PhD THESIS

Improving reproductive indices by molecular and hormonal methods in native sheep breeds

(SUMMARY OF PhD THESIS)

PhD student **Alexandru-Marius Deac**

Scientific coordinator **Prof. dr. Marius Zăhan**

INTRODUCTION

The tradition of sheep breeding in Romania is intricately intertwined with the country's culture, especially in the mountainous and hilly regions. Considering these aspects, but also due to the fact that Romania is in 2nd place in the European Union in terms of sheep herds, for the year 2022 Eurostat reporting a herd of 10.24 million individuals (16.7% of the E.U.'s total), it is necessary to improve the productive and reproductive performances of native breeds.

Lamb meat production is the primary source of income for sheep farmers on a global scale (Young și colab., 2014). A flock's prolificacy, which is the number of lambs produced from an adult female at one lambing, plays a key role in determining the annual lamb production of the entire flock.

The methods of increasing the number of lambs obtained annually from a specific number of females can be molecular or hormonal. Thus, genetic methods can be applied in order to permanently improve prolificacy, a process that is long-lasting, and hormonal methods can be applied immediately, resulting in an increase in the quantity and quality of lamb meat deliverable per unit of time.

Studying genes associated with prolificacy is of particular importance, as it can lead to an efficiency of the selection process in breeding programs by including the genetic information of the animals. Over time, mutations in a group of closely related genes that are part of the transforming growth factor-β (TGFβ) superfamily have been shown to significantly increase the ovulation rate in sheep (Davis, 2005).

Reproductive biotechnologies are an effective tool for accelerating genetic progress in sheep (Nicholas, 1996). These techniques include various approaches, some of which improve the selection process (artificial insemination, embryo transfer), while others accelerate genetic progress by shortening the interval between generations, by obtaining products from infertile females through the in vitro fertilization technique (Zăhan and Miclea, 2020).

This PhD thesis is structured in two distinct parts, the first being represented by the "Current stage of knowledge", which includes two chapters, respectively "**Ch. 1**. Molecular methods for improving reproduction indices" and "**Ch. 2.** Methods for endocrine management of reproductive function in sheep". The second part, entitled "Personal contribution" includes seven chapters: "**Ch. 3.** Objectives", "**Ch. 4.** Materials and methods - general aspects", "**Ch. 5.** Identifing single-nucleotide polymorphisms within prolificacy-associated (BMP15) gene in three autochthonous sheep breeds", "**Ch. 6**. Testing a protocol for synchronizing estrus during the breeding season in Tsigai sheep breed", "**Ch. 7.** Establishing an optimal protocol for induction and estrus synchronization protocols during non-breeding season in anestrous Cluj Merino ewes ", "**Ch. 8.** General conclusions and recommendations" and "**Ch. 9.** Originality and innovative contributions of the thesis". A summary of these chapters will be presented below. Also, this doctoral thesis includes a number of 13 tables and 25 figures.

1. Molecular methods of improving reproductive indices

Chapter 1 presents the main molecular means of improving the reproductive indices in sheep, especially the prolificacy trait. The main genes previously documented to be involved in folliculogenesis, referred to as major genes, wich are all members of the transforming growth factor-β (TGFβ) superfamily, are briefly described. The functional implications of these genes on reproductive function are also described. The problem of establishing an optimal prolificacy according to the farming system is illustrated in this chapter. The three fecundity genes described in the BMP family are: bone morphogenetic protein receptor type IB (BMPR1B; FecB) located on chromosome 6 (Souza și colab., 2001), growth differentiation factor 9 (GDF9; FecG) on chromosome 5 (Hanrahan și colab., 2004) and bone morphogenetic protein 15 (BMP15; FecX) on the X chromosome (Hanrahan și colab., 2004).

1.1. BMP15 (bone morphogenetic protein 15)

The first fecundity gene described is represented by bone morphogenetic protein 15 (BMP15; FecX). During this subchapter, the genomic structure of this gene is described, specifying data such as the genomic interval, the number of base pairs (bp), the number of exons and introns, as presented in the NCBI (National Center for Biotechnology Information) database . The main functional characteristics of BMP15 are also described and how it influences ovarian activity, ovulation rate and implicitly prolificacy. All previously documented mutations associated with sheep hyperprolificacy are described. Thus, for each of these genetic variants, the nucleotide changes of the coding sequence and it's impact on the amino acid structure of the BMP15 protein is presented. The mutations described in this subchapter are: $FecX^{I}$ (c.896T>A; p.V299D), $FecX^{H}$ (c.871C>T; p.Q291*), $FecX^{B}$ (c.1100G>T; p.S367I), $FecX^{G}$ (c.718C>T; p.Q238*), $FecX^{L}$ (c.962G>A; p.C321Y), $FecX^{R}$ (c.525_541delTGGGTCCAGAAAAGCCC; p.W154Nfs* 55), $FecX^{O}$ (c.1009A>C; p.N337H), $FecX^{Bar}$ (c.301_304delGCTAinsT; p.A101Cfs*113), c.755T>C (p.L252P), $FecX^{RA}$ (c.1172C>T; p.T400).

1.2. GDF9 (growth differentiation factor 9)

Another major autosomal gene affecting prolificacy, essential for normal folliculogenesis, is growth differentiation factor 9 (GDF9), also called FecG. Like in the previous subchapter, the genomic structure of this gene is described, specifying data such as the genomic interval, the number of base pairs (bp), the number of exons and introns, as presented in the NCBI database (National Center for Biotechnology Information). The main functional characteristics of GDF9 are also described, how it

influences ovarian activity, ovulation rate and prolificacy. The mutations described in this subchapter are: FecG^{H} (c.1184C>T; p.S395F), FecG^{T} (c.1279A>C; p.S427R), FecG^{E} (c.1034T>G; p.F345C).

1.3. BMPR-1B (bone morphogenetic protein receptor type 1B)

The BMPR-1B gene (also known as ALK6 or FecB), encodes the bone morphogenetic protein 1B receptors in the ovaries (Davis, 2005). Like for BMP15 and GDF9, throughout this subchapter the main functional characteristics of BMPR-1B are described, with details related to the genomic structure of this gene, the genomic interval, the number of base pairs (bp), the number of exons and introns, as presented in the National Center for Biotechnology Information (NCBI) database. To date, only one variant associated with prolificacy has been identified in BMPR-1B gene, namely the Booroola mutation (FecB^{B}: c.746A>G; p.Q249R).

2. Methods for endocrine management of reproductive function in sheep

Throughout this chapter, the main methods used for endocrine management of reproductive function in sheep are presented. Also, the advantages of using assisted reproduction techniques (ART's) in order to increase the productivity of the farm, by improving the reproductive performance of sheep, are described. Thus, both the "natural methods" of inducing estrus in sheep, as well as the hormonal methods, achieved by the administration of exogenous hormones, are presented.

2.1. Natural methods

Most domestic animals, including sheep, were domesticated from wild species that evolved in temperate climates (Gupta, 2004). They have preserved seasonal reproduction to this day, being an adaptation of mammals that evolved to live in cold and temperate regions. In this subchapter are presented the methods that do not require the administration of exogenous hormones, but involve the establishment of natural conditions to stimulate reproductive activity, either by subjecting them to an artificial photoperiod regime (increasing the duration of exposure to darkness, which causes an increase of blood levels of melatonin), or by introducing one or more individuals into the herd in order to stimulate the reproductive activity of the females, an effect also known as biostimulation.

2.1.1. Artificial manipulation of photoperiod

In sheep, during short days, increased melatonin levels serve as an internal code for external photoperiod perception, with high levels of melatonin coding for short (stimulatory) days and low blood concentrations of melatonin coding for long (inhibitory) days (Malpaux și colab., 1996). Also, for rams, the short photoperiod is associated with an increase in the proportion of live and mobile spermatozoa and a reduction in the frequency of acrosomal anomalies (Zăhan and Miclea, 2020). Thus, by using light-insulated buildings or additional artificial lighting, conditions specific to the breeding season can be created.

2.1.2. Biostimulation

One of the methods that require lower infrastructure costs is represented by the biostimulation of females by exposing them to the male (also known as the "ram effect" or "male effect"). The induction of estrus in seasonally anestrous ewes by introducing rams into the flock has long been documented (Underwood și colab., 1944). The methods of stimulating estrus through the "female-female effect" are also presented, and for stimulating the sexual activity of males, the "female effect" and the "male-male effect" are described. Because the costs of biostimulation are negligible, this technique has long been successfully adopted in order to induce estrus outside the breeding season in sheep (Martin și colab., 1986).

2.2. Hormonal methods

Considering that the so-called "natural methods" are either difficult to apply (artificial manipulation of the photoperiod), or do not always give satisfactory results (biostimulation), this subchapter presents the hormonal methods of inducing and synchronizing sexual cycles in sheep. In order to induce estrus, exogenous hormones play an essential role, with agents such as progestagens, prostaglandins, gonadorelins, gonadotropins and melatonin being commonly used.

2.2.1. Progestagens

The use of progestogen hormones to induce and synchronize estrus cycles in sheep is based on the principle of simulating the presence of the corpus luteum (Zăhan and Miclea, 2020). Progesterone treatments are used for both short-term protocols (5-7 days of progesterone exposure) and long-term protocols (12-14 days), in combination with gonadotropins (Menchaca Alejo și colab., 2017), females showing estrus approximately 48 h after device removal (Abecia și colab., 2012). Also, throughout this

sub-subchapter, the main advantages and side effects associated with the use of this hormone are presented.

2.2.2. Prostaglandins

An alternative method of controlling reproductive function in sheep is the induction of luteolysis, which has the effect of triggering the follicular phase, followed by ovulation. The prostaglandins involved in the regulation of reproductive function are PGF2α and PGE2α (Miclea și colab., 2010). The use of prostaglandins as luteolytic agents has revolutionized the management of sexual cycles, as they have the role of rapidly inducing the regression of the corpus luteum (especially in its decline phases), thus allowing the manifestation of the stimulating action of gonadotropic hormones, but also a standardization of the ovarian response (Zăhan and Miclea, 2020). Also, the main advantages and negative effects associated with prostaglandins administration in sheep are described.

2.2.3. Gonadorelins

GnRH (Gonadotropin-releasing hormone) is secreted by the hypothalamus, which later reaches the capillaries of the median eminence of the hypothalamic-pituitary portal system (Miclea și colab., 2010). The secretory activity of gonadotropins (Gn-RH) is controlled endocrine and neurally. Endocrine, steroid hormones (estrogens and testosterone), prostaglandins, melatonin and some central neurotransmitters (acetylcholine, adrenaline, dopamine, serotonin, neurotensin, endorphins, enkephalins) act on the hypothalamus (Miclea și colab., 2010).

Polyovulation induction through gonadorelins (GnRH) is possible in some species, including sheep due to their role in stimulating the pituitary gland to release FSH and LH (Zăhan and Miclea, 2020).

2.2.4. Gonadotropins

In order to maximize the effect of the administration of progestogens and prostaglandins, the administration of extrapituitary gonadotropins such as PMSG (pregnant high serum gonadotropin) or eCG (equine chorionic gonadotropin) and hCG (human chorionic gonadotropin human) are frequently used (Zăhan and Miclea, 2020). The role of gonadotropins in estrus induction and timing in sheep is intended to stimulate follicular growth, maturation and ovulation (Hameed și colab., 2021).

2.2.5. Melatonin

Melatonin stimulates GnRH and LH secretion by reducing tyrosine hydroxylase activity (Viguié și colab., 1997). Continuous exogenous administration of melatonin via subcutaneous implants (18 mg melatonin) allows mimicking the short days specific to the autumn breeding season, even in the spring or summer season (Pădeanu, 2011). The timing of implant application is important in order to guarantee good effectiveness. Thus, their administration around the summer solstice is widely applied as a means of advancing the breeding season in sheep from areas further north than N45◦ (Haresign și colab., 1990) or south than S45◦ (McMillan and Sealey, 1989).

3. Objectives

The objectives pursued in this work aimed to identify some molecular and hormonal methods for improving the reproductive indices in native sheep breeds. Thus, in this chapter, the main 3 objectives of this PhD thesis were precisely described, namely: "3.1. Identifing genetic variants in major genes, members of the transforming growth factor-β (TGFβ) superfamily associated with sheep prolificacy in autochthonous sheep breeds", " 3.2. Testing a protocol for synchronizing estrus during the breeding season in Tsigai sheep breed", "3.3. Establishing an optimal protocol for induction and estrus synchronization protocols during non-breeding season in anestrous Cluj Merino ewes".

4. Materials and methods - general aspects

This chapter presents the aspects related to the biological material used in this work, the bioethical standards, the materials, reagents and hormones used, work equipment used as well as the software used.

5. Identifing single-nucleotide polymorphisms within prolificacy-associated (BMP15) gene in three autochthonous sheep breeds

The improvement of the reproductive traits of animals is of great interest for livestock production. Due to its positive impact on the sheep industry's profitability, prolificacy is one of the most economically significant biological traits, showing variation between and within breeds of domestic sheep (*Ovis aries*). Different mutations in BMPR-1B, BMP15 and GDF9 genes coding for the transforming growth factor-β (TGFβ) superfamily have been shown to influence the ovulation rate and litter size.

Numerous single-nucleotide polymorphisms (SNPs) in the bone morphogenetic protein 15 (BMP15) gene have been linked to ewe prolificacy. In order to achieve this objective, blood samples were collected from thirty prolific females (2–3 years old), belonging to the Cluj Merino (n = 10), Tsurcana (n = 10) and Tsigai (n = 10) from different farms in Transylvania, Romania. The selected ewes showed only twin or multitwin labings. Using targeted PCR amplification and Sanger sequencing, we were able to identify heterozygous SNPs in exon 2 of BMP15 in three sheep breeds reared in Romania: Tsigai, Cluj Merino and Tsurcana. The sequence analysis revealed three previously documented mutations, namely the missense mutation c.755T>C (L252P), which is predicted to change the tertiary structure of the BMP15 protein, and two silent mutations, c.747T>C (P249P) and c.1047G>A (V349V). In addition, we also identified one novel silent mutation, c.825G>A (S275S). Based on our findings and publicly available data, we indicate four putative mutational hotspots within exon 2 of BMP15 that could be considered for improving the indigenous sheep breeds through targeted gene editing and SNP genotyping strategies.

6. Testing a protocol for synchronizing estrus during the breeding season in Tsigai sheep breed

There are a variety of hormonal protocols and products on the market, but it is still unclear how they will work on specific field conditions. The efficacy of using different forms of prostaglandin F2α analogues such as dinoprost and cloprostenol in a FGA-based estrus synchronization protocols were compared in sheep. For this purpose, on the basis of a completely randomized design, 60 ewes (Tsigai breed, Rusty variety; 2–4-years old, mean body score of 2.5 ± 0.5) were divided into two estrus synchronization treatment groups, which included: fluorogestone acetate (FGA) sponges for 11 days, with the administration of an intramuscular injection of 5 mg of dinoprost on the 9-day, followed by 300 IU PMSG at the time of sponge withdrawal (FGA-D-PMSG group, n=30), and for the other group, the same synchronization protocol was followed, with the difference that instead of dinoprost, ewes received 75 µg of cloprostenol (FGA-C-PMSG group, n=30). The estrus response rate percentage (%ERR) ranged between 76.66% (FGA-C-PMSG group) and 93.33% (FGA-D-PMSG group). In this study, it is shown that the type of PGF2α (natural or synthetic) can influence the results of a 11-day FGA-PGF2α-PMSG synchronization protocol, in terms of occurrence of estrus behaviour. Additionally, hormonal treatments cost for each protocol was calculated in order to determine the most cost-effective method and whether it can be implemented in small and large-scale sheep farming.

7. Establishing an optimal protocol for induction and estrus synchronization protocols during non-breeding season in anestrous Cluj Merino ewes

The aim of this study was to compare three protocols used for induction and estrus synchronization followed by fixed-time artificial insemination (FTAI) during non-breeding season in anestrous Cluj Merino ewes. Estrus was synchronized in 80 healthy multiparous ewes using intravaginal sponges containing 20mg of fluorogestone acetate (FGA) for 14 day. Ewes were previously divided into three groups. At the time sponge removal, ewes in groups II and III received a dose of 500 IU PMSG, while those in group I received a half dose, respectively 250 IU PMSG, followed by the administration of 50 μg of GnRH 30 hours later. Ewes from group III were previously implanted with subcutaneous mini-implants of melatonin, 20 days before progestogens treatment. We could not find significant differences in terms of reproductive performances between group I and group II, results showing that group I had a conception rate of 47.05%, which is lower than that of group II (56.52%). However, there were significant differences ($P<0.05$) between melatonin-supplemented group III and group I (63.33% vs. 47.05%). Thus, the potential substitution of PMSG with GnRH still needs to be optimized, while supplementation with melatonin can increase conception rate during non-breeding season in anestrous ewes.

8. General conclusions and recommendations

Regarding the first objective, genetic analyzes revealed the presence of a total of four mutations in exon 2 of the BMP15 gene of the Tsurcana and Cluj Merino breeds. Unfortunately, for the Tsigai sheep breed, we were not able to identify any variant, all alleles being wild type. One of the mutations identified is novel (c.825G>A; p.S275S), wich has not been identified in any sheep breed in the world to date. Thus, we registered this novel mutation in the GenBank/NCBI database with the accession number OQ593381. The c.755T>C (p.L252P) mutation has been documented to increase prolificacy in sheep, and this variant does not induce sterility in the homozygous state.

For the second objective, the protocol used for the FGA-D-PMSG group resulted in a higher estrous response rate (%ERR) than that used for the FGA-C-PMSG group (93.33% vs. 76 .66%), which could be influenced by a superior luteolytic efficiency of dinoprost compared to cloprostenol. The costs of the protocols were similar (approximately 5.70 euros/sheep).

Finally, for the third objective, the study conducted revealed that all three tested protocols are suitable for induction and estrus synchronization for fixed-time artificial insemination (FTAI) during non-breeding season in anestrous Cluj Merino ewes. Supplementation with subcutaneous melatonin mini-implants resulted in the best

conception rate and GnRH has the potential to replace PMSG outside the breeding season.

Following these conclusions, it is recommended to include the c.755T>C (p.L252P) mutation as a genetic marker in future breeding programs that aim to improve the prolificacy of native sheep breeds. Further studies could aim to determine the genetic penetrance of the identified variants, respectively c.747T>C (p.Pro249=), c.755T>C (p.Leu252Pro), c.1047G>A (p.Val349=), c.825G>A (p.Ser275=) but also their association with prolificacy considering a larger number of animals. Also, based on the results obtained, it is recommended to use the protocols for synchronizing and induction and estrus tested in this study, both during and outside the breeding season.

9. The originality and innovative contributions of the thesis

The originality of this work derives from the fact that the gene associated with prolificacy (BMP15) was characterized for the first time in three native Romanian sheep breeds. The innovative contributions of the thesis result from the sequencing for the first time and the identification of four mutations in exon 2 of this gene, which can lead to an improvement in reproductive indices. One of these mutations was novel, which was not previously identified in any breed worldwide. Thus, we registered this novel mutation in the GenBank/NCBI database with the accession number OQ593381. Also, in this work, the effectiveness of the natural (dinoprost) and the synthetic (cloprostenol) form of prostaglandin F2α (PGF2α) used as a luteolytic agent was tested for the first time in a protocol that also involves the administration of a progesterone analogue, respectively fluorogestone acetate (FGA) and pregnant mare serum gonadotropin (PMSG). Another aspect of originality results from testing the possibility of replacing half of the dose of PMSG with GnRH, but also the influence of supplementing the protocols with subcutaneous mini-implants of melatonin on the reproductive performance of native sheep breeds outside the breeding season for for fixed-time artificial insemination (FTAI).

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