Chapter 12

Genes Involved Litter Size in Olkuska Sheep

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Additional information is available at the end of the chapter

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Abstract

Major genes increasing litter size were identified in certain sheep breeds. These genes include *BMPR-1B*, *BMP15*, *GDF9* and *B4GALNT2*, *FecX2*. Polish Olkuska sheep is a high-fecundity sheep breed; while some animals might give birth to just one or two lambs, there are Olkuska ewes which have six or even seven lambs/lambing. Fertility of this breed is caused by mutation in the major gene $FecX^O$ (*BMP15* gene), but analysis of polymorphism at the locus *GDF9* revealed presence of four polymorphisms: G447A (L159L), A978G (G326G), G994A (V332I) and G1111A (V371M). Substitutions V371 and V332I are missense mutations found in the sequence encoding active GDF9 protein. V371 polymorphism has also an effect on litter size in Olkuska breed ewes. Study of genes associated with litter size in Olkuska sheep is of high importance, as they could be used in breeding programmes as selection markers for increasing production efficiency.

Keywords: fecundity, genes, litter size, Olkuska sheep

1. Introduction

Currently, more than 1200 sheep breeds differing in many production features, including fertility, are known globally. Ewes usually give birth to one or two lambs/litter. However, there are certain highly prolific breeds—such as *inter alia* Cambridge, Thoka, Javanese, Belclare, Lacaune, Woodland, Booroola, Aragonesa, Romney (Inverdale and Hanna), Garole (Bengal), Belle-ile, small-tailed Han, Hu and Kendrapada—whose litter size ranges from three to six lambs. Genetically conditioned differences in number of maturing and ovulating follicles in different breeds of sheep have been researched already since 1980 [1], when an attempt was made to explain the genetic basis for multiple births within a litter in various breeds around the world. It has been shown that sheep fertility depending on breed may be determined either polygenic or by a major segregating gene named the *Fec* gene.

Most mutations increasing ovulation levels and the number of lambs born to an ewe were identified in genes encoding proteins belonging to the family of transforming growth factors TGF-β: BMPR1B (bone morphogenetic protein receptor-1B), BMP15 (bone morphogenetic protein 15) and GDF9 (growth differentiation factor 9). In addition, mutations $FecL^{L}$ in Lacaune breed on chromosome 11 and FecX2W (fecundity gene *X2*) in Woodland sheep were identified. $FecX^{L}$ locus contains two genes *IGF2BP1* (insulin-like growth factor 2 mRNA binding protein1) and *B4GALNT2* (beta-1, 4-N-acetyl-galactosaminyl transferase 2). It has been shown that *B4GALNT2* is responsible for high prolificacy of Lacaune sheep [2]. The mechanism of action of the gene FecX2 on X chromosome in Woodland sheep is not yet exactly understood [3].

1.1. FecB mutation in the bone morphogenetic protein receptor 1B (*BMPR1-B*) gene

Booroola was the first major gene (FecB) significantly influencing level of ovulation and litter size in sheep to be identified [4]. The effect of FecB mutation (Q249R) identified in Booroola Merino breed in the gene of bone morphogenetic protein receptor *BMPR1-B* on chromosome 6 is an increase in ovulation level and litter size in carriers of one or two copies of the gene FecB. This mutation was also identified in Asian breeds: Garole, Javanese, thin-tail Hu and Han. With regard to level of ovulation, the observed increase is additive in nature, while as concerns litter size—it is partly dominant. In ewes with heterozygous $FecB^{B+}$ ovulation level increased by 1.3 CL (corpus luteum), while in ewes with $FecB^{BB}$, the observed increase was up to 3.6 CL. The effect on litter size between homozygous++ and BB was +0.8 [6].

Many authors have shown high fertility of carriers of $FecB^{B+}$ genotype, among others in Garole-223% lamb production [6], Hu-210%, Han-240% [7] and Javanese breeds-259% [8]. Research results in Indian sheep of Muzaffarnagari breed engendered some discussion, as presence of ewes with FecB gene that still had single births was proven, and share of sheep with genotypes $FecB^{BB}$and $FecB^{B+}$ was identified as 3.47 and 41.73%, respectively [9].

In antral follicles of $FecB^{B+}$ ewes, a decrease in granulosa cell proliferation and premature expression of LH receptors were observed [10]. Appearance of FecB mutation in sheep compared with ewes devoid of mutation is associated with higher number of growing small cavitary (antral) follicles on the ovaries. Both total number of granulosa cells and total secretion of estradiol and inhibins in the ovaries of $FecB^{BB}$ ewes still remain the same as in ewes with $FecB^{++}$ genotype. Significant impact of gene FecB on development of ovaries during foetal life is also observed, including, *inter alia*, a reduced number of oocytes from the 35th to the 50th day of gestation, a smaller number of class 1 follicles from 75th to 90th day of gestation and class 2 follicles (on 135th day). A small number of class 2 follicles were also observed in early neonatal life of $FecB^{BB}$ sheep [10, 11]. Significant effect of FecB gene on function of the hypothalamic-pituitary system has been proven as well. It has been found that it may lead to increased secretion of follicle-stimulating hormone (FSH) in $FecB^{BB}$ homozygotes, although it has not been confirmed in all of the animals investigated. No FecB influence on release of luteinizing hormone (LH) has been found [12].

1.2. Mutations in the gene of bone morphogenetic protein 15 (*BMP15*)

1.2.1. Protein BMP15

BMP15 protein together with growth factor 9 (GDF9) plays an important role in folliculogenesis [13, 14]. Its paracrine action affects granulosa cells, theca cells and the oocyte itself [15]. *In vitro* cultures indicate that recombinant protein BMP15 is a potent stimulator of granulosa cell proliferation, suggesting that both cell growth and their subsequent division and differentiation require BMP15 action [16]. The protein inhibits expression of FSH and LH receptors, as decrease in activity of BMP15 increases cell sensitivity to action of gonadotropins [17]. The conducted experiments showed that BMP15 inhibits expression of StAR protein, responsible for transport of cholesterol—specifically low density lipoprotein (LDL)—into cells. LDL is the substrate for production of steroid hormones. In rats, BMP may modulate the process of steroidogenesis in granulosa cells, modifies biological effects of FSH by inhibition of progesterone-induced FSH secretion, without affecting production of estradiol [16]. BMP15 mRNA transcripts are observed from the primary follicle stage, in secondary, tertiary and dominant follicles stages, ending with atretic antral follicles [18]. In sheep, BMP15 affects proliferation rate and inhibits baseline and FSH-mediated secretion of progesterone by granulosa cells of small antral follicles [11]. Presence of BMP15 mRNA was determined in the oocytes (class 1, 2, 3, 4 and 5 follicles), but it has not been demonstrated in granulosa cells and follicle theca [19].

1.2.2. Polymorphism in the BMP15 gene

In mammals, formation of BMP15 protein is determined by action of a single gene on chromosome X [20]. An encoded sequence with a length of 1179 bp is contained in two exons separated by an intron of 5400 bp in length. mRNA translation produces a peptide built of 393 amino acids, and the resulting mature protein has a length of 125 amino acids [21]. Mutations identified in the *BMP15* locus ($FecX^{I}$, $FecX^{H}$, $FecX^{L}$, $FecX^{G}$, $FecX^{B}$ and $FecX^{R}$) result in appearance of the same phenotype in different sheep breeds, and have different molecular basis. Heterozygous ewes give birth to more lambs, while homozygotes with two copies of a gene are infertile. Action of mutations marked $FecX^{O}$ and $FecX^{Gr}$ identified in Olkuska and Grivette breeds differs from the above-mentioned, as in the case of ewes with both one and two copies of the gene, an increase in prolificacy is observed (**Table 1**).

Substitution of $FecX^{H}$ detected in Hanna sheep causes introduction of a stop codon in place of glutamine in position 23 of amino acid residue of mature protein. Transition C>T observed in the case of $FecX^{G}$ in Galoway and Cambridge breeds, also introduces a premature stop codon in place of glutamine at position 31 of the polypeptide chain. As concerns carriers of these mutations, heterozygotes show increased levels of ovulation, while homozygotes with two copies of the gene are infertile [17].

A similar phenotype is observed for $FecX^{I}$ mutation, where hydrophobic valine is substituted for asparagine at position 31 of mature protein. This leads to changes in electrostatic potential of the region involved in formation of dimers—this, in turn, interferes with dimerization and

Variant	Base change	Coding base bp	Coding residue*	Amino acids change	Mutation/reference
V299D	T>A	897	299	Val>Asp	*FecX*^{I}/[12]
Q291-	C>T	873	291	Gln>STOP	*FecX*^{H}/[12]
C321Y	G>A	963	321	Cys>Tyr	*FecX*^{L}/[24]
Q239STOP	C>T	718	239	Gln>STOP	*FecX*^{G}/[21]
S367I	G>T	1100	367	Ser>Izo	*FecX*^{B}/[21]
Del	Deletion	525–541	175–180		*FecX*^{R}/[26]
175–180	17 bp				
T317I	C>T	950	317	Thr>Ile	FecX^{Gr}/[27]

*Position of the coding residue in the *Ovis aries* bone morphogenic protein 15; BMP15 protein GenBank accession number AAF81688.1.

Table 1. Mutations identified in sheep at the *BMP-15* gene locus.

consequently leads to the elimination of biological effects of BMP15. Homozygous ewes are infertile due to folliculogenesis being arrested at initial stage of the process [10].

FecX^{B} mutation found in *BMP15* gene locus in Belclare sheep is a result of polar serine being substituted for non-polar isoleucine at position 99. This substitution has a significant impact on the ability of a protein to bind to type II membrane receptor; it determines the structure of the ligand-receptor complex with BMP2, BMP7, activin A proteins. This polymorphism abolishes biological activity of BMP15, most likely by prejudicing the binding of protein and receptor [21]. Homozygous ewes are infertile due to hypoplasia of ovaries. Admittedly, presence of antral follicles was observed, but with oocytes of anomalous size, surrounded by a single layer of abnormal granulosa cells. Morphology of ovaries of such sheep is similar to image of ovaries of FecX^{I} Inverdale ewes.

One copy of the allele FecX^{I} (Inverdale) or FecX^{H} (Hanna) results in ovulation level increase of +0.8–1.0 CL, significantly affecting size of the litter by +0.6 lamb. Homozygous FecX^{II} and FecX^{HH} Hanna ewes are sterile; in their small and poorly developed ovaries, follicles are provided with a single layer of granulosa cells [22]. In FecX^{I+} sheep, follicles were determined to be of a smaller diameter than those produced in ovaries of homozygotes devoid of mutation. However, FecX^{I+} ewes present a greater number of mature preovulatory dominant follicles of smaller diameter, and therefore do not differ in the level of estradiol and inhibin from non-mutated ones [23]. Preovulatory follicles are also characterized by early maturation, which determines greater secretion of FSH and early formation of LH receptors [12].

Studies by Bodin et al. [24] on prolific sheep of Lacaune breed showed presence of FecX^{L} mutation, leading to substitution of cysteine for tyrosine at position 53 of the BMP15 chain. Just as observed in the case of polymorphisms (FecX^{I}, FecX^{H}, FecX^{G} and FecX^{B}), also this change in gene structure causes increased level of ovulation in heterozygous ewes and infertility in homozygotes. *In vitro* studies revealed that the mutation FecX^{L} is responsible for defective

secretion of both precursors and active forms of BMP15. It is believed that this may be caused by damage to structure of one of the disulphide bonds whose formation involves cysteine, and that play an important role in stabilizing the structure of BMP15 [24]. In the case of presence of one copy of $FecX^{L}$ in ewes, ovulation level increase of +1.9–2.17 CL is observed [2, 25].

Lacaune sheep are, next to Hanna, Inverdale, Cambridge, Belclare and small-tail Han breeds, an example where increase in litter size is determined by the presence of two major genes: $FecX^{L}$ and FecL on autosomal chromosome 11 [2].

In Aragonesa $FecX^{R}$ ewes, a 17 bp deletion results in a frameshift and appearance of a stop codon, even before the coding region of mature protein. The consequence of this mutation is an 85% modification in the propeptide sequence, specifically limitation of its size to 45 amino acids out of 245 present in the original propeptide. Heterozygous ewes are very highly fecund, while the presence of two copies of the mutated gene leads to a blockage of follicular development at initial growth state and female infertility. $FecX^{R+}$ ewes have an average of 2.66 lamb/litter compared to flock average of 1.36. Presence of one copy of the gene resulted in litter size increase of +1.3 lamb [26].

A mutation in the gene *BMP15* changing the amino acid sequence in the amino acid chain p.T317I was identified in French Grivette sheep. Ewes of $FecX^{GrGr}$ genotype have 2.5 lamb/litter, while $FecX^{++}$ and $FecX^{Gr+}$give birth to 1.83 and 1.93 lamb in a litter, respectively. Sterilization of homozygotes with the $FecX^{GrGr}$ mutation has not been identified in the studied population [27].

Recent research in phenomenon of high fertility of ewes of the African breed Barbarine (167% annual lamb crop) showed presence of another substitution in the gene encoding *BMP15*, resulting in A119T mutation in the coding sequence of mature protein. Frequency of the mutated allele in the population of these sheep, or its impact of prolificacy are unknown [28].

1.2.3. Mutations in the growth differentiation factor 9 (GDF9) gene

1.2.3.1. Protein GDF9

Growth differentiation factor 9 (GDF9) is another protein belonging to TGF-β family. This peptide regulates development of ovarian follicles in rodents and ruminants (sheep), as well as humans [12, 29]. It has been shown that its synthesis, similarly as in the case of BMP15, occurs in the oocyte [30]. Mice lacking functional protein GDF9 (GDF9KO) were infertile, their follicle growth was arrested at primary follicle stage, with granulosa cells layer not properly formed, and changes in the zona pellucida. Oocytes were excessively enlarged and surrounded by a single layer of deformed granulosa cells, or oocyte was not observed in the follicle whatsoever [30, 31]. Growth differentiation factor 9 as a multi-functional protein is responsible for follicular growth from its early stage—it initiates and regulates folliculogenesis and oocyte development. It has autocrine effects on oocytes, plays a role in their development and maturation, and paracrine effects on somatic cells, inhibits expression of luteinizing hormone receptor gene and stimulating synthesis of hyaluronic acid [32, 33]. *GDF9* gene expression in sheep occurs in oocytes, with the presence of the transcript and protein demonstrated in the oocyte during formation of the follicle, in class 1 primary follicles as well as

in follicles in the phase of intensive growth. *In vitro* experiments demonstrated that GDF9 is also found in degraded oocytes and in malformed follicular structures [34, 35]. In humans, GDF9 plays an important role in pathogenesis of polycystic ovary syndrome and premature ovarian failure [36].

1.2.3.2. Polymorphism in the GDF9 gene

The gene encoding protein GDF9 was found to be located in sheep on chromosome 5 [37]. The gene consists of two exons, with length of 397 and 968 bp, respectively. Its total length is 5644 bp, with coding sequence comprised of 1359 nucleotides [35].

A number of mutations (**Table 2**) were identified in gene *GDF9*; it was determined that the mutation FecG^{H} (G8, Ser1184Phe) causes sterility in homozygous ewes. Impact of the mutations G1 (G260A, R87H), G2 (C471T), G3 (G477A), G4 (G721A, E241K), G5 (A978G), G6 (G994A, V332I) and G7 (G1111A, V371M) on fertility of sheep had not been initially analysed, but fertile homozygous animals with G1, G4 and G7 mutations were found [21, 30]. Effects of FecG^{H} mutation are due to lack of active form of the protein, leading to arrest of follicular growth in the early stages of development [38, 39] (**Table 2**).

Mutation FecG^{H} (G8) occurs within the sequence responsible for coupling a protein to the receptor and is a missense mutation. As a result, synthesized protein exhibits less affinity for the cell surface receptor. Ewes with one copy of the gene with FecG^{H} mutation have higher ovulation level. Early maturation of small secondary follicles, inhibition of their growth and earlier ovulation of a larger number of oocytes were noted [21]. Most likely, early maturation of the developing follicles is associated with inhibition of FSH receptor expression at mRNA level due to the absence of biologically active GDF9 [19]. The fact that the FecG^{H} mutation

Variant	Base change	Coding base bp	Coding residue*	Amino acids change	Mutation/reference
A87H	G>A	260	87	Ala>His	G1/[21]
V157V	C>T	471	157	Val>Val	G2/[21]
L159L	G>A	477	159	Leu>Leu	G3/[21]
Q241L	G>A	721	241	Gln>Leu	G4/[21]
Q326Q	A>G	978	326	Gln>Gln	G5/[21]
V332I	G>A	994	332	Val>Ile	G6/[21]
V371M	G>A	1111	371	Val>Met	G7/[21]
S395F	C>T	1184	395	Ser>Phe	G8 (*FecG*^{H})/[21]
F345C	T>G	1034	345	*Phe>Cys*	*FecG*^{E}/[41]
S109R	A>C	1279	109	*Ser>Arg*	*FecG*^{T}/[40]

*Position of the coding residue in the *Ovis aries* growth differentiation factor 9; GDF9 protein GenBank accession number AAC28089.2.

Table 2. Mutations identified in sheep at the *GDF9* gene locus.

determines the decrease in quantity of active form of GDF9 was confirmed by immunization of ewes, which caused increase in ovulation level [30]. Presence of $FecG^H$ resulted in increased litter size in Belclare sheep, from 1.98 in animals lacking the mutation to 2.67 lamb/litter in heterozygotes; for Cambridge sheep, the litter size surged from 2.27 to 4.28 lamb/litter.

Phenotypic effect similar to $FecG^H$ was observed for $FecG^T$ mutation in Icelandic Thoka sheep breed [40].

The results of research conducted on Brazilian Santa Ines breed indicate that presence of a mutation called $FecG^E$ (Embrapa) in *GDF9* gene has a completely different phenotypic effect than the mutations in the locus of this gene listed above, because ewes with identified two copies of the gene are prolific [41].

2. Genes determining litter size in Olkuska sheep

2.1. Mutations in the *BMP15* gene

Analysing reasons behind high fertility of the prolific Olkuska sheep breed, neither $FecX^I$ mutation in *BMP15* gene nor $FecB^B$ mutation in gene *BMPR-1B* was identified [42]. However, a number of new mutations were detected in the *BMP15* gene locus: A77A, L110L, P101, V135G [27] and N237K and N337H, defined as $FecX^O$ (synonyms: N69H or A1009C or p.Asn69His) (**Table 3**) [27, 43].

Substitution of N237K was identified outside the coding region of mature peptide in most studied ewes, and no connection with their fertility was demonstrated. $FecX^O$ was located in exon 2, in the coding sequence of mature protein (position 69 aa), right next to the sites of the mutations $FecX^I$, $FecX^H$, $FecX^L$ and $FecX^B$ found, respectively, at positions 39, 23, 53 and 99 aa of mature protein.

Two alleles (A and C) and three genotypes (AA, AC and CC) were found for the A1009C mutation identified in the sequence encoding BMP15 mature protein. The C allele (with

Variant	Base change	Coding base (bp)	Coding residue (bp)	Amino acid change	Reference
A77A	T>G	231	77	Ala>Ala	[27]
P101A	G>C	301	101	Pro>Ala	[27]
L110L	C>T	330	110	Leu>leu	[27]
V135G	T>G	404	135	Val>Gly	[27]
$FecX^O$	**A>C**	**1009**	**337**	**Asn>His**	[27]
N337H					[43]
N237K	A>T	711	237	Asn>Lys	[43]

Table 3. Mutations identified in **Olkuska sheep** in the *BMP15* gene.

N337H mutation) had a frequency of 0.55 and ewes with one (AC) and two (CC) copies of the gene constituted 56 (AC) and 27% (CC) of the animals, respectively [43].

2.1.1. Effects of N337H mutation on litter size of Olkuska sheep

Analysis of effects of mutation N337H on litter size of ewes showed a significant impact of polymorphism on prolificacy, which in sheep of genotype $FecX^{++}$ was 1.74 ± 0.55 lamb/litter, with 2.47 ± 0.77 and 2.98 ± 1.50 lamb/litter for $FecX^{+O}$ and $FecX^{OO}$ genotypes, respectively [43]. Very similar results showed Demars et al. [27] for genotypes $FecX^{++}$, $FecX^{+O}$ and $FecX^{OO}$ namely, 1.84, 2.46 and 3.05, respectively.

Changes in litter size in subsequent lambings of Olkuska ewes show an increase in fecundity correlated with increasing age of a mother. The maximum size of litter in $FecX^{OO}$ ewes was noted in their third lambing, with ewes giving birth to an average of 3 lambs/litter. However, for mothers with genotype $FecX^{++}$, litter size continued to increase up to their fourth lambing, when the litter size reached 2.25 lambs [43]. Increase in the number of lambs in the first three consecutive lambings, and then subsequent decrease in litter size has been demonstrated in studies on other highly prolific sheep breeds. Liu et al. [44] showed that average litter size for $FecB^{BB}$ homozygotes in small-tail Han sheep was 2.47 in the first lambing of an ewe, and 3.17 for older mothers. Increase in litter size as ewes were aging was also observed in Chinese Hu breed. Carriers of *FecB* gene bore an average of 1.92 lamb/litter in their first lambing, compared to 2.56 lamb in the third lambing [45]. The number of lambs in a litter was also positively affected by the number of prior lambings, an ewe has undergone in hybrids Garole × Marpura; heterozygous mothers gave birth into 1.51 ± 0.06, 1.55 ± 0.07, 1.70 ± 0.09 lamb/litter in their first, second and third lambing, respectively [46].

2.1.2. Litter size in Olkuska sheep population

In all herds of sheep breeds with a segregating major gene, distribution of litter size similar to the one determined in Olkuska sheep, that is, with high proportion of twin births and large share of triplets and larger litters, was determined. Distribution analysis of litters of Olkuska ewes showed that 29% of $FecX^{OO}$ mothers gave birth to four or more lambs in a litter, including sextuplets and septuplets. In the most numerous group of $FecX^{+O}$ ewes, only 14% of animals showed similar litter size, and among sheep of genotype $FecX^{++}$ no litters of such size were observed (**Table 4**).

In the case of $FecX^{++}$, the proportion of triplets was also only 11.2% [43]. A much smaller share of quadruplet and lager litters was found in Javanese $FecB^{BB}$ ewes (16%) [8]. The most common litter sizes in Garole $FecB^{B+}$ sheep were twins, single births and triplets; accounting for, respectively, 65, 21 and 5% of the total [47]. In turn, share of quadruplet and triplet litters in $FecB^{BB}$ ewes of Javanese breed was, 34 and 20%, respectively, with an average litter size of 2.5 lamb/litter [48]. Interestingly, in the flock of Olkuska sheep, for mothers with genotypes $FecX^{OO}$ and $FecX^{+O}$ singleton births were twice less frequent than for $FecX^{++}$ ewes (16 vs. 33%). Share of twin births decreased along with appearance of additional alleles with the mutation; among $FecX^{++}$ewes they accounted for 55% of litters, and for genotype $FecX^{OO}$

Gene/genotype	Litter size of ewes (lambs)				
	1	**2**	**3**	**4**	**5, 6, 7**
***BMP15* gene-N337H (%)**					
$FecX^{++}$ (AA)	37	51.8	11.2		
$FecX^{+O}$ (AC)	15.6	40.8	29.8	8.3	5.2
$FecX^{OO}$ (CC)	18.6	23.9	28.3	9.7	19.4
***GDF9* gene-V371M (%)**					
GG	20.8	40.6	26.8	6.6	5.2
GA	8.3	20	28.3	15	33.2
***GDF9* gene-V332I (%)**					
GG	17.4	39	28.9	7	7.3
GA	25.7	37	21.9	9	6.7

Table 4. Percentage of litter types of ewes with N337H (*BMP15* gene) and V371M, V332I (*GDF9* gene) mutations.

only for 27%. In the population of Olkuska sheep with average annual lamb production of 218%, the distribution of litters of various sizes was: 21.7% of singletons, 41% of twins, 24.6% of triplets and 12.7% of quadruplets and larger. Thus, the share of litters larger than triplet is as high as 37.1%. In Garole sheep, characterized by slightly lower prolificacy (168–187%), the proportion of mothers with twins, triplets and quadruplets was 65, 21 and 5%, respectively [6]. Similar differences in distribution of litter size were observed in a herd of Chevoit-Thoka sheep [49]. With an average litter size of 2.23 lamb/litter, the authors found similar share of twin litters (56.5%) in this population, but noted a much smaller share of births with quadruplet and larger litters (3.1%). High frequency of twin births, reaching 47% of the studied population, was demonstrated in Thoka-Chevoit sheep; this breed was also characterized by a high share of singleton births (35%) [50]. A much smaller share of multiple births in comparison with data collected for Olkuska sheep was demonstrated in Aragonese breed with 120–150% fecundity: 66.4% of singleton litters, 28.4% of twin ones, but only 1.9% of triplets. It should be noted that among more than 2000 ewes studied, only three gave birth to quadruplets and only one to quintuplets. For sheep lacking the $FecX^{R}$ mutation, only singleton births were noted [26].

2.1.3. Effect of mutation N337H on litter size

In studies on effects of mutation N337H ($FecX^{O}$) on litter size, it was shown that the effect on ovulation levels in O+ and OO ewes was an increase of +2 and +3.3 CL [27]. Effect of the O+ copy of the gene was measured at +0.73 lamb, with the effect of two copies of the gene estimated at +1.07 lamb/litter. Analysing litter size over three first lambings of an ewe, the effect on O+ ewes was +0.62 lamb/litter, and +1.07 lamb for the OO genotype (**Table 5**) [43].

Litter size of ewes	Genotype *BMP15* (N337H)			Mutation effect [43]
	AA	CA	CC	
Reproductive life span	1.74	2.47	2.98	O+: +0.73
				OO: +0.34
Lambings: I–III	1.74	2.36	2.81	O+: 0.62
				OO: +0.45
I	1.76	2.36	2.51	O+: +0.60
				OO: +0.15

Table 5. Estimated mutation effect for number of lambs born to ewes.

Comparative analysis carried out on the basis of studies by many authors between litter size for carriers of one copy of the gene versus wild sheep genotypes for populations with a major segregating gene revealed that the data vary depending on breed, age and environment. In the presence of one copy of the gene ($FecB^{B+}$), effect varies from +0.48 in Booroola × Dorset hybrids in Australia [5] to +1.16 lamb/litter for offspring of Booroola × Romney and Booroola × Perendale hybrids in New Zealand [51]. Ewes with two copies of the gene had litters larger by +0.64 lamb for the Israeli Affec × Awassi hybrids, +1.61 for Chinese Merino meat strain [52, 53]. Garole × Malpura ewes of genotypes $FecB^{B+}$ and $FecB^{BB}$ gave birth to, on average, 1.73 and 2.17 lamb, respectively, while litter size of $FecB^{++}$ sheep was 1.03 lamb. Litter size for $FecB^{B+}$ and $FecB^{BB}$ ewes compared to sheep of $FecB^{++}$ genotype was higher by +0.70 and +1.14, respectively. The effect of one copy of the gene was increasing in consecutive lambings, from +0.52 in the first lambing to +1.03 in the ewes' third lambing [46, 54].

2.2. Mutations in the gene *GDF9*

Identification of variations in the gene sequence of *GDF9* revealed presence of point mutations G3, G5, G6 and G7—detected in 2004 in Belclare and Cambridge sheep by a team led by Hanrahan et al. [21]—also in Olkuska sheep (**Table 6**).

Presence of $FecG^{H}$ (G8) mutation was excluded [43]. Thus, the Polish Olkuska breed can be classified as one of the few breeds in the world, where presence of polymorphisms has been confirmed both in the gene *BMP15* and the autosomal gene. Next to the N337H mutation in *BMP15* gene, missense mutations V371M (G7) and V332I (G6) have been identified in the region encoding mature protein GDF9. It should be noted that in Olkuska sheep, the allele with mutation V371M occurs with very low frequency of 0.06, but GA heterozygotes demonstrate high annual lamb production of 346%, with the same for GG ewes at 236% (**Table 7**) [43].

Analysis of litter size distribution in sheep with GG and GA genotypes in the locus V371M (G7) revealed that the most frequently occurring litter size in GG ewes was twins (40.6%), followed by singletons (20.8%). Litters of quadruplets and larger accounted for 11.8% of all births. Among mothers with identified one allele with the GA mutation, the share of twin

Mutation	Base change	Coding base (bp)	Coding residue (bp)	Amino acid change (aa)	Reference
G3	G→A	477	159	Leu>Leu	[43]
G5	A→G	978	326	Gly>Gly	[43]
G6	G→A	994	332	Val>Ile	[43]
G7	G→A	1111	371	Val>Met	[43]

Table 6. Mutations identified in Olkuska sheep in the *GDF9* gene.

Variant	Allele		Genotype	
	G	A	GG	AG
V371M	0.94	0.06	0.89	0.11
V332I	0.83	0.17	0.69	0.29

Table 7. Allele and genotype frequencies in the population of Olkuska sheep, (mutations V371M, V332I) in *GDF-9 gene*.

litters was twice smaller (20%), while litters with four and more lambs accounted for almost 50%. The share of single births was only 8.3% [43].

Also mutation V332I (G6) was found to be present in Olkuska sheep; with two alleles (G and A) and three genotypes (GG, AG and AA) found (**Table 7**).

The G allele appeared with a very high frequency of 0.84, and ewes of genotype GG accounted for 70% of all animals. Mothers with the GG genotype did not differ in average litter size from GA sheep, both over total duration of their productive life (2.24 ± 0.87 vs. 2.13 ± 0.91 lamb), and in the first three lambings (2.39 ± 0.90 vs. 2.30 ± 0.87) [43].

2.2.1. Effect of mutation V371M (G7) on litter size

Ewes with one allele with V371M substitution showed an increase in litter size of +0.55 lamb, while those with the V332I mutation showed a decrease of 0.18 lamb/litter [43]. Thus, only in the presence of the A allele, the mutation V371M resulted in an increase in litter size. Mutations described earlier by Hanrahan et al. [21], namely: G3, G4, G5 and G6 were also detected in the case of Olkuska breed. However, impact of these mutations on prolificacy of ewes has not been studied.

In ewes of the Brazilian breed Santa Ines, a mutation that does not cause infertility has been identified in gene *GDF9*. Melo et al. [55] and Silva et al. [41] identified substitution F345C ($FecG^{E}$) to a significant degree determining fecundity of sheep. Alleles $FecG^{+}$ and $FecG^{E}$of frequencies 0.48 and 0.52, and genotypes $FecG^{++}$, $FecG^{+E}$ and $FecG^{EE}$ with frequencies 0.17, 0.61 and 0.22, respectively, have been identified. Association between the genotype and level of ovulation and litter size has been confirmed. $FecG^{EE}$ ewes bore an average of 1.78 lambs/litter, $FecG^{+E}$ sheep 1.44 and $FecG^{++}$ mothers 1.13 lambs/litter. Level of ovulation in $FecG^{EE}$ ewes (2.22 ± 0.12)

was 82% higher than that observed for the $FecG^{++}$ genotype. No statistically significant difference was identified between $FecG^{+E}$ heterozygotes and $FecG^{++}$ ewes (1.34 ± 0.08 vs. 1.22 ± 0.11). As concerns litter size distribution, share of twin litters for ewes with the identified three genotypes was 44 and 12% for $FecG^{EE}$ and $FecG^{+E}$ ewes, respectively, while no twin litters were noted for $FecG^{++}$ sheep [41].

Presence of the mutation G1 was also confirmed in Iranian Moghani and Ghezel breeds. Presence of three possible genotypes was identified, and all the ewes were fertile. Infertility was found in only one sheep, carrying also an additional copy of the gene $FecX^{G}$ in gene *BMP15*. Ewes with heterozygous genotype were more prolific compared to those with wild sheep genotype. Share of twin litters was small (6.3%) also among animals with the wild sheep genotype, while among heterozygotes twin births amounted to as much as 53.8%. Four homozygotic mothers with two copies of the gene G1 were fertile, but gave birth to singletons [56]. Mutation G1 was als identified in Indian Garole sheep, and frequency of the wild allele and of that carrying G1 mutation was, G-0.82 and A-0.18, respectively, with respective genotype frequency of 0.64 for GG and 0.36 for AG ewes [57]. Also in this case, presence of genotype with two copies of the AA gene was not detected. Mutation in the gene *GDF9* was accompanied by the presence of *FecB* gene. In Chinese breeds, similar to the case of Olkuska sheep, several mutations in several different genes have been identified. In addition to gene FecB, also presence of mutations G2 (C471T) [58], and G3 (G477A) was revealed. Entirely new mutations G729T (Q243H) and T692C [59] were identified. The authors, however, found no impact of G3 on the number of lambs bore by ewes. While identifying mutation G729T outside the region coding mature protein, the researchers have found the allele T (0.091) as well as genotypes GG and GT (respective frequency of 0.817 and 0.183). In GT ewes, estimated litter size was 2.88 ± 0.19, while for GG sheep, it was only 2.11 ± 0.11. Effect on litter size was also recorded for the T692C mutation, where litters of CC mothers were larger by 0.63 lamb than litters of CG ewes [59]. In Hu sheep, A154G mutation at position 51 of the amino acid located outside the region coding mature protein GDF9 (Asn51Asp) was identified [60].

3. Effect of N337H and V371M (G7) mutations on litter size

Determining effect of simultaneous presence of N337H (A1009C) and V371M (G7) mutations on fertility of Olkuska breed ewes showed that the largest number of lambs was born to $FecX^{OO}$ mothers that were carriers of V371M mutation (3.32 ± 0.26 lamb/litter) (**Table 8**).

Effect of presence of both the allele N337H and V371M in ewes was similar to how presence of two copies of gene N337H affected the sheep, and amounted to +0.92 lambs/litter [43].

Studies conducted so far on interaction and potential interdependencies between different mutations within the loci of genes encoding transforming growth factors TGF-β have shown that presence of one copy of the gene with a mutation in the locus of *BMP15* led to an increase in litter size; the result was sterilization in the case of homozygous genotype with two copies of the gene. This issue has thus far been researched only within a very narrow range. Presence of several alleles of various genes in the same animal was demonstrated by Hanrahan et al.

Trait	Combined genotype in *BMP15* and *GDF9* loci				
	AA^{BMP-15}/GG^{GDF-9}	CA^{BMP-15}/GG^{GDF-9}	CA^{BMP-15}/GA^{GDF-9}	CC^{BMP-15}/GG^{GDF-9}	CC^{BMP-15}/GA^{GDF-9}
Litter size of ewes (LSM ± SE) (reproductive life span)	1.64 ± 0.11	2.26± 0.08	2.56 ± 0.19	2.64 ± 0.08	3.32 ± 0.26

Table 8. Estimated effect of N337H mutation in the *BMP15* gene and V371M mutation in the *GDF9* gene on number of lambs born to Olkuska ewes (combined genotype) [43].

[21] and Davis et al. [42], sheep carrying a copy of both $FecX^B$ and $FecX^G$or $FecX^H$ and $FecX^I$ were infertile. Sheep carrying one copy of any of the above mutations on chromosome X and the mutation FecB (*BMPR-1B*) had fully functional ovaries and ovulation levels higher than observed when these factors were occurring independently. The effect of simultaneous presence of one copy of FecX in *BMP15* and one copy of $FecG^H$ (*GDF9*) was varied. Most observations showed that the effect of these two genes was additive, but in some cases the demonstrated impact of *GDF9* was weakened when the FecX mutation was present alongside it. Estimated effect on level of ovulation due to the presence of $FecX^G$ in Belclare and Cambridge sheep amounted to 0.77 and 1.18 CL, respectively, while presence of $FecX^B$ in Belclare sheep resulted in 2.38 CL. The effect of carrying one copy of $FecG^H$ in Belclare and Cambridge ewes was much higher, and amounted to 1.79 and 2.35 CL, respectively. Generally, it can be assumed that the average stimulating effect on level of ovulation in case of mutations $FecX^G$, $FecX^B$ and $FecG^H$was 0.70, 0.97 and 1.39 CL, respectively. Simultaneous appearance of different copies of a gene with a mutation in one animal resulted in a much greater increase in the observed number of corpora lutea in the ovaries. In sheep with genotypes $FecG^H/FecX^G$ and $FecG^H/FecX^B$, the number of ovulatory follicles was 5.8 and 6.09, respectively, while for carriers of only one copy of the gene $FecX^G$ it was estimated at 2.69 CL, with 3.26 CL for $FecX^B$ ewes, and 2.67 CL for $FecG^H$ genotype [21]. Presence of $FecX^I$ and FecB genes in ewes resulted in high level of ovulation (4.4 CL), suggesting multiplicative effect of these mutations resulting from interactions between the genes *BMP15* and *BMPR-1B* [20].

4. Effect of N337H, G6 and G7 mutations on body weight

Mutations N337H, G6 and G7 detected in Olkuska sheep have no effect on body weight at 2, 28 and 56 days after birth [43]. These results are consistent with observations conducted for sheep breeds with the major gene FecB, such as hybrids Rambouillet × Booroola [61]. No effect of FecB on body weight at birth and weaning was identified for this population. Also, Kleemann et al. [62] and Abella et al. [63] found no impact of FecB on body weight and daily gains in the initial period of a lamb's life. Visscher et al. [64] identified the weak effect of the gene FecB on initial and final body weight of lambs from 7 to 12 weeks of age in Booroola × Texel hybrids. In contrast to these observations, in nine breeds of Chinese sheep with FecB gene, its significant impact on litter size, body weight and body size has

been identified. On day 90 after birth, the body weight of lambs with genotypes $FecB^{BB}$ and $FecB^{B+}$ was higher than those of lambs with the genotype $FecB^{++}$. However, these differences were age dependent and have not been detected with respect to weight measured at 2 and 120 days of age [45]. Gootwine et al. [65] have demonstrated lower body weight in lambs that were carriers of $FecB^{B+}$ gene compared to $FecB^{++}$ lambs that did not carry the mutation. Body weight at birth for lambs with various genotypes, but from the same birth type differed significantly; $FecB^{BB}$ lambs compared to $FecB^{++}$ and $FecB^{B+}$ ones had lower body weight. Comparing weight of Assaf breed sheep with different genotypes, lower body weight was identified in the case of lambs with two copies of the gene FecB [66]. Studies on Garole-Malpura sheep aimed at assessing the impact of FecB genotype on body weight have shown that this gene significantly affects body weight at birth and at 12 months of age, as well as weight gains up to 3 months of life of a lamb. There was no effect on body weight at 3 and 9 months of age [46].

5. G617A polymorphism in inhibin-α gene (*INHA*)

Proteins encoded by the genes *GDF9* and *BMP15*, necessary for an oocyte to gain cytoplasmic maturity, are a group of more than 35 proteins in the transforming growth factor β family. Inhibin A and B is one of these proteins. A missense-type substitution 617G > A—not causing amino acid substitution of proline—was identified in Olkuska sheep in exon 2 of the inhibin-α gene (*INHA*) [43]. The highest frequency was found for allele G (0.86) and genotype GG (0.71), and no ewes with genotype AA were identified. No effects of the mutation on litter size were determined; while the difference between mothers of GG and AG genotypes did indeed amount to +0.3 lamb/litter, it was not statistically significant. The impact of the mutation was dependent on the age of the animals, and for an ewe's first litter was only +0.1 lamb. This result confirms conclusions from other studies, which demonstrated the association among INHA, INHBA and INHBB and litter size of sheep [67]. Influence of variation in the inhibin gene on litter size of Merino and Friesian sheep at the level of, respectively, +0.04 and +0.09 lamb had been demonstrated previously [68]. The effect of *Taq*I/*INHA* polymorphism on the number of offspring has also been confirmed for Merino, East-Friesian and Romanowska breeds [69]. After comparing frequency of allele A in sheep with varying prolificacy, the above authors found that with increasing fertility, an increase in the frequency of allele A could be observed as well. *Ovis ammon* and *Ovis vignei* presented only allele B, while high frequency of allele A (0.65) has been determined in romanowska sheep. Studies in fecund small-tail Han and Hu breeds, as well as in low fecundity breeds Dorset, Texel and German Mountain Merino showed presence of polymorphism in the locus of inhibin βB A276G, in the untranslated region 3′-UTR, only in Hu sheep. Presence of genotypes AA, AB and BB with respective frequencies of 0.636, 0.046 and 0.318, as well as alleles A (0.659) and B (0.341), has been identified. Evaluation of genotype influence on litter size of Hu ewes showed that sheep with genotype BB gave birth to +0.58 lambs more than sheep with AA genotype [59].

6. Conclusions

To summarize, Olkuska sheep are among the few breeds with significant polymorphisms in genes coding proteins of the TDF-beta family. Fecundity of this breed is determined not only by the presence of major gene FecXO (N337H,1009 > C) in *BMP15* locus, but also by the presence of a missense mutation V371M (1111G>A) in the gene locus of *GDF9*. Considerable polymorphism in *GDF9* locus —where three other, low-frequency mutations G3, G5 and G6 can be found—is particularly noteworthy. Ewes of Olkuska breed are highly prolific, both in the case of carriers as well as $FecX^{OO}$ animals, in contrast to sterilization observed in the majority of known homozygotes with mutations in the *BMP15* gene. Research in genes determining prolificacy is an important factor for increasing fecundity of ewes and thus profitability of sheep production as well.

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