GA and AA in GDF9 locus recorded in ewes born in 2005–2015 in private flocks and in experimental flocks of Wrocław and Kraków universities

The effect of mutations in the BMP15 locus on reproductive performance of ewes

Table 1 presents parameters of reproductive performance in homozygous, heterozygous and non-carrier ewes of the FecX⁰ mutation.

A significant increase in litter size was observed along with an increase in the number of copies of the FecX⁰ mutation. The average prolificacy in a group of ewes carrying a single copy of the FecX⁰ mutation (AC genotype) was 230.5%, while in a group of non-carriers 194.9%. Ewes with two copies of the mutant allele (CC genotype) were characterized by extremely high prolificacy, 282.2% on average. Two copies of the FecX⁰ mutation (CC) resulted in an increase in mean litter size by 0.87 lambs compared to non-carrier ewes (AA), and by 0.51 lambs compared to heterozygous carriers (AC) (Table 1).

Table 1. Reproductive performance of ewes of different genotypes in BMP15 gene (Genotype AA – wild, AC – heterozygous carriers, CC – homozygous carriers)

Genotype	No of ewes	No of lambings	Fertility (%)	Mean litter size ± sd	Lamb's rearing (%)	Fecundity %
AA ^A	129	571	97.7	1.948 (0.672)	77.7	147.6
AC ^B	217	880	96.4	2.310 (0.851)	81.7	182.3
CC ^C	106	323	93.8	2.818 (1.206)	70.5	186.4
Total	453	1774	96.4	2.279 (0.921)	77.6	176.9

A – AA, wild; B – AC, heterozygous carriers; C – CC, homozygous carriers.

Statistical analysis showed a significant effect of the ewe genotype on litter size. A single copy of the gene in heterozygous carriers increased litter size by 0.255 (0.063) (P-val <0.001), while the effect of two copies in homozygous genotypes increased litter size by 0.874 (0.081) (P-val <0.001).

Figure 6. A distribution of litter size in ewes of three genotypes in BMP15 gene

Despite the very high litter size observed in homozygous carriers of the *FecX⁰* mutation, their fecundity was similar to that observed in heterozygous ewes (Table 1), due to lower rearing of lambs born in numerous litters, which is much more common in homozygous dams.

The distribution of litter size in three genotype groups shows a substantial shift towards higher litter size with an increase in the number of copies of the *FecX⁰* mutation (Figure 6).

In this study, non-carrier ewes had litters up to three lambs, the highest litter size in heterozygous carriers was five, and in homozygous carriers, seven. The share of litters consisting of three or more lambs in ewes of AA, AC and CC genotype, was 17.9%, 35.6% and 54.8% respectively.

The ovulation rate was only recorded in the Żelazna flock. The number of corpora lutea observed increased with the number of copies of the *FecX⁰* mutation, although the increase was not linear (Table 2). One copy of the mutant allele resulted in an increase in the number of corpora lutea by 0.502 (0.169) (P-val = 0.004), while two copies by 1.825 (0.174) (P-val <0.001), in comparison with ewes of the homozygous wild genotype. The maximum number of corpora lutea varied from 3 in non-carrier ewes to 8 in homozygous CC carrier ewes. The average ovulation rate was 3.430, 2.055, 1.560 for homozygous and heterozygous carriers, and the wild type genotype respectively, as shown in Table 2.

Table 2. Average ovulation rate in ewes of three genotypes in BMP15 gene in the Żelazna flock

Genotype	N	Number of records	Mean ovulation rate (SD)	Maximum ovulation rate
AA ^A	17	109	1.560 (0.586)	3
AC ^B	56	379	2.055 (0.763)	5
CC ^C	49	254	3.430 (1.373)	8
Total	122	742	2.456 (1.231)	8

A – AA, wild; B – AC, heterozygous carriers; C – CC, homozygous carriers.

The effect of mutations in the GDF9 locus on reproductive performance of ewes

Table 3 presents the reproductive performance of ewes of three genotypes in the GDF9 locus. Due to the very small number of homozygous carriers of the mutation (N = 3), it was not possible to estimate the effect of two copies of the mutation at the GDF9 locus. The fact that homozygous ewes in the population had litters, only proves that the V371M mutation does not result in infertility. Statistical results obtained so far, based on a small sample, indicate that one copy of the V371M mutation increased litter size of heterozygous carriers by 0.137 ± 0.076 , but the gene effect was not statistically significant (P-val = 0.0734).

Table 3. Reproductive performance of ewes of different genotypes in GDF9 gene

Genotype	N	No of lambings	Fertility (%)	Mean litter size (SD)	Lamb's rearing (%)	Fecundity %
GG ^A	202	741	97.2	2.129 (0.783)	76.9	1.638
GA ^B	37	154	97.5	2.260 (0.730)	72.7	1.643
AA ^C	3	15	100.0	2.467 (0.516)	86.5	2.133
All	242	910	97.3	2.282 (0.922)	76.4	1.603

A – GG, wild; B – GA, heterozygous carriers; C – AA, homozygous carriers.

Figure 7. Distribution of litter size in ewes of three genotypes in GDF9 gene

Two loci carriers

Table 4 presents the preliminary results on litter size in ewes that were genotyped for both the GDF9 and BMP15 mutations. Out of 314 ewes genotyped for GDF9 (Table 3), 285 also had genotypes established for the BMP15 gene. Within this group, 242 ewes had information on average litter size. This group contained 90 AA homozygous ewes, 111 heterozygous carriers and 41 CC homozygous carriers. Three ewes that were homozygotes for the GDF9 mutation were also heterozygous carriers of the BMP15 mutation. The presence of the second mutation increased litter size by about 0.2–0.3 lambs born in AA and AC ewes. Based on the available data, it was not possible to detect such increases for ewes of CC genotype due to the small sample size.

Table 4. Number of samples genotyped on GDF9 and BMP15 mutations with available phenotype information on litter size (LS)

Genotype	AA ^A			AC ^B			CC ^C			All	
	N	N LS	Mean LS	N	N LS	Mean LS	N	N LS	Mean LS	N	N LS
GG ^D	70	66	1.894	124	100	2.176	45	36	2.793	239	202
GA ^E	25	24	2.169	12	8	2.364	6	5	2.667	43	37
AA ^F	0	0	–	3	3	2.467	0	0	–	3	3
Total	95	90	–	139	111	–	51	41	–	285	242

A – AA, wild, BMP15; B – AC, heterozygous carriers, BMP15; C – CC, homozygous carriers, BMP15; D – GG, wild, GDF9; E – GA, heterozygous carriers, GDF9; F – AA, homozygous carriers, GDF9.

Discussion

Over the years, the frequency of the *FecX^O* mutation in the BMP15 gene in the Olkusa sheep population has been maintained at a comparable level. However, significant differences in the frequency of mutation were observed between flocks.

Particularly high frequency was observed in the Żelazna experimental flock, and in the Imbramowice private flock, which were subject to continuous selection to improve litter size. In the Żelazna flock, the continuous selection of replacement ewes and rams, potential carriers of a major gene, was carried out since the mid-1990s. The classification of ewes was based on laparoscopy results, with thresholds of 3 and 5 corpora lutea for hetero- and homozygous carriers, while carrier rams were identified on the basis of dam/daughters ovulation rates. Since the discovery of the *FecX^O* mutation (Demars et al., 2013), the carriers of both sexes were identified through genotyping. In the Imbramowice flock, high frequency of the *FecX^O* mutation resulted from successful selection to increase litter size, with many high litters resulting, up to seven lambs.

The results are quite different in comparison with other BMP15 mutations, where estimated frequency of segregating alleles was rather low. For example, the frequency of the Grivette (*FecX^{Gr}*) mutation in the Mouton Vendéen breed was low, with only 10.3% of carrier ewes in the population (Chantepie et al., 2018). The heterozygous carriers of Barbarine mutation constituted 39%, and homozygous carriers only 3% of the W flock, selected for litter size (Lassoued et al., 2017).

In the current study, a significant increase in litter size was observed with the increase in the number of copies of the *FecX^O* mutation, with the effect of one copy +0.255, and two copies, +0.874 lambs born. A higher gene effect was shown by Kaczor (2017) in a group of over 100 Olkusa ewes located in southern Poland. A single copy of the gene increased litter size by +0.73 lambs born, and two copies by +1.04 lambs, in comparison with non-carriers.

There was no evidence of inbreeding depression affecting litter size in Olkusa sheep (Drobik and Martyniuk, 2016). In comparison with other BMP15 mutations, the impact of *FecX^{Gr}* mutation in the Grivette breed was much higher, +1.9 and +2.5 respectively (Demars et al., 2013), while interestingly, the effect of the same mutation in the Mouton Vendéen breed was only +0.3 lamb born (Chantepie et al., 2018).

With the exception of the *FecX^O* and *FecX^{Gr}* mutations, and also the T755C mutation found in four Iranian sheep breeds (Amini et al., 2018), all other BMP15 mutations led to infertility in homozygous carriers. To compare the phenotypic impact of mutations, a single copy of Hanna and Inverdale mutations resulted in increased litter sizes by 1.0 and 0.6 lambs born respectively (Davis, 2005), and the Barbarine mutation increased litter size by 0.3 (Lassoued et al., 2017). The highest effect of the Rasa Aragonesa mutation increased litter size by 1.3 lambs born by heterozygous ewes (Drouilhet et al., 2009). In Lori and Mehraban sheep, no significant effect on litter size was observed in case of the novel mutation identified in exon 1 (Nadri et al., 2016), while exon 2 carrier heterozygous ewes had significantly higher litter sizes than homozygous wild type ewes, 1.56 and 1.08 respectively (Zamani et al., 2015). As no homozygous carriers were found in either breed or the mutation loci,

and the population was not in Hardy-Weinberg equilibrium, a sterility or lethal effect was suggested in the case of Lori and Mehraban homozygous carriers.

The T755C mutation found in Afshari, Ghezel, Lori-Bahtiyari and Shal sheep had a significant effect on litter size, with heterozygous carrier CT ewes having larger litter sizes than both homozygous and wild and carrier ewes (Amini *et al.*, 2018). Also, sterile ewes, and ewes with triplets, were heterozygous for this mutation. This suggests a synergistic effect with other unknown mutations (Amini *et al.*, 2018).

In the current study, one copy of the mutant *FecX^o* allele increased the ovulation rate by 0.502, and two copies by 1.825, in comparison with ewes of the homozygous wild genotype. This is a slightly higher effect than initially estimated for this mutation (Demars *et al.*, 2013), where the ovulation rate in homozygous carriers *FecX^o/FecX^o* was 3.28 ± 0.85 , in comparison with heterozygous carriers *FecX⁺/FecX^o* 2.02 ± 0.47 . In comparison, the heterozygous carriers of Belclare and Galway mutations increased the ovulation rates by 1.0 and 0.7 respectively (Davis, 2005); the Barbarine mutation increased the ovulation rate by 0.7 (Lassoued *et al.*, 2017); while in the Lacaune sheep, one copy resulted in an increase of +1.5 CL (Bodin *et al.*, 2007). However, all these mutations (*FecX^l*, *FecX^h*, *FecX^B*, *FecX^G*, *FecX^L*, *FecX^R* and *FecX^{Bar}*), resulted in dysfunctional ovaries and sterility in homozygous carriers (Galway *et al.*, 2000; Hanrahan *et al.*, 2004; Bodin *et al.*, 2007; Martinez-Royo, 2008; Monteagudo, 2009; Lassoued *et al.*, 2017 respectively). In the case of the Lacaune sheep, due to the discovery of a much more promising mutation at the B4GALNT2 locus (Drouilhet *et al.*, 2013) in 2005, a decision was taken to cull the *FecX^L* mutation in the Lacaune active population.

Three mutations in the GDF9 locus: *FecG^H* in Belclare and Cambridge (Hanrahan *et al.*, 2004), *FecG^T* in Icelandic sheep (Nicol *et al.*, 2009), and *FecG^V* in Brazilian Ile de France population (Souza *et al.*, 2014), resulted in sterility in homozygous carriers. Their effect on the ovulation rate, as observed only in heterozygous carriers, was +1.4, +1.2 and +0.9 respectively.

In contrast, other GDF9 mutations result in high reproductive potential in homozygous carriers (Silva *et al.*, 2011; Bravo *et al.*, 2016). The *FecG^E* mutation in Santa Inês sheep increases the ovulation rate by 1.0, and litter size by 0.7 in homozygous carriers, in comparison with the wild type (Silva *et al.*, 2011). The frequency of the mutation in prolific selected flocks was 0.522, while in randomly chosen flocks from the breed population, only 0.192. The *FecG^A* mutation in Chilean Araucana creole sheep increases litter size in homozygous carriers by 0.66 lambs born, in comparison with heterozygous or wild type genotypes (Bravo *et al.*, 2016). However, the frequency of this mutation was very low, 0.06 in Chilota and 0.02 in Araucana populations (Paz *et al.*, 2014), thus with little effect on production output. It was also shown that neither the G1 or G4 mutations in GDF9 had any impact on litter size in Afshari, Ghezel, Lori-Bakhtiyari and Shal sheep breeds in Iran (Eghbalsaid *et al.*, 2017).

The mutation in the GDF9 locus segregating in Olkusa sheep is the same as originally identified by Hanrahan *et al.* (2004), as GDF9/G7. The site is responsible for a Val→Met substitution at position 371 (V371M, g.1111G>A), with no significant phenotypic effect on ovulation rates in Belclare and Cambridge sheep. The frequency of this mutation in Olkusa sheep was very low, and in some flocks there

are no single carriers of the mutation. In the present study, one copy of the V371M mutation increased litter size of heterozygous carriers by 0.137, but the difference was not significant. The fact that three Olkuska ewes were homozygous fertile carriers proves that the GDF9/G7 mutation does not lead to sterility. To estimate accurately the effect of GDF9 mutation, and to detect possible interactions between both mutations segregating in Olkuska sheep, more animals should be included in future studies. In earlier studies on the prolific Olkuska population, the frequency of the A allele was 0.06, the GA genotype 0.11 and the effect of one copy of G7 was +0.55 lambs born (Kaczor, 2017).

Interestingly, the same GDF9/G7 mutation found in the Norwegian White Sheep (NSW) breed, resulted in 0.46–0.57 additional lambs born by ewes sired by AI rams homozygous for this mutation, in comparison to daughters of wild-type rams (Våge et al., 2013).

It might also be observed that for ewes that were examined for both mutations, the increase in litter size in double heterozygous carriers GA/AC in comparison with double non-carriers GG/AA was 0.47, while the increase observed in single carriers (GA versus GG and AC versus AA was about 0.3 (Table 4). The single analysis on BMP15 effect, based on much larger samples (Table 2), indicates similar differences between heterozygous carriers and wild genotypes. In Olkuska sheep, as in the case of other super prolific ewes (e.g. Booroola, Davis et al., 2002; Davis, 2005), due to the lower percentage of lambs reared from numerous litters, fecundity in homozygous carriers is much lower than expected. There was also a lower fertility rate observed in carrier Olkuska ewes, which may be associated with more frequent miscarriages at an early stage of multiple pregnancies.

The B4GALNT2 locus, identified in Lacaune breed, and proved to be involved in ovulation rate regulation in sheep (Drouilhet et al., 2013), initially had a low frequency in the female population composed of 71% homozygous wild type ewes (++), 27% heterozygous (L+) ewes and 2% homozygous mutant (LL) ewes (Martin et al., 2014). The FecL^L mutation increases litter size by 0.5 lamb per lambing. However, lambs from ++ dams were heavier (+300 g) and the mortality of twin lambs born from ++ was lower than those from L + dams (Martin et al., 2014). As the mutation is easy to manage, the objective in the Lacaune OVI-Test selection nucleus flocks is to achieve 50% of carrier ewes (Chantepie et al., 2018). Segregation of the FecL^L mutation was also found in Noire du Velay meat breed. The frequency of ewes carrying FecL^L in the Noire du Velay population was estimated at 21.2%, and the estimated mutated allele effect of FecL^L on litter size, at +0.4 lamb per lambing (Chantepie et al., 2018).

The preliminary comparison of litter size in ewes genotyped for both BMP15 and GDF9 mutations, showed an increase in litter size in double carriers, which suggests the additive effect of both mutations. However, the limited number of ewes in respective groups does not allow statistical confirmation of this observation.

Conclusions

After the discovery of the mutant FecX^o allele in BMP15 locus (Demars et al., 2013), and the identification of the phenotypic effect of the GDF9/G7 mutation (Kac-

zor, 2011), it became important to better understand the impacts of both mutations on reproductive traits, and estimate their frequencies in the Olkuska sheep population.

The study showed that the $FecX^0$ mutation was well-fixed in the flocks where long-term selection to increase litter size had been conducted and was at the medium level in the remaining flocks. In contrast, the frequency of the GDF9 mutation was very low in the entire population of the breed. The impact of the $FecX^0$ mutation was high, with the first copy increasing litter size by 0.255 ± 0.063 , and two copies by 0.874 ± 0.081 , at already high levels of performance in wild genotypes, 1.948 mean litter size and 0.672 sd. The GDF9 mutation in heterozygous ewes increased litter size by only 0.131 lambs born, but the mean litter size was already at a high level in both non-carriers and heterozygous carriers, 2.129 and 2.260 respectively.

The low frequency of the GDF9/G7 mutation has not allowed estimations of either the impact of the mutation in homozygous carriers (only three ewes were found), or if there is any interaction between the two mutations carried by the same individual. Nevertheless, it was proved that neither mutation leads to sterility (no streak or anatomically changed ovaries), as in both cases, homozygous ewes were hyperprolific. This is a positive characteristic of both mutations, allowing their utilization in selection programmes. The current production system in general does not support very high prolificacy, a high share of ewes with litters of 3 or more lambs, which are more difficult to rear than smaller litters. Maintaining a substantial proportion of heterozygous females seems to be the best solution to facilitate flock management.

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